Oxidative Stress in Applied Basic Research and Clinical Practice

Stephen Bondy
Kenneth Maiese
Editors

Aging and Age-Related Disorders

Humana Press

Oxidative Stress in Applied Basic Research and Clinical Practice

Volume 3

Editor-in-Chief
Donald Armstrong

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Note from the Editor-in-Chief

All books in this series illustrate point-of-care testing and critically evaluate the potential of antioxidant supplementation in various medical disorders associated with oxidative stress. Future volumes will be updated as warranted by emerging new technology, or from studies reporting clinical trials.

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Aging and Age-Related Disorders

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Preface

Some of the features that characterize the aging process include the gradual accumulation of damage to cells consequent to prolonged exposure to oxidative and inflammatory events over a lifetime. In addition to the accretion of lesions that often cannot be resolved, the intrinsic levels of pro-oxidant and aberrant immune responses are elevated with age. These adverse events are often further enhanced in the chronic and slow-progressing diseases that characterize the senescent brain and cardiovascular system. The incidence of some disorders such as Alzheimer's disease and vascular diseases becomes sufficiently prevalent in the extreme elderly so that these disorders can arguably be considered "normal." The chapters of this volume examine the interface between normal and pathologic aging and illustrate how this border can sometimes be diffuse. In organs with a very low rate of cell division such as cardiac and nervous tissues, the immune "memory" of early insults can be very prolonged. This can lead to poor reversibility of heightened inflammatory responses in such tissues, leading to oxidative stress and cell death. This volume explores and illustrates the processes underlying the means by which aging becomes increasingly associated with inappropriate levels of free radical activity and how this can serve as a platform for the progression of age-related diseases.

With these observations that oxidative stress plays an important role during aging and age-related disorders, it becomes imperative to gain further knowledge into the pathways that may regulate aging. This volume, *Aging and Age-Related Disorders*, relies on the knowledge of internationally recognized experts and provides chapters that examine the interactive relationship between systems in the body, such as the nervous system and vascular system, that can enhance or sometimes even limit cellular longevity. In addition, specific redox mechanisms in cells are discussed that ultimately influence the development of disorders, such as diabetes and cardiovascular insufficiency. With this, energy mechanisms that rely upon proper mitochondrial function are seen as key players during both normal physiologic processes and during age-related disorders. Another important aspect for aging that this volume describes is the close relationship between the systems of the body and exposure to environmental influences of oxidative stress that can affect both cellular senescence and destruction of a cell's nuclear DNA. What may be even more interesting to note is that these external stressors are not only confined to illnesses usually associated

with aging but also can be evident early in maturing and young individuals. As the editors, we are extremely enthusiastic about this volume and honored by the breadth of collaborators that have worked with us to highlight emerging knowledge and therapy for the understanding of the basis and development of age-related disorders.

Irvine, California — Stephen Bondy
Newark, New Jersey — Kenneth Maiese

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Part I
General Aspects of Aging

Protein Redox-Regulation Mechanisms in Aging

Ufuk Çakatay

Abstract The perspicuity of the general mechanisms of in vivo protein oxidation was achieved in the 1980s and that of the redox-homeostasis mechanisms of reactive oxygen species (ROS)/antioxidants in the 1990s. Publications in the scientific literature dealing with protein redox-regulation mechanisms in aging have appeared only within the past 10–15 years. As is well known, the group of protein disulfide oxidoreductases, such as thioredoxin (Trx), glutaredoxin, and Trx-dependent oxidoreductases, as well as methionine sulfoxide reductase (Msr), and the mechanisms related to these systems work synergistically to regulate the level of oxidized proteins and to repair mildly oxidatively modified proteins, keeping a balanced redox potential to maintain the function of aging cells. The proteolytic enzyme systems such as proteasome complexes, caspases, and the Lon protease, which are regulated by redox mechanisms, eliminate oxidized proteins. These mechanisms, in turn, affect redox homeostasis of proteins in aging cells. The ubiquitination and sumoylation of proteins are other mechanisms by which selectively oxidized proteins are targeted for degradation and compartmentalization with such specificity believed to be necessary for maintenance of cellular redox homeostasis. However, some of the extensively oxidized proteins of an unrepairable nature can escape degradation pathways and form high-molecular-weight aggregates that accumulate with age. Such oxidized protein aggregates can become cytotoxic and have been associated with a large number of age-related disorders, including Alzheimer's disease, Parkinson's disease, cataractogenesis, and cancer. Considering the variations that have emerged in redox-regulation mechanisms and antioxidant systems related to age-related disorders, it is found that these are of an extremely complex nature. Work communicated to us in the current scientific literature now shows the extent of oxidative protein damage in aged subjects and in age-related disorders. Future research will probably be concerned with understanding the relationship between

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the aforementioned redox-regulatory proteins and age-related disorders. Such scientific progress will bring preventive and therapeutic approaches to control altered redox homeostasis in these disorders.

Keywords Aging · Protein oxidation · Oxidative protein damage · Redox regulation

1 Introduction

Since the completion of the Human Genome Project in 2002 and the recognition that this cannot provide all the answers to the etiology of age-related diseases, attention has turned to assessing changes in the expressed proteins of a given genome. Proteins are highly sensitive to oxidative modifications by reactive oxygen species (ROS) and reactive nitrogen species (RNS). In addition, native proteins can be modified by highly reactive aldehydes and ketones produced during ROS-mediated oxidation of lipids and glycated proteins during the aging process [1]. Most of our understanding about the modification of proteins by ROS comes from the pioneering studies of Garrison [2, 3], Swallow [4], and Schuessler and Schilling [5], who studied the effects of ionizing radiation on the modification of amino acids, peptides, and proteins. The results of these studies demonstrate that the oxidation of proteins by ROS can lead to oxidation of amino-acid-residue side chains, cleavage of peptide bonds, and formation of covalent protein-to-protein cross-linked derivatives. In later years, mechanisms of chemical modification of proteins by ROS/RNS were investigated until the 1990s, notably by E.R. Stadtman, R.L. Levine, R.T. Dean, K.J. Davies, and others, and were demonstrated extensively [6].

On the other hand, a broad community of investigators focused on the role of protein oxidation in the etiology and/or progression of several age-related diseases [7]. As a result of all these studies, it was determined that oxidized protein levels in tissues of subjects having such diseases are higher than those in normal subjects. Publications in the scientific literature dealing with protein redox-regulation mechanisms in aging appeared only within the past 10–15 years. As is well known, the group of protein disulfide oxidoreductases, such as thioredoxin (Trx)/Trx reductase and glutaredoxin (also known as thioltransferases), as well as methionine sulfoxide reductase (Msr), and the antioxidant mechanisms related to these enzymes work synergistically to regulate and repair oxidatively modified proteins, keeping a balanced redox potential to maintain the function of aging cells [8]. The proteolytic enzyme systems such as proteasome complexes, caspases, Lon protease, and the small ubiquitin-like modifier (SUMO), which are regulated by redox signaling mechanisms, that affect redox homeostasis of proteins in aging cells [7, 9–11]. However, some of the extensively oxidized proteins of an unrepairable nature can escape from the degradation pathways and form high-molecular-weight aggregates that accumulate with age. Such oxidized protein aggregates can become

cytotoxic and have been associated with a large number of age-related disorders, including Alzheimer's disease, Parkinson's disease, cataractogenesis, and cancer [12].

Because protein oxidation mechanisms and their markers in aging and age-related disorders have been treated extensively in various reviews and textbooks, including those by the aforementioned leading authors, we have chosen not to deal with the same material in this chapter and concentrate instead on protein redox-regulation mechanisms in the aging cell.

2 Postmitotic Aging and Redox Homeostasis

Aging is a progressive decline in an organism's adaptability and consequent increase in morbidity and mortality. It largely depends on changes occurring in long-lived postmitotic cells (nondividing cells), such as neurons, cardiac myocytes, and retinal pigment epithelial cells [13, 14]. Available data from various studies support the development of mitochondrial failure in old age in fixed postmitotic cells suggesting that mitochondrial failure may be central to the senescent process. It is now generally accepted that aging in such long-lived cells is induced by endogenously formed (primarily from mitochondria) ROS causing irreversible damage (destined mainly to mitochondria) with increased oxidative protein damage and triggering apoptotic cell death [14, 15]. The delicate balance between beneficial/harmful effects of free radicals is a very important aspect of living organism and is achieved by a mechanism called *redox regulation* (Fig. 1). The process of redox regulation protects living organisms from various oxidative stresses and maintains *redox homeostasis* by controlling the redox status in vivo [14, 16].

Postmitotic tissues of high energy demand are at greater risk of being damaged by free radicals, consistent with the notion that signs of oxidative damage usually start to appear at these body sites [17]. Due to the differing efficacy of redox homeostasis mechanisms, postmitotic tissues are generally much more vulnerable to oxidative protein damage than the mitotic cells. Whereas the liver is an organ with high mitotic rate and the major organ of antioxidant enzyme release, the brain, which is of a postmitotic nature, produces more ROS per gram of tissue than any other organ, because of its high lipid content, high oxygen consumption (20% of all oxygen), and relatively poor antioxidant defense [7, 18].

These cells are very rarely (or never) replaced because of the division and differentiation of stem cells, causing the accumulation of biological waste materials (e.g., lipofuscin, irreversibly damaged mitochondria, and aberrant proteins) that gradually replace normal structures, leading to functional decay and cell death [19, 20]. The free radical theory of aging, as already proposed in 1956 by Denham Harman [21], has recently been extended to the oxidative "garbage-catastrophe theory" by Alexei Terman [22] according to which ROS or reactive oxygen intermediates are responsible for the accumulation of oxidatively damaged biomolecules in aging. According to garbage-catastrophe theory, aging may

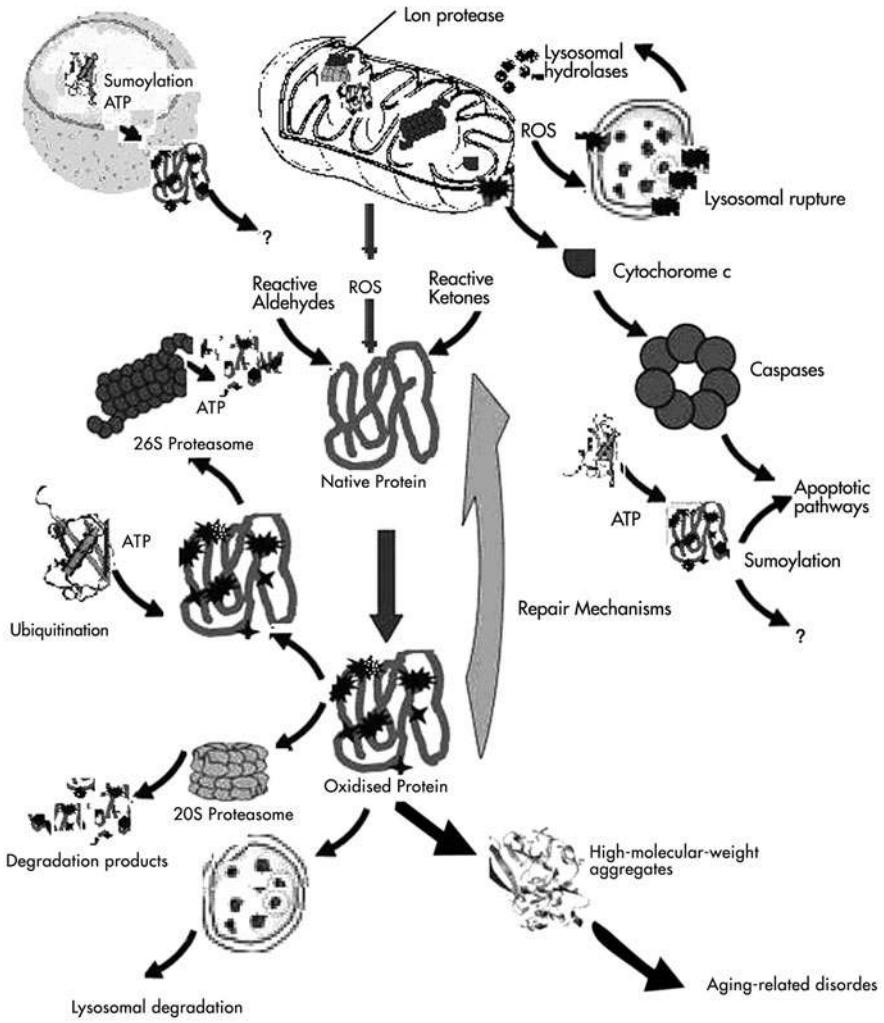

Fig. 1 Proteins can become oxidatively modified by a large number of reactions involving ROS, reactive aldehydes, and ketones. Intracellular degradation pathways and repair systems maintain redox homeostasis of the proteins in aging postmitotic cells. Oxidized proteins are generally destined for proteolysis by the lysosomal system, proteasome, small ubiquitin-like modifier (SUMO), and the Lon protease but can escape degradation and form high-molecular-weight aggregates that accumulate with aging. Depending on the type and degree of the oxidative modification, intracellular elimination of oxidatively modified proteins materializes in the different cellular compartments by either degradation or repair systems including the group of protein disulfide oxidoreductases, such as Trx/Trx reductase, glutaredoxin, and Msr. The accumulation of oxidized proteins is known to be linked to age-related diseases such as Alzheimer's disease, Parkinson's disease, and cancer

derive from imperfect elimination of oxidatively damaged, relatively indigestible cellular material, the accumulation of which further hinders metabolic functions and mainly affects such cells [22–24]. Because of inherently imperfect lysosomal degradation (autophagy) and other self-repair mechanisms, damaged structures

(biological "garbage") progressively accumulate within such cells, both extra lysosomally and intralysosomally. Defective mitochondria and aggregated proteins are the most typical forms of extralysosomal "garbage," whereas lipofuscin that forms due to iron-catalyzed oxidation of autophagocytosed or heterophagocytosed material represents intralysosomal "garbage" [19]. This proteolytic process seems to be largely due to an unfolding process resulting from oxidation. An increase in surface hydrophobicity of oxidized protein takes place during unfolding as hydrophobic amino acids that are normally buried within the interior of all soluble proteins are exposed to the aqueous environment [24]. ROS-mediated destabilization of lysosomal membranes releases hydrolytic enzymes to the cytosol, eventually leading to cell death (either apoptotic or necrotic depending on the magnitude of the oxidative attack), whereas chelation of the intralysosomal pool of redox-active iron prevents these effects. In relation to the onset of oxidant-induced apoptosis, but after the initiating lysosomal rupture, cytochrome c is released from mitochondria and caspases are activated. Mitochondrial damage follows the release of lysosomal hydrolases, which may act either directly or indirectly, through activation of phospholipases or other proapoptotic proteins. Additional lysosomal rupture seems to be a consequence of a transient oxidative stress of mitochondrial origin that follows the attack by lysosomal hydrolases and/or phospholipases, creating and amplifying a loop system [19]. The mitochondrial-lysosomal axis theory of aging describes the relationship between lipofuscin accumulation, decreased autophagy, increased ROS production, and mitochondrial damage in senescent, long-lived, postmitotic cells [19, 20].

A variety of age-related diseases, such as neurodegenerative disease, and the physiologic aging process are characterized by the intracellular or extracellular accumulation of oxidized proteins [7, 13, 14, 24]. Although damaged proteins may partially preserve their functions, their enzymatic activity per unit mass declines. Lewy bodies and neurofibrillary tangles (composed of α-synuclein or amyloid β peptide, respectively) are characteristic examples of such aggregates. Nonenzymatic protein modifications and oxidation is a continuous process occurring in all cells. Therefore, one has to assume that oxidized proteins accumulate with age because they are not perfectly turned over by the cellular proteolytic enzyme systems such as 20S and 26S proteasome complexes in cytoplasm and nucleus, caspases in cytosol, Lon protease in mitochondria, and lysosomal enzymes [7, 9, 11, 19, 20, 24]. The ubiquitination [7, 11, 12, 24] and sumoylation [10, 11] of proteins are other crucial mechanisms by which selectively oxidized proteins are targeted for degradation with such specificity believed to be necessary for maintenance of cellular redox homeostasis in the aging cell.

Although the SUMO system was only discovered 10 years ago, extensive studies in the past few years have demonstrated that sumoylation is a remarkably versatile regulatory mechanism of protein functions involved in the regulation of diverse cellular process. SUMO can either regulate the functional activity of a target protein by direct sumoylation or indirectly regulate a signaling pathway via sumoylation of a key signaling molecule. SUMO proteases may also act as redox sensors and effectors modulating the desumoylation pathway and specific cellular responses to oxidative stress. Recent evidence indicated that equilibrium between

SUMO conjugation–deconjugation under high oxidative stress could be affected by ROS [10].

Other evidence was provided previously by the finding that overexpression of antioxidant enzymes that prevent the generation of excess free radicals, such as superoxide dismutase (SOD) and catalase [25], reduce the levels of oxidized proteins. On the other hand, the oxidation of methionine plays an important role in oxidative protein damage in the aging cell. Depending on the nature of the oxidizing species, methionine may undergo a two-electron oxidation transforming it into methionine sulfoxide or a one-electron oxidation into methionine radical cations. Methionine radical cations will be destined to predominately irreversible reaction channels, which ultimately yield carbon-centered and/or peroxyl radicals. These may become starting points for chain reactions of protein oxidation [26]. Methionine sulfoxide levels may increase as a result of insufficient active Msr and/or the required cofactors as a consequence of pathologies and biological aging. Msr [27], which catalyzes the repair of oxidized methionine in proteins by reducing methionine sulfoxide back to methionine, extends the life span of *Drosophila melanogaster* markedly. In agreement with this, mutations in the age-1 gene of *Caenorhabditis elegans* result in an age-specific increase in the activity of catalase and CuZn-SOD and double the life span [28]. It is worthwhile to mention that the germ line of egg and sperm has been maintained alive and safe from senescence and oxidative damage for more than a billion years. Because multicellular organisms are able to reproduce with germ-line cells before senescence of their soma, which incapacitates them, there was never any evolutionary impetus to develop biochemical mechanisms of preventing senescence in their postmitotic cells. Notably, both *Drosophila melanogaster* and *Caenorhabditis elegans* are mostly composed of postmitotic cells; the results from these invertebrates are much more supportive of the free radical theory of aging than are results from rat.

Our understanding of the intricate and delicate redox-regulation mechanisms of cellular proteins and antioxidant systems in postmitotic tissues in aging and age-related diseases is much less advanced although many detailed studies have been performed in recent years.

3 Redox-Regulation Pathways and Repair of Proteins

3.1 General Principles

Although excessive oxidative damage in proteins and nucleotides may occur, physiologic amounts of oxidative stress transduce intracellular signals for activation, differentiation, and proliferation. Special pathways exist in aging cells to accompany redox regulation of proteins including redox-regulating enzymes, proteolytic enzyme systems, and tagging systems. Several possibilities may be the cause of an increase in the steady-state level of oxidatively modified proteins [23]. These include [1] an increase in the formation of oxidizing species [2], a decreased

antioxidant capacity to scavenge those species [3], an increased susceptibility of the proteins to become oxidized as a consequence of transcriptional and translational errors, and [4] a decrease in the levels or activities of the proteasome or proteases that selectively degrade oxidized proteins. Moreover, it has been shown that the activity of the redox-regulating enzymes catalyzes the repair of oxidized proteins, and tagging systems affect the steady-state level of oxidatively modified proteins [8, 10, 11, 27]. On the other hand, gender-related hormonal status and other possible regulatory events are other important factors that contribute to the type and extent of oxidative protein damage in various tissue proteins in aged subjects [29–31]. It is still obscure how gender-related factors affect the redox-regulating mechanisms and, in turn, the steady-state level of oxidatively modified proteins.

Reversible oxidation–reduction reactions of sulfur atoms in cysteine and methionine provide a common mechanism for the control of physical and functional properties of cellular proteins. Cys-based redox signaling mechanisms are an essential cellular response on oxidative stress. Oxidation of cysteine residue side-chains in proteins forms disulfides, sulfenic acids, sulfinic acids, and sulfonic acids. Many amino acids can undergo oxidation, and sulfur atoms can be oxidized to a number of different oxidation states; but only Cys and Met undergo reversible oxidation reactions, and these involve only a limited number of oxidation states. The cellular redox measurements largely reflect the cytoplasmic compartment. The restricted movement of biomolecules imposed by both the plasma membrane and intracellular membrane systems creates multiple compartments with different redox status. Based on current data, which are limited for some organelles, the redox status of the secretory pathways and lysosomes appears to be relatively oxidizing, whereas that of the nucleus is relatively reducing [32]. The *i*ntegrated mechanisms for controlling redox homeostasis in different cellular compartments need to be elucidated in future aging studies.

Low-molecular-weight thiols such as reduced glutathione (GSH) and protein thiol (–SH) groups undergo reversible oxidation to form disulfides [32]. Cellular thiol systems are important in the control of redox regulation of proteins, both by protecting the aging cells against oxidative damage and serving in redox signaling mechanisms to sense danger and to repair damage [33]. Studies by a number of research groups show that the redox state of the central tissue antioxidant, GSH, can be measured in plasma and provide a quantitative systemic indicator of oxidative stress. The GSH/GSSG (oxidized glutathione) redox couple in humans and experimental animals tends to oxidize with age [29, 30]. However, the GSH/GSSG redox couple is not in equilibrium with the larger plasma cysteine/cystine (Cys/CySS) pool, and the Cys/CySS redox couple varies with age in a pattern that is distinct from that of the GSH/GSSG redox couple. Furthermore, in vitro studies show that variation in Cys/CySS redox-couple status over the range found in vivo affects signaling pathways that control cell proliferation and oxidant-induced apoptosis. The results point to the conclusion that free radical scavenging antioxidants are of increased importance when thiol/disulfide redox states are oxidized. Because thiol/disulfide redox states, per se, function in redox signaling and control as well as antioxidant protection, GSH/GSSG and Cys/CySS redox states may provide central parameters

to link environmental influences and progression of changes associated with aging [33].

The current knowledge on the oxidized protein repair systems and degradation mechanisms in aging is reviewed herein. The possible interactions between the ubiquitin-proteasome system, the SUMO system, the protein repair mechanisms, and other antioxidative defense strategies are highlighted without going into extensive detail.

3.2 Intracellular Mechanisms

3.2.1 Role of Thiol-Based Repair Systems

Oxidized protein repair systems are limited to the reduction of certain oxidation products of sulfur-containing amino acids. Two principal systems foresee cellular thiol/disulfide redox state: GSH and Trx. These systems are complementary but also have overlapping activities that provide a partial redundancy in their functions. GSH is a low-molecular-weight thiol present at millimolar concentrations in cells and is well suited for functions in detoxification, interorgan cysteine homeostasis, and redox control, whereas Trx is a key molecule for redox regulation and small multifunctional protein (12 kDa) and is present at micromolar concentrations and has a redox–active disulfide/dithiol within the conserved active site sequence: Cys-Gly-Pro-Cys [32, 34, 35]. Trx is induced by a variety of oxidative stress conditions and plays crucial roles as a redox-regulator of intracellular signal transduction and as a radical scavenger. The dithiol motif at its active site is ideally suited for reduction of protein disulfides, sulfoxides, and sulfenic acids, but also is capable of peroxide elimination [32].

Methionine is first oxidized into methionine sulfoxide, which can be further oxidized into methionine sulfone. The oxidation of methionine into methionine sulfoxide is accompanied by a decreased hydrophobicity and flexibility and has been associated with the impairment of protein function. Disulfide bridge and sulfenic acid reduction is achieved by the Trx/Trx reductase system, whereas the glutaredoxin/glutathione/glutathione reductase system can reduce both disulfide bridges and low-molecular-weight mixed disulfides, including glutathione. Trx and glutaredoxin are small ubiquitous proteins belonging to the thiol/disulfide oxidoreductase family, the members of which contain a redox-active disulfide at their active centers. Oxidized Trx, which carries a disulfide bridge, is subsequently reduced in an NADPH-dependent manner by Trx reductase, an enzyme containing selenocysteine and flavin that is present in both the cytosol and mitochondria of mammalian cells. The inactivation of Trx-dependent peroxidases and peroxiredoxins as the result of cysteine-sulfinic acid formation was found to be reversible in mammalian cells [36].

Oxidation of methionine residues leads to the formation of two diastereoisomers, Met-S(O) and Met-R(O), which can be enzymatically reduced back to methionine by the ubiquitous enzymes methionine sulfoxide reductase A (MsrA) and B (MsrB), respectively. MsrA and MsrB are both found in the cytosol, nucleus, and

mitochondria, and endoplasmic reticulum provides an additional location for MsrB izoenzyme. Oxidized MsrA and MsrB are then reduced by the Trx/Trx reductase system in vivo [36, 37]

3.2.2 The Roles of Proteasome, Ubiquitin, and SUMO

Modification of proteins by the covalent attachment of small polypeptides is an important regulatory mechanism. The best-known molecule of this type of modification is ubiquitin: Ubiquitination is critical for targeting proteins to be degraded by proteasome complexes. Proteasome-mediated protein degradation is responsible for a large percentage of bulk protein turnover in the aging cell [7, 24]. The proteasomes are multicatalytic protease complexes that play an important role in the degradation of short-lived, oxidatively damaged proteins, including mutant or misfolded proteins, and are localized in the cytosol, the nucleus, as well as being attached to the endoplasmic reticulum. Generally, the proteasome operates in the following manner: The oxidatively damaged protein is recognized by the protease as it enters the proteasome complex through the narrow opening (α-annulus) in the middle of the outer α-ring and is processed in the catalytic chamber between the two β-rings, and the small polypeptides leave the proteasome through the opening of the second α-ring. Within the catalytic chamber of the proteasome between the two β-rings are located the six active centers that exhibit different catalytic activities [7]. The core proteasome degrades the protein only partially to single amino acids. The resulting oligopeptides from proteasome activity are further hydrolyzed by several intracellular peptidases to single amino acids. Proteins must be deaggregated and unfolded in order to be able to enter the proteasome. Most aggregated proteins, particularly cross-linked aggregates, may no longer "fit" into the proteasome. The cross-linking of these proteins may thus result in restricted entry into the core particle of the proteasome and incomplete degradation [24]. The proteasome also plays a pivotal role in several other cellular activities including cell cycle regulation, antigen presentation, and apoptosis [7]. It has been described that the 20S proteasome is activated under oxidative stress conditions and is able to degrade oxidized proteins in an ATP- and ubiquitin-independent manner [38], different from the 26S proteasome. The latter contains a central, barrel-like core particle (the 20S proteasome) composed of four stacked seven-membered rings, with the subunit stoichiometry $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ [7]. The ubiquitin-dependent 26S proteasome as well as ubiquitin-activating and -conjugating enzymes are very sensitive to direct oxidative inactivation, and cells deficient in ubiquitin-conjugating activity are able to degrade oxidatively damaged proteins at near normal rates. The key role of the 20S proteasome in the clearance of oxidized proteins is supported by data showing that several genes encoding 20S proteasome subunits are upregulated in cells exposed to oxidative stress and also during recovery after oxidative damage [39]. Within the 20S proteasome, subunits β1, β2, and β5 of both adjacent β-rings expose their proteolytically active sites, exhibiting post–glutamyl peptide hidrolyzing, trypsin-like and chymotrypsin-like cleavage specificity, respectively. As the proteasomal system consists of several proteasomal forms and regulatory particles, which themselves

are composed of different subunits with distinct functions, it seems to be likely that regulation of the proteasomal activity is very complex [7]. The knowledge of how and why the 20S and 26S proteasomes are regulated differently under oxidative stress can be of great importance for the development of new therapeutic strategies in age-related disorders. As aging cells have an altered activity of proteasomes in their nuclei and cytosol, the regulation of proteasomal systems is also at least a co-candidate for understanding age-related disease mechanisms. More information about the redox regulation of the proteasomal system within the aging cell is needed to clarify all these questions.

Similar to ubiquitin, members of the SUMO family of proteins are conjugated to lysine residues in target proteins. A short sequence containing the consensus Ψ-K-X-D/E (where Ψ is a large hydrophobic residue, K is the lysine conjugated to SUMO, X is any amino acid, D or E is an acidic residue) is thought to be necessary for this in vitro protein sumoylation to occur, however sumoylation has also been observed in cases where the consensus site is not conserved lysine residues on the target protein. SUMO proteins are relatively low-molecular-weight proteins. Most of them are approximately 100 amino acids in length and 12 kDa molecular weight. In the SUMO conjugation (sumoylation) process, cysteine residues are crucial for the formation of E1-SUMO and E2-SUMO thioester intermediates as well as catalysis of the isopeptide bond (Fig. 2). Isopeptide bond between SUMO and its target protein can be formed using the sumoylation enzyme cascade and can also be cleaved by SUMO-specific proteases, making the sumoylation process reversible [10, 11, 40, 41]. Although SUMO has very little sequence homology with ubiquitin at the amino acid level, it has a nearly identical molecular architecture with respect to ubiquitin. SUMO proteins are covalently attached to and detached from other cellular proteins to modify their biological function. Sumoylation is a posttranslational modification and may affect many cellular processes such as signal transduction, nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to oxidative stress, and progression through the cell cycle and modulation of ubiquitination. The sumoylation process (Fig. 2) is realized by an enzymatic cascade analogous to that involved in ubiquitination. Ubiquitin is conjugated to target proteins in three steps. These steps are catalyzed by enzymes known as E1 (or ubiquitin-activating enzyme), E2 (or ubiquitin-conjugation enzyme), and E3 (or ubiquitin-protein ligase), respectively. The E1 catalyzes the ATP-dependent activation of the ubiquitin C-terminal carboxyl group, resulting in a covalent

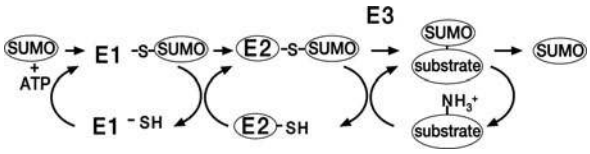

Fig. 2 The formation of the SUMO-substrate (oxidized protein) isopeptide bond. The isopeptide bond between SUMO and its substrate can also be cleaved by SUMO-specific proteases, making the sumoylation process reversible

intermediate in which ubiquitin is linked to an active site Cys residue in the E1 via a high-energy thiolester bond. Activated ubiquitin molecule is then transferred from the E1 to a Cys residue in the E2, yielding ubiquitin-E2 thiolester intermediate. Ubiquitin is then transferred from the E2 to the Lys residue in the target protein with the help of the E3, which is primarily responsible for substrate recognition and regulation of the ubiquitination process. The polyubiquitin chain is finally removed and hydrolyzed into free ubiquitin, which can be reused in a further cycle.

Although little is known about the molecular mechanism of SUMO function in aging cells, it is clear that SUMO does not target proteins for proteasome-dependent proteolysis. In some cases, SUMO appears to act by altering the subcellular localization of the SUMO-modified target protein, whereas in other cases, SUMO prevents ubiquitin-dependent proteolysis of the modified protein.

Xu et al. [10] demonstrate that the SUMO proteases may serve as redox sensor and effector undergoing both reversible and irreversible covalent modification upon exposure to various degrees of oxidative stress. Unlike most of the redox-regulatory switches previously identified, the reversible modification of SUMO proteases is at the intermolecular level [10]. It is anticipated that the seven human SUMO proteases identified to date and its substrate specificities would generate a diverse but specific intracellular redox response. On the other hand, Zhang and colleagues' study for the first time demonstrates that the amount of sumoylated protein increases with age in the spleen, and that in contrast with dietary restriction-mediated effects on the levels of ubiquitinated protein, age-related increases in the sumoylation process are not significantly affected by dietary restriction. Because sumoylation is known to have effects on protein function/localization, their data suggests a new role for sumoylation potentially contributing to altered protein function in the aging spleen [11]. Little is known of the regulation of the SUMO in physiologic aging, but any failure in its function seems to be important in age-related diseases.

3.2.3 The Roles of Mitochondrial Antioxidant Systems and of ATP-Dependent Proteases

As complexes I and III are known to be the major sites of ROS production in the cell, mitochondrial proteins are particularly exposed to oxidative damage. The first line of defense against oxidative injury is composed of a complex network of ROS detoxifying enzymes and nonenzymatic antioxidants. In the mitochondria, the ROS-detoxifying enzymes include manganese superoxide dismutase, catalase, glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, and two isoforms of peroxiredoxins. Other enzymatic systems such as Trx/Trx reductase and glutaredoxin/glutathione/glutathione reductase also play an important role in mitochondrial antioxidant defenses [36].

Mammalian mitochondria contain three major ATP-dependent proteases: Lon, Clp-like, and AAA proteases. Clp-like and AAA proteases are hetero-oligomeric complexes located in the matrix and the inner mitochondrial membrane, respectively. These proteases contribute to the degradation of misfolded and damaged proteins and/or the maintenance of mitochondrial genome stability as a second

line of defense. Mitochondrial proteases contribute to the degradation of misfolded and oxidatively damaged proteins and/or the maintenance of mitochondrial genome stability. Both proteolytic systems appear to exert chaperone activity [36]. The physiologic function of Clp-like protease has still to be determined. Currently, information regarding the redox regulation of each of the ATP-dependent proteases and/or the identities of specific protein substrates is limited.

Irreversibly oxidized proteins are mainly targeted to degradation by mitochondrial matrix proteolytic systems such as the Lon protease. As with numerous mitochondrial proteins, Lon is encoded by the nuclear genome [36, 42]. The *lon* gene encodes a 963-amino-acid protein. The ATP-stimulated Lon protease is believed to play a crucial role in the degradation of oxidized proteins within the mitochondria. Age-related declines in the activity and/or expression of this mitochondrial proteolytic system have been previously reported [7, 9, 36, 42, 43]. Age-related impairment of mitochondrial protein maintenance may therefore contribute to the age-associated build up of oxidized proteins and impairment of mitochondrial redox homeostasis. The Lon protease seems to play a critical role in the removal of oxidized protein. Indeed, aconitase, a Krebs cycle enzyme known to be susceptible to oxidative inactivation, has been shown to be a substrate of the Lon protease when the enzyme is inactivated upon treatment with oxygen radicals. The expression of the Lon protease increased with age in the heart of a rat in comparison with the younger ones, suggesting compensation for loss in specific activity. Although the ATP-stimulated protease activity remained unchanged in the heart's mitochondrial matrix during aging, a decrease was observed in the level of aconitase activity. These results indicate that the Lon protease undergoes age-dependent alterations leading to the accumulation of oxidatively modified substrates of Lon such as aconitase, although the effects are tissue-specific [7, 9, 36, 44].

3.3 Extracellular Mechanisms

Extracellular age-accumulated molecular damage by glycation, oxidation, and cross-linking of long-lived extracellular proteins, mainly collagen and elastin, is a major cause of several important human aging pathologies. Cross-linking increases mechanical stiffness of blood vessels and urinary bladder. Cross-linking impairs the functioning of the kidney, heart, retina, and other tissues and organs. Glycation adducts trigger inflammatory signaling, provoking oxidative damage and cancer [45].

Major differences between cellular and extracellular compartments exist both in terms of the concentrations of thiol/disulfide systems and their relative redox states. Many proteins present on cell surfaces and located in extracellular fluids contain cysteine and methionine residues that are subjected to oxidation. These proteins, which include transporters, receptors, and enzymes, respond to oxidative variations in the extracellular thiol/disulfide redox environment [32]. Changes in the activity of these proteins can alter the ability of organs to function normally in aging.

Perhaps the most frequently recognized difference is that the major low-molecular-weight thiol/disulfide system in cells, GSH/GSSG, is principally in the reduced form, whereas the major low-molecular-weight system in the extracellular compartment, Cys/CySS, is principally in the disulfide form, cystine. On the other hand, recent in vivo data have shown that the redox state of the GSH/GSSG couple in plasma of aged rats varies between genders and may in turn affect the redox state of the postmitotic tissue proteins [30].

4 Altered Redox-Regulation Pathways and Age-Related Disorders

The concept of "redox regulation of proteins" is emerging as an understanding of the novel mechanisms in aging [46–48] and of the pathogenesis of a large number of age-related disorders, including Alzheimer's disease, Parkinson's disease [48–51], cataractogenesis [52, 53], and cancer [54], which were chosen here by this author from among such diseases because the others have not been sufficiently clarified to treat in this instance (Table 1). The decreased efficiency of the autophagic system with age has gained renewed attention. The contents of an increasing number of reports stipulate clues for defective autophagy in the pathogenesis of different age-related diseases. The accumulation of autophagic vesicles and inclusion bodies (protein aggregates or aggresomes) is commonly observed in many protein-conformation disorders and consequent age-related pathologies. In all these disorders, insoluble oligomeric complexes of misfolded or unfolded proteins accumulate in the cytosol as aggregates [55].

Table 1 Protein redox regulation mechanisms in age-related disorders

Diseases	Redox regulation mechanisms
Alzheimer's disease	*Thiol-based repair systems:* Msr and Trx *Ubiquitin-dependent degradation pathway and SUMO*
Parkinson's disease	*Thiol-based repair system:* Msr *Ubiquitin-dependent degradation pathway and SUMO*
Cataracts	*Thiol-based repair systems:* Glutathione, NADPH-dependent Trx system, and thioltransferase *Ubiquitin-dependent degradation pathway*
Diabetes	*Thiol-based repair systems:* Glutathione and Trx system *Ubiquitin-dependent degradation pathway and SUMO*

The neurons of the brain cannot be renewed or replaced, and with time irreversible changes occur. These include the accumulation of defective proteins or protein fragments called peptides, and memory is thereby impaired. Alzheimer's disease is characterized by two hallmark pathologic features: neurofibrillary tangles mainly composed of hyperphosphorylated tau protein aggregates and senile plaques containing deposits of amyloid β peptide (Aβ). These proteins are localized within

both the intracellular and extracellular environment [49]. The accumulation of Aβ in senile plaques is one of the main events in the neuropathology of Alzheimer's disease. Aβ is a peptide derived from the β-amyloid precursor protein (βAPP) by proteolytic cleavage [49]. On the other hand, Parkinson's disease involves a selective loss of dopaminergic neurons from the substantia nigra [56]. The postmortem brains of Parkinson's disease patients are characterized by reduced activity of mitochondrial complex I, an enzyme of the mitochondrial electron transport chain. In turn, this defect may cause a "leakage" of electrons from mitochondria, leading to the accumulation of oxidatively damaged proteins [51, 57]. Dopaminergic neurons also show evidence of impaired proteasomal function (increased oxidative stress and decreased elimination of oxidatively damaged polypeptides). Surviving neurons in the brains of Parkinson's disease patients contain Lewy bodies, which are cytosolic inclusions enriched with aggregated forms of the presynaptic protein α-synuclein. Oxidative stress may play a role in α-synuclein neurotoxicity in two ways: First, oxidative modifications promote the formation of α-synuclein oligomers but not mature fibrils. Second, aggregated forms of α-synuclein may cause an accumulation of ROS, thereby triggering a vicious cycle [51, 58].

Lenses are subject to age-related changes simply because there is no mechanism to replace their protein molecules. The lens is mainly made up of specialized proteins known as crystallins and is constantly subjected to oxidative stress due to environmental radiation and other sources. Cataracts are a common pathologic abnormality of the lens characterized by the loss of lens transparency. They are the leading cause of blindness worldwide and of undue financial burden in the developing countries. The lens has several mechanisms to protect its components from ROS and to maintain its redox state, including enzymatic pathways and high concentrations of ascorbate and GSH [53]. With aging, accumulation of oxidized lens components and decreased efficiency of antioxidant and repair mechanisms (GSH peroxidase, GSH reductase, thioltransferase, and Trx) can contribute to lens opacities or cataracts [52].

4.1 Role of Thiol-Based Repair Systems

Trx plays an essential role in cell function by limiting oxidative stress directly with antioxidant effects and indirectly with protein–protein interactions. Increased experimental evidence demonstrates that, in mammals, cellular redox regulation of many processes is provided by an interaction of the Trx and GSH systems. In fact, Trx and GSH systems are involved in a variety of redox-dependent pathways such as supplying reducing equivalents for ribonucleotide reductase, the first step in DNA biosynthesis, and peptide Msr, an enzyme involved in antioxidant defenses and the regulation of cellular redox state. Together, they form a powerful system controlling signal transduction, protection against oxidative protein damage, as well as regulation of the redox state of the extracellular environment [50].

4.1.1 In Alzheimer's Disease

Previous studies have shown that the pathophysiology of Alzheimer's disease is linked to decreased activity in the peptide Msr. A decline in peptide MsrA activity could reduce antioxidant defenses and increase the oxidation rate of critical neuronal proteins [26, 59]. On the other hand, Trx is a crucial protein for antioxidative defense mechanisms, as well as a redox regulator of the intracellular and extracellular signaling pathways and the transcription factors [60]. Both in vivo and in vitro studies demonstrate that Trx and Trx reductase have protective roles against neurotoxicity mediated by the generation of ROS. The decrease in Trx may contribute to the increased oxidative stress and subsequent neurodegeneration observed in the brains of Alzheimer's disease patients. In contrast with low Trx protein levels, thioredoxin reductase activity was significantly elevated in the amygdala and cerebellum of Alzheimer's disease brain. It is likely that the expression of the Trx cycle enzymes must be tightly regulated to maintain optimal neuronal function and to mount appropriate defenses in response to oxidative stress conditions. Based on the evidence of neuroprotective effects of Trx, upregulation of Trx may be an effective strategy for the prevention and treatment of Alzheimer's disease [50, 60, 61].

4.1.2 In Parkinson's Disease

MsrA is present throughout the brain, including the substantia nigra. Cells with increased or decreased levels of MsrA are relatively vulnerable but resistant to oxidative attacks, respectively. MsrA protects cells from oxidative stress not only by repairing proteins damaged by methionine oxidation but also by engaging the cycle of methionine oxidation and reduction that ultimately results in ROS scavenging. The findings of the recent study by Liu et al. indicate that MsrA protects dopaminergic neurons from the toxic effects of complex I inhibition and synuclein expression [51].

Because soluble α-synuclein lacks Trp and Cys residues, mild oxidation of α-synuclein in vitro with hydrogen peroxide selectively converts all four methionine residues to the corresponding sulfoxides. Both oxidized and nonoxidized α-synucleins have similar unfolded conformations; however, the fibrillation of α-synuclein at physiological pH is completely inhibited by methionine oxidation. Furthermore, the Met-oxidized protein also inhibits fibrillation of unmodified α-synuclein. The degree of inhibition of fibrillation by Met-oxidized α-synuclein is proportional to the number of oxidized methionine residues [62].

4.1.3 In Cataracts

Oxidative protein damage has been observed in cataractous lenses. The lens depends on a balanced redox state for maintaining its transparency. A large percentage of lens proteins are the structural proteins called crystallins, which contain a high level of free thiol (–SH) groups that are in a necessarily reduced state to maintain clarity of the lens [52, 53]. The high content of GSH in the lens plays a vital role as

the first line of defense and is believed to protect –SH groups in structural proteins and enzymes for optimum biological function. The second line of defense for the health of the lens is its intrinsic repair enzymes that constantly dethiolate the protein–thiol mixed disulfide (protein thiolation) or protein–protein disulfides induced by oxidative stress so that lens proteins gain their –SH groups again, thus restoring lens protein and enzyme functions and activities. Protein–thiol mixed disulfides exist in various forms such as protein-S-S-glutathione (PSSG), protein-S-S-cysteine (PSSC), and protein-S-S-γ-glutamylcysteine. Repair enzymes – NADPH-dependent Trx system and thioltransferase can dethiolate protein disulfides and thus are extremely important regulators for redox homeostasis in the lens [52].

4.1.4 In Diabetes

Aging is associated with impaired insulin activity, which may lead to alterations in energy homeostasis and increasing blood glucose levels in the elderly.

Signal transduction mechanisms of mammalian insulin and insulin-like growth factor (IGF-1) have a major role in the control of longevity. Studies of physiologic characteristics and polymorphisms of insulin-related genes in exceptionally long-lived people suggest a role of insulin signaling in the control of human aging [63]. Oka et al. recently reported that Trx binding protein-2/Trx interacting-protein couple is a critical regulator of insulin secretion [64].

The higher glycation rate in diabetic individuals is undoubtedly related to the fact that diabetes greatly resembles accelerated aging. Glycation, thiol oxidation, and aggregation of lens crystalline proteins show parallel changes in streptozotocin-diabetic and aging rats [65]. Disturbances of thiol-related homeostatic mechanisms such as GSH/glutathione disulfide have been observed in both diabetes [66] and aging [31, 67, 68]. On the other hand, it is well known that Trx contributes to the regulation of glucose metabolism and glucose transporter-1 (Glut1) expression [69]. A tangible link between Trx and glucose metabolism is the effect of Trx on mitochondrial membrane potential [70]. In diabetes, glucose toxicity affects different organ systems, including pancreatic islets where it leads to deterioration of beta-cell function during the progression of diabetes via oxidative stress, but the exact molecular mechanisms are not fully understood. Recently, it has been reported that Trx is overexpressed in pancreatic beta-cells and can protect beta cells from destruction in diabetes [71].

4.2 The Roles of Proteasome, Ubiquitin, and SUMO

Oxidatively damaged proteins are first recognized by molecular chaperones, which facilitate protein refolding/repairing process. If the oxidative damage is too extensive for repair or cellular metabolism is unfavorable for protein repair, damaged proteins are targeted for degradation pathways. Three major proteolytic systems are responsible for most of the intracellular protein turnover: the lysosomal system, the

ubiquitin-proteasome system, and the SUMO system. A functional ubiquitin proteasome system is essential for all eukaryotic cells, and any alteration in its components has therefore potential pathologic consequences. Thus, age-related proteasomal dysfunction could be regarded as a factor in these disease processes, which involve the formation of plaques, filaments, and aggregates. Once generated, these protein inclusions have been found to further inhibit proteasome activity and thus amplify the formation of inclusion bodies. Although the exact underlying mechanisms are unclear, an age-related decrease in proteasome activity weakens cellular capacity to remove oxidatively modified proteins and favors the development of age-related diseases [7, 55].

4.2.1 In Alzheimer's Disease

The ubiquitin-dependent degradation pathway plays an important role in the modulation of levels of short-lived regulatory proteins and in the removal of abnormal or damaged neuronal proteins [7, 49, 55]. A growing body of evidence suggests that the ubiquitin-dependent degradation pathway may be altered in brains affected by Alzheimer's disease in several ways. βAPP-soluble isoforms have been shown to be degraded after ubiquitin tagging, and Aβ itself seems to bind to the 20S proteasome and inhibit its chymotrypsin-like activity. On the other hand, β-peptides inhibit the proteolytic activities of the 26S proteasome. When the proteolytic activity of the 26S proteasome is inhibited with lactacystin, there is a marked decrease in Aβ degradation, suggesting that the peptide, in both astrocytes and neurons, could be a possible substrate for this enzymatic complex [49]. On the other hand, Cecarini et al. reported that proteasomes from Alzheimer's disease brain exhibited increases in protein carbonyl groups, 4-hydroxynonenal conjugation, and neuroprostane conjugation [72]. Together, these data confirm that impairment in the function of purified proteasomes occurs in the earliest stages of Alzheimer's disease in the brain and directly support a role for oxidative inactivation contributing to declines in proteasome function in Alzheimer's disease.

The importance of sumoylation in this analogous modification is becoming increasingly apparent in age-related neurodegenerative pathologies [40, 73–77]. Sumoylation is gaining growing interest in aging and age-related disorders in the scientific literature. Many substrates for sumoylation have been identified, and the number is still expanding. Among them, βAPP and the microtubule-associated protein tau-protein are particularly interesting. Sumoylation of tau protein is supposed to be implicated in the pathologic process of Alzheimer's disease in the brain and tauopathies from cell-culture experiments [40, 73, 76]. Sumoylation of protein domains that are exposed to the lumen of the endoplasmic reticulum and other compartments of the secretory pathway had been previously reported by Zhang and Sarge [76]. Their results also provide the first demonstration that the SUMO E2 enzyme is present within the endoplasmic reticulum, indicating how βAPP and other proteins enter this compartment. On the other hand, Takahashi et al. have examined the relationship between hyperphosphorylated tau protein and SUMO protein in the brains of transgenic mice [40]. In this study, authors reported that SUMO-1

immunoreactivity is observed in phosphorylated tau aggregates in βAPP transgenic mice, an Alzheimer's disease model.

4.2.2 In Parkinson's Disease

The brains of Parkinson's disease patients show evidence of impaired proteasomal function, a defect resulting in increased oxidative stress and decreased elimination of oxidatively damaged polypeptides. Dopaminergic neurons of the substantia nigra contain relatively high basal levels of ROS, resulting from dopamine metabolism and auto-oxidation. Therefore, these neurons may be selectively vulnerable to assault that increases oxidative stress in Parkinson's disease brain, including complex I inhibition and proteasome impairment [51, 57].

DJ-1 is a multifunctional protein that plays roles in transcriptional regulation and against oxidative stress, and loss of its function is thought to result in the onset of Parkinson's disease. The condition of patients carrying DJ-1 mutations demonstrate reduced dopamine uptake indistinguishable from that of patients with sporadic Parkinson's disease. Consistent with the role of DJ-1 in transcriptional regulation, several protein interaction studies suggest a potential functional link between DJ-1 and SUMO. DJ-1 interacts with SUMO-1, SUMO-2, and SUMO E3 ligases. However, the functional significance of the link between sumoylation and DJ-1 and the potential relevance to the pathogenesis of Parkinson's disease is still unclear as in other contexts related to sumoylation [73–75, 77].

4.2.3 In Cataracts

Age-related decreasing proteasome content and peptidase activities is associated with the formation of cataracts [78, 79]. Murakami et al. [78] were first to indicate that the lens proteasomes can degrade mildly photo-oxidized lens proteins, but proteins that are extensively damaged are not degraded but may accumulate. In particular, increasing levels of carboxymethylation were observed with age in the proteasome. Viteri et al. concluded that the lower levels of soluble active enzymatic complex present in lenses of the elderly and the posttranslational modifications affecting the proteasome may at least partly explain the decrease in proteasome activity. The concomitant accumulation of carboxymethylated and ubiquitinated proteins occurs with the aging process [79].

4.2.4 In Diabetes

Diabetes-induced oxidative stress can lead to protein misfolding and degradation by the ubiquitin-proteasome system [80]. Exposure to chronic high glucose induces oxidative stress and causes an increase in ubiquitin-protein aggregates in β-cells. Autophagy delivers the ubiquitin-protein aggregates to the lysosome for its degradation [80]. Glucose-dependent insulinotropic polypeptide (GIP) is a gastrointestinal hormone that is a potent stimulator of insulin release under normoglycemic conditions. However, its insulinotropic effect is reduced or even absent entirely in type

2 diabetic patients. Interference with the binding of GIP to its receptor (GIP-R) results in impairment of insulin secretion and variable degrees of glucose intolerance. GIP-R is continuously degraded when islets are exposed to high glucose for a long period. The results of Zhou et al. suggest that the GIP-R is ubiquitated, resulting in downregulation of the actions of GIP [81].

In contrast with polyubiquitinylation that targets modified proteins for the proteasome degradation pathway, the biological consequences of sumoylation include the increase of protein stability [82]. Aberrant SUMO regulation is a likely cause of a variety of human disease including diabetes [83, 84]. SUMO-4 has recently been cloned in an attempt to identify genes susceptible to human type 1 diabetes mellitus. SUMO-4 expression is primarily restricted in pancreatic islets, immune tissues, and kidneys. Further extensive investigation into these SUMO-4 target proteins is expected to lead to better understanding of the mechanisms underlying the role of SUMO-4 in the pathogenesis of diabetes. Furthermore, proteins that regulate glucose levels in the blood are also regulated by the sumoylation process. Sumoylation promotes the membrane accumulation of GLUT-4, presumably by enhancing the protein stability and facilitating its trafficking [84].

How is the SUMO signaling deregulated in age-related disorders and diabetes? Studies in the years to come will certainly generate exciting answers to many of these questions.

5 Concluding Remarks

It seems clear that most of the effects of ROS in aging cells are related to signaling pathways and redox regulation mechanisms rather than to nonspecific damage of proteins. In fact, the relation of molecular events, such as the control of protein-redox regulation mechanisms, with intracellular signaling pathways are still under extensive investigation. Molecular mechanisms controlling the redox-regulation systems related to proteins and variation in the regulation of these controlling systems can be expected to contribute to the susceptibility of postmitotic tissues to oxidative stress during aging and disease. As the redox regulation mechanisms in aging and age-related disorders become clearer, new therapeutic approaches and prospective solutions are coming into view. A major research and development effort is required to bring forth novel therapies as related to redox regulation of proteins and make these available to the aging population.

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Nitrosative Stress in Aging – Its Importance and Biological Implications in NF-κB Signaling

Predrag Ljubuncic, Einat Gochman, and Abraham Z. Reznick

Abstract The free radical theory of aging is supported by the findings that overexpression of CuZn superoxide dismutase (SOD) and catalase or the enhancement of reductive capacity by overexpression of glucose-6-phosphate dehydrogenase extends the life span of *Drosophila melanogaster*. Furthermore, treatment with small-molecule antioxidant SOD mimetics, which successfully increases the life span in multicellular models of aging such as *Drosophila melanogaster* and *Caenorhabditis elegans*, gave additional needed credibility to the free radical theory of aging. Also, nitration of proteins by reactive nitrogen species and 4-hydroxy-2-nonenal production by lipid peroxidation were found to increase with age, lending additional support to the oxidative stress hypothesis of aging. A number of research groups have reported alterations in transcriptional activity of nuclear factor kappa B (NF-κB) outside the immune system that was characterized by striking increases in constitutive NF-κB activity in different tissues of aging humans and animals. On the other hand, reactive nitrogen species are signaling molecules that modulate NF-κB activity. In this way, the concept of nitrosative stress has emerged from an understanding that interactions between nitrosants and oxidants may produce products that are more toxic than either reactants alone. Thus, the proinflammatory peroxynitrite molecule presents such a unifying link between reactive oxygen species and reactive nitrogen species by virtue of its generation from the reaction between superoxide, a reactive oxygen species, and nitric oxide, a reactive nitrogen species. Importantly, peroxynitrite effects on NF-κB signaling can be considered in light of the relation that exists between molecular inflammation and nitrosative stress and with the aging process itself. This will reflect on the pro-aging signaling of redox-sensitive NF-κB and on the development of age-related diseases.

Keywords Aging · Peroxynitrite · Nitrosative stress · Nuclear factor kappa B · Age-related diseases

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1 Introduction

1.1 The Oxidative Stress Hypothesis of Aging – Historical Account

In the mid-1950s, Denham Harman, M.D., Ph.D., scientist from the Donner Laboratory of Biophysics and Medical Physics, University of California, Berkeley, proposed in his insightful article "Aging: A Theory Based on Free Radical and Radiation Chemistry" that the damaging effects of reactive oxygen species (ROS) such as superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) may play a key role in the mechanism of aging [1]. He wrote "it seems possible that one factor in aging may be related to deleterious side attacks of free radicals (which are normally produced in the course of cellular metabolism) on cell constituents" [1]. He proposed that oxygen free radicals, specifically hydroxyl and hydroperoxyl radicals, are formed endogenously from normal oxygen-utilizing metabolic processes and play an essential role in the aging process. He concluded, "Aging and the degenerative diseases associated with it are attributed basically to the deleterious side attacks of free radicals on cell constituents and on the connective tissues. The free radicals probably arise largely through reactions involving molecular oxygen catalyzed in the cell by the oxidative enzymes and in the connective tissues by traces of metals such as iron, cobalt, and manganese" [1]. At first incomplete, this theory became more attractive in concert with the discoveries of free radicals by Commoner and co-workers [2, 3] and findings that erythrocuprein is an antioxidant superoxide-radical dismutating CuZn superoxide dismutase (CuZn-SOD) enzyme, which is a protector of oxygen-metabolizing organisms in concert with Mn-SOD [4]. Also, the in vivo presence of hydrogen peroxide was documented [5]. Furthermore, the fact that all aerobic organisms containing cytochrome systems have both SOD and catalase [6, 7] and that their overexpression or treatment with small-molecule antioxidant SOD mimetics successfully increases life span in standard multicellular models of aging such as *Drosophila melanogaster* [8, 9] and *Caenorhabditis elegans* [10] or murine life-span [11] strongly contributed to the validity of the free radical theory of aging. Additionally, the finding that enhancement of reductive capacity by overexpression of glucose-6-phosphate dehydrogenase (G6PD), a key cytosolic enzyme for NADPH biosynthesis that is susceptible to oxidative damage, could protect against oxidative stress and extend the life span of transgenic *D. melanogaster* [12] strongly supported the oxidative stress hypothesis of aging and gave the free radical theory of aging a needed credibility. In addition, Harman later proposed his modified mitochondrial theory of aging [13], which was based on the fact that the mitochondria generate large amounts of ROS in cells. Generally, the results from the more correlative genetic studies of distantly related species such as *C. elegans, D. melanogaster*, and mice and studies "from yeast to men" support the free radical hypothesis of aging. The evidence included claims that:

1. Variation in species life span is correlated with metabolic rate and protective antioxidant activity.
2. Enhanced expression of antioxidative enzymes in experimental animals can produce a significant increase in longevity.

3. Cellular levels of free radical damage increase with age.
4. Reduced calorie intake leads to a decline in the production of reactive oxygen species and an increase in life span [14–20]. Also, the free radical theory may also be used to explain many of the structural features that develop with aging including protein oxidation, the lipid peroxidation of membranes, formation of age pigments, and cross-linkage of proteins, DNA damage, and decline of mitochondrial function.

Generally, it is agreed that there is a correlation between aging and the accumulation of oxidatively damaged proteins, lipids, and nucleic acids. Protein carbonyls, 8-oxo-2′-deoxyguanosine, acrolein, or 4-hydroxy-2-nonenal (4-HNE), are well established biomarkers of protein, DNA, and lipid oxidation, respectively. Basic principles that govern the oxidation of proteins by ROS were established in several pioneering studies that characterized reaction products formed by performing studies in which proteins were exposed to ionizing radiation under conditions where only hydroxyl radical ($OH^{\bullet}$), superoxide anion (O_2^{-}), or a mixture of both was made available [21–24].

Oxidatively modified proteins have been shown to increase as a function of age, demonstrated by an age-related increase in the level of protein carbonyl content, oxidized methionine, protein hydrophobicity, and cross-linked and glycated proteins, as well as the accumulation of less active enzymes that are more susceptible to heat inactivation and proteolytic degradation [25]. For the most part, oxidatively modified proteins are not repaired and must be removed by proteolytic degradation, and a decrease in the efficiency of proteolysis will cause an increase in the cellular content of oxidatively modified proteins. The carbonyl content of proteins increases almost exponentially in widely different animal species and tissues as a function of animal age. Within the protein molecule, both the peptide bond or side chain may be targeted by free radicals in a site-specific fashion, and these reactions are often influenced by redox metal cycling cations, such as iron copper [26]. Protein carbonylation may occur due to direct oxidation of amino acid side chains (e.g., proline and arginine to γ-glutamyl semialdehyde, lysine to amino adipic semialdehyde, and threonine to aminoketobutyrate) [27]. It can also be induced through the interaction of proteins with oxidative by-products of other molecules, such as lipid peroxidation derivatives like 4-HNE and malondialdehyde (MDA) [26, 28]. Collectively, oxidation of amino acid residue side chains, formation of protein–protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation can be observed.

It should also be noted that specific proteins may be particularly susceptible to oxidative damage exemplified by key mitochondrial enzymes adenine nucleotide translocase and aconitase [29]. The oxidative modifications of key metabolic enzymes such as aconitase and its role in the citric acid cycle may have a profound effect on the cellular burden of oxidatively modified proteins. Of particular interest is oxidation of methionine residues by ROS to methionine sulfoxide (MetO). The oxidation of Met residues is readily reversed by the action of methionine sulfoxide reductase (Msr), which catalyzes the thioredoxin-dependent reduction of MetO residues of proteins back to Met. This cyclic interconversion of Met and

MetO residues of proteins is an important antioxidant mechanism for the scavenging of ROS and is likely involved in the regulation of enzyme activities and cell signaling and can target proteins for proteolytic degradation [30]. Furthermore, a loss in the ability to catalyze the reduction of protein MetO to Met residues leads to a decrease in the maximum life span, whereas overexpression of this activity leads to an increase in the life span of animals. In addition, a decrease in Msr activities in brain tissues is associated with the development of Alzheimer's disease [30].

The levels of oxidatively modified proteins increases with age, not only in whole cell but also in mitochondrial fractions, and this change correlates with a decline in the intracellular ATP level [31]. Also, age-related decline in the ATP level reduces the cell capacity to induce apoptosis and promotes necrotic inflammation. This switch may trigger a number of age-dependent disorders. A number of age-related diseases and pathologic disorders have been shown to be associated with elevated levels of oxidatively modified proteins including amyotrophic lateral sclerosis, respiratory distress syndrome, muscular dystrophy, cataractogenesis, rheumatoid arthritis, progeria, and Werner's syndrome [24]. Also, oxidative modification of proteins has been implicated in atherosclerosis, diabetes, Parkinson's disease, essential hypertension, cystic fibrosis, cancer, arteriosclerosis, rheumatoid arthritis, lupus erythematosus, chronic inflammatory diseases of the gastrointestinal tract, cataract, diabetes, diabetic retinopathy, Parkinson's disease, Alzheimer's disease, as well as aging itself [32, 33].

Oxidation of proteins can be induced by direct oxidation of proteins by ROS as well as by indirect modification of proteins by the secondary by-products of oxidative stress. The secondary modifications include oxidatively modified carbohydrates and lipids, which may react with the proteins by cross-linking. The most abundant lipid peroxidation products are MDA and HNE, which when cross-linking with proteins may have inactivation effects on these molecules [34, 35]. Proteins can also be damaged by glycation, also called Maillard reaction, or nonenzymatic glycosylation resulting in reducing sugars becoming chemically attached to proteins. The reaction occurs through the formation of Schiff base (i.e., an imine double bond between the aldehyde group of glucose and the epsilon amino group of lysine residues in proteins). The imine can quickly rearrange to form a ketoamine and is called an Amadori product. The Amadori products can be oxidized to form advanced glycation end products, and the formation of advanced glycation end products is irreversible [36]. Both reactive carbonyl compounds (RCCs) formed during lipid peroxidation and sugar glycoxidation, namely advanced lipid peroxidation end products (ALEs) and advanced glycation end products (AGEs), accumulate with aging and oxidative stress–related diseases, such as atherosclerosis, diabetes, or neurodegenerative diseases [37]. RCCs induce the "carbonyl stress" characterized by the formation of adducts and cross-links on proteins, which progressively leads to impaired protein function and damage in all tissues, and pathologic consequences including cell dysfunction, inflammatory response, and apoptosis. Because lipids are a major component of living organisms and probably the first easy target of free radicals once they are produced, lipid peroxidation

might play an important role in initiating and/or mediating some aspects of the aging process. It has been widely demonstrated that there is an age-associated increase in the steady-state concentrations of lipid peroxidation products [38]. In that way, proteomic techniques prove that many serum proteins are modified by HNE as well as by the reactive proinflammatory reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$). Importantly, nitration and HNE adduction were found to increase with age, lending additional support to the oxidative stress hypothesis of aging [39].

To summarize, the link between oxidative stress and aging has been indicated by the fact that aging is associated with accumulation of oxidized forms of protein [40], nucleic acids [41], and lipids [42], and also by the fact that there is an inverse relationship between the maximum life span of organisms and the age-related accumulation of oxidative damage [40, 43, 44]. Protein carbonyls, 8-oxo-2′-deoxyguanosine, acrolein, or 4-HNE, are well established biomarkers of protein, DNA, and lipid oxidation, respectively. The intracellular level of protein carbonyl has become one of the most widely accepted measurements of oxidative stress–dependent cellular damage [45].

1.2 The Concept of Nitrosative Stress

As previously mentioned, oxidative stress can also be caused by RNS. The concept of nitrosative stress has emerged from an understanding that interactions between nitrosants and oxidants may produce products that are more toxic than either reactant alone. In other instances, nitrosative mechanisms of cellular injury may predominate [46, 47]. Under such conditions, nitrosylation may directly inhibit critical protein functions [47, 48] and/or promote deleterious oxidative modifications [46]. At the cellular level, nitric oxide (NO) has been widely implicated in nitrosative stress, which was linked to inhibition of cell growth and apoptosis [49].

Higher concentrations of oxidative species promote the conversion of NO to higher oxide forms, such as nitrogen dioxide and peroxynitrite. One consequence of the production of such species is the formation of nitrotyrosine [50]. Peroxynitrite is a highly reactive molecule that induces many changes in proteins by oxidizing the sulfhydryl groups of cysteine and methionine as well as tryptophan residues and selectively nitrating tyrosine residues [51, 52]. The detection of nitrotyrosine (NO_2-Tyr) formation in various inflamed tissues and during the process of aging is recognized as a peroxynitrite-triggered mechanism of nitrosative injury [53–58].

Peroxynitrite can cause cell necrosis as well. This process is mediated by a complex process involving activation of the DNA repair enzyme poly(ADP-ribose) polymerase-1 (PARP-1). Activated PARP consumes NAD to build up poly(ADP-ribose) (PAR) polymers, which are themselves rapidly metabolized by the activity of poly(ADP-ribose) glycohydrolase (PARG). Some free PAR may exit the nucleus and travel to the mitochondria, where they amplify the mitochondrial efflux of

apoptosis-inducing factor (AIF) – nuclear to mitochondria cross-talk. Mild damage to DNA activates the DNA repair machinery, whereas excessive oxidative and nitrosative stress–induced DNA damage ends in apoptosis in case of moderate permeability transition pore (PTP) opening and PARP activation with preservation of cellular ATP or in necrosis in the case of widespread PTP opening and PARP overactivation, leading to massive NAD consumption and collapse of cellular ATP [59]. The peroxynitrite-PARP pathway is relevant in a wide variety of disparate diseases, ranging from myocardial ischemia/reperfusion injury, myocarditis, heart failure, circulatory shock, and diabetic complications to atherosclerosis, arthritis, colitis, and neurodegenerative disorders [60].

Consequently, besides presenting the historical background to the development of the free radical theory of aging and the effects of the oxidative stress caused by the ROS, we aim to present here the role of oxidative stress caused by RNS, particularly the role of proinflammatory peroxynitrite. By virtue of its origin, which reflects a change in redox state homeostasis and which results in its generation from the reaction between superoxide, ROS, and nitric oxide, the RNS peroxynitrite molecule presents a unifying link between ROS and RNS. We will also discuss its effects in light of the relation that exists between molecular inflammation and oxidative stress in aging and pro-aging signaling of redox-sensitive nuclear factor kappa B (NF-κB). We will also relate NF-κB signaling to different age-related diseases with emphasis on loss of muscle mass in old age. We will discuss the importance and clinical implications of NF-κB signaling in muscle loss having in mind that muscle mass comprises about 40% of total body weight in an average adult person. Conclusions and perspectives will also be presented.

2 Reactive Nitrogen Species and Nitrosative Stress

2.1 First Come Definitions

Nitration – Attachment of an $–NO_2$ (nitro) group to a compound. Usually, it is not easily reversible.

Nitrosation – A process converting compounds into nitroso derivatives containing R-NO (nitroso) functionality. We can recognize C, N, O, and S nitrosations in biological systems.

Nitrosylation – The attachment of an NO (nitroso, nitrosyl) group to a thiol (S-nitrosylation) or a metal. It is usually reversible.

2.1.1 "Nitrosation" and "Nitrosylation" – It Obviously Needs Some Clarification

From the chemical point of view, "nitrosation" means the addition of a nitroso group (i.e., the NO diatomic group). "Nitrosylation" means the addition of a nitrosyl group NO, stressing the concept of the addition of a chemical group that, if it were free,

would be a radical (in analogy to other chemical additions containing the "-yl-" particle: acetylation, phosphorylation, etc.).

The fact is that in the nitric oxide species, both atomic groups and the radicals are the same, as the radicals themselves are relatively stable. Even so, some authors prefer to distinguish the incorporation of the NO radical to a metal by a complex (or coordinating bond) as "nitrosylation" and the covalent incorporation of an NO diatomic group to another chemical group (regardless of the reaction mechanism) as "nitrosation" (in accordance with the nomenclature, which uses the term "nitroso" when defining the names of the resulting compounds). Also, the increasing recognition of the functional importance of this posttranslational modification and the widespread inclusion of the "-yl-" particle in terms describing other posttranslational modifications (glycosylation, phosphorylation) has led pioneer investigators in the field to make a case for the use of nitrosylation both for thiols and metals [61, 62]. In any case, incorporation to a thiol can be clearly distinguished because of the prefix "S-," referring to the incorporation of the NO moiety to a sulfur atom to form the S–NO bond: "S-nitrosation" or "S-nitrosylation."

2.2 Reactive Nitrogen Species – Chemistry and Availability

The family of nitric oxide synthase (NOS) isoenzymes [endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS)] [63, 64] catalyze the five-electron oxidation of one Nω-atom of the guanidino L-arginine to produce NO and L-citrulline through cofactors including NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH4) [65]. The NOS isoforms are denoted by descriptive terms, based on the requirement of intracellular calcium transients for full activity. Constitutive NOS enzymes, such as eNOS and nNOS, are activated by a transitory increase generally in cytosolic calcium, which promotes the release of NO over several minutes. A cytokine-inducible NOS isoform is expressed in many cells including macrophages and hepatocytes after the stimulation of immunologic or inflammatory reactions. This produces large amounts of NO for several days [66, 67].

The physiologic actions of NO at its nanomolar concentrations include regulation of vascular tone and blood pressure, prevention of platelet aggregation, and inhibition of vascular smooth muscle proliferation. They are a result of the activation by NO of the soluble guanylate cyclase and consequent generation of cyclic guanosine monophosphate (cGMP). In contrast, inflammatory cells such as macrophages produce local concentrations of nitrogen monoxide that are two or three orders of magnitude higher than its nanomolar concentrations [68]. The biological lifetime of nitrogen monoxide is close to 5 s, and during this time it can diffuse over several cell diameters and thereby carry out its function as an intracellular as well as extracellular messenger [69].

When produced in the presence of appropriate reactive targets, NO can be readily converted into other nitrogen oxide moieties. For example, one of the reactive

targets of NO is the cytochrome c oxidase, the terminal enzyme in the electron transport chain, which is inhibited by NO in a manner that is reversible and competitive with oxygen. The consequent reduction of cytochrome c oxidase activity leads to the release of superoxide anion, which under certain circumstances may react with NO to form another RNS species, the peroxynitrite (ONOO) [70]. In that way, the concept of "reactive exposure" was introduced by Joseph Beckman with his consideration of the formation of peroxynitrite from the reaction of NO with superoxide. According to the proposed principle [71], the relative amount of a compound (e.g., NO) that reacts with its respective targets is determined by the relative concentrations of those targets and the reaction rates of the compound with each of them. For example, the reaction of NO with superoxide is high ($\sim 10^{10}$ μM/s), but the physiologic concentration is low (~0.1–1 nM) [71]. However, because the reaction rate for NO with superoxide is high, even a small increase in the concentration of this reactant will result in a large increase in reactive exposure; a 1 nM increase would lead to a 10-fold increase in reactive exposure [72]. Given that the rate constant for reaction of NO with superoxide is higher than that for reaction of superoxide with any of the three SOD isoforms, peroxynitrite will be formed in any cell or tissue where both radicals exist simultaneously [73].

2.3 Nitrosylation and Nitration Are Mediators of Cell Signaling

2.3.1 Nitrosylation

In addition to the mediation of cytotoxicity, the RNS serve as important mediators and intracellular signaling molecules [74, 75]. NO can affect the cellular functions through posttranslational modifications of proteins directly (i.e., nitrosylation and nitration) and indirectly (i.e. methylation and ribosylation). The list of cGMP-independent effects of NO is growing at a rapid rate in relation to the importance and relevance of nitrotyrosine formation [76, 77]. For example, the inhibition of mitochondrial complex I–mediated respiration was demonstrated in cells after incubation with activated macrophages [78]. This inhibition of complex I activity was found to be due to NO [79]. S-Nitrosation of the reactive thiols on the surface of proton-translocating NADH:ubiquinone oxidoreductase (mitochondrial complex I), which is responsible for oxidation of matrix NADH and presents the major entry point for electrons to the respiratory chain, has been proposed to play a significant role in disorders such as Parkinson's disease and sepsis [80]. Also, S-nitrosation of insulin receptor has been proposed as a mechanism of insulin resistance in diabetes [81]. Within mammalian tissues, the concentration of S-nitrosothiols can vary from nanomolar to micromolar levels [82, 83], and thiol S-nitrosylation and NO transfer reactions (transnitrosation reactions) are involved in virtually all classes of cell signaling, including apoptotic cell death pathway. The work of Moran Benhar and Jonathan S. Stamler [84] revealed a key signal transduction pathway through which nitric oxide regulates apoptosis induced by disparate cellular stresses: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is S-nitrosylated

by NO, which initiates an interaction with the E3 ligase Siah1, leading to nuclear translocation and ubiquitin-mediated degradation of nuclear target proteins [84]. In another example, S-nitrosylation of two key apoptosis-regulatory proteins of the intrinsic and extrinsic death pathways, namely B-cell lymphoma-2 (Bcl-2) and FLICE-inhibitory protein (FLIP), has also been described [85]. These proteins have been shown to be upregulated in a variety of tumors and have been implicated in cancer chemoresistance through dysregulation of apoptosis. S-Nitrosylation of these proteins precludes their ubiquitination and subsequent degradation by the proteasome, thus accentuating their antiapoptotic effect, which is critical in the context of tumorigenic potential and cancer progression. Such posttranslational modifications of proteins by NO may be a general mechanism that tumor cells exploit to tilt the scales toward survival and proliferation by evading cell death.

2.3.2 Nitration

A balanced analysis of existing evidence indicates that (a) different nitration pathways can contribute to tyrosine nitration in vivo and (b) most, if not all, nitration pathways involve free radical biochemistry with carbonate radicals ($CO_3^{\bullet-}$) and/or oxo-metal complexes oxidizing tyrosine to tyrosyl radical followed by the diffusion-controlled reaction with $\bullet NO_2$ to yield 3-nitrotyrosine [86]. Peroxynitrite-mediated tyrosine nitration plays a key role in inflammation and pain. Nitration can be focused on specific tyrosine residues in proteins and potentially results in modification, loss, or gain of function.

Protein tyrosine nitration has three major effects: It may affect protein function, modulate phosphorylation cascades, and induce an immunologic response. The nitration of tyrosine residues was considered already 10 years ago as being of particular importance as nitration precludes the ability of tyrosine residues to undergo cyclic introversions between phosphorylated and unphosphorylated forms [87] or between nucleotidylated and unmodified forms [88]. To be considered a cellular signaling mechanism, protein nitration must meet four basic criteria: (1) controlled rates of formation, (2) specificity, (3) modification of target protein and cell function, and (4) reversibility. The specificity of protein nitration and modification of protein and cell functions by protein nitration have been demonstrated; it has also been suggested that protein nitration by 3-nitrotyrosine (3-NT) can be a reversible process [89, 90], and 3-NT may disappear without the need for a proteolytic pathway similar to what is observed for phosphorylation–dephosphorylation or methionine oxidation enzymatic repair. Koeck et al. [91] have shown that rat liver mitochondria are capable of completely eliminating 3-nitrotyrosyl adducts during 20 min of hypoxia–anoxia and of a selective partial reduction after only 5 min. They identified the modified proteins, having verified that all main nitrated proteins before hypoxia–anoxia and after reoxygenation are identical, thus markedly increasing the possibility that nitrotyrosine clearance is indeed a protein denitration process involved in a nitrative-signaling mechanism. Recently, it has been shown that a macrophage lipopolysaccharide (LPS)-inducible denitrase activity is capable

of specifically acting on nitrated calmodulin, a calcium signaling protein, forming native tyrosine calmodulin without the formation of any aminotyrosine [92].

Usually, nitrated proteins are recognized and degraded by the proteasome system. However, tyrosine nitration and dimerization may promote assembly of protein filaments or protein aggregates that become poor proteasome substrates and can accumulate as intracellular or extracellular amyloids. In this way, metabolism of nitrated proteins includes the potential reduction by yet-to-be-established biological reductants or the removal of the nitro group by putative denitrase activities. Although the concept of denitrase activity is attractive, the gene(s) or the specific protein(s) have not yet been identified, and more work is needed to more solidly prove its existence [93].

3 NF-κB, Aging, and Nitrosative Stress

3.1 NF-κB Signaling

The activities of a variety of nuclear regulatory proteins are affected by proinflammatory signals, RNS, S-nitrosylation, and tyrosine nitration chemistry. One of these RNS-sensitive transcription factors is NF-κB [94–96]. Although the transcription factor NF-κB was originally recognized in regulating gene expression in B-cell lymphocytes [97], subsequent investigations have demonstrated that it is one member of a ubiquitously expressed family of Rel-related transcription factors that serve as critical regulators of many genes, including those of proinflammatory cytokines.

In the classic (canonical) pathway of activation, which is the main NF-κB signaling pathway, stimulating cells with an agonist such as tumor necrosis factor-α (TNFα) or interleukin-1β (IL-1β) activates the I kappa B Kinase (IKK) complex, which is composed of two catalytic subunits, IKKα and IKβ, and a regulatory subunit, NEMO (IKKγ). The IKβ, but not IKKα, phosphorylates IκB proteins at two amino-terminal serine residues (Ser-32 and Ser-36) in vivo. This signal-induced phosphorylation targets IκB for ubiquitination and subsequent degradation by the proteasome [98].

After the release from IκB, NF-κB is transported to the nucleus where it binds specifically to κB enhancer elements of DNA and promotes gene transcription by binding to responsive elements in the DNA, called κB motifs [76]. The binding of specific κB moieties sets off target gene transcription that encode cytokines such as interleukin-1 (IL-1), IL-2, IL-6, IL-8, IL-12, and interferon-β and -γ (IFN-β, IFN-γ), TNFα, growth factors, membrane proteins such as major histocompatibility complex classes I and II, intracellular adhesion molecule 1 (ICAM-1), E-selectin, inhibitors of apoptosis (e.g., c-FLIP and Bcl-XL), and iNOS [16, 17]. The iNOS enzyme isoform is usually associated with malignant tissue transformation [99].

A hallmark of the noncanonical (nonclassic) NF-κB pathway is inducible p100 processing, leading to liberation of the mature transcription factor p52 in complex with RelB [100]. Also, one of the manifest characteristics of the noncanonical pathway is the slow kinetics of p100 processing, lasting several hours, which stands

in apparent contrast with that of the canonical pathway in which the process of IκBα degradation occurs within minutes. In addition, the IKK-independent pathway of NF-κB activation has also been recognized; it is based on casein kinase-2 (CK2) activation in response to DNA damage [101, 102]. The operating mechanism includes CK2-induced IκBα degradation through IκBα phosphorylation at a cluster of serine residues contained in its C-terminus [103]. Finally, it is worthwhile to mention that the localization of NF-κB within mitochondria has been reported [104–106].

3.2 NF-κB Signaling Increases in Aging

The NF-κB signaling pathway is a key molecular link between inflammation and tumor initiation and progression and provides a mechanistic link between inflammation and cancer [107, 108]. In addition, NF-κB is implicated in promoting proliferation [109–111], survival [112–116], and angiogenesis [117–119] of a variety of tumors. Importantly, NF-κB is considered the master regulator of innate immunity, which is activated during aging [120]. Accordingly, the NF-κB system induces a proinflammatory condition called "inflamm-aging" [121–123]. Recent studies have revealed that SIRT1 (Sir2 homolog) and FoxO (DAF-16), the key regulators of aging in budding yeast and *C. elegans* models, regulate the efficiency of NF-κB signaling and the level of inflammatory responses [124]. FoxOs are transcription factors, and Sirtuins are the protein deacetylases that regulate the activity of FoxOs and NF-κB [125].

Aging-associated signaling may represent life-span extending, longevity regulation or age-related degenerative, pro-aging signaling. Accordingly, it is proposed that the signaling cascades mediated via Sirtuins and FoxO represent the life-span extending, anti-aging type of regulation. Conversely, NF-κB signaling enhances tissue atrophy and inflammation and supports inflamm-aging [124]. In addition, imposed calorie restriction (CR) is shown to result in the most reproducible end point of life-span extension in all animal models tested [126]. Calorie restriction results in the suppression of NF-κB signaling [127], thus providing evidence that NF-κB signaling may be involved in regulation of aging and longevity [128].

Kumar et al. [129] have listed the genes whose expression is regulated by NF-κB, as well as the major diseases associated with functional changes in the NF-κB system. Most of the diseases are chronic and age-associated degenerative diseases such as atherosclerosis, dementia (both vascular and Alzheimer's types), and cancer. Several serum proinflammatory markers have been shown in relation to dementia and cognitive decline, and inflammatory responses are hypothesized to modulate the pathogenic processes associated with Alzheimer's disease (AD) [130, 131]. In this way, pathology evidence indicates that major, chronic age-related diseases are inflammation-related, and a proposal for the molecular inflammation hypothesis of aging is based on the observation that the redox derangements that occur during aging are the major factor for increased risk for age-related inflammation [132]. Accordingly, continuous (chronic) upregulation of proinflammatory

mediators is induced during the aging process due to an age-related redox imbalance that activates many proinflammatory signaling pathways, including the NF-κB signaling pathway [133, 134]. However, this position of inflamm-aging should not be viewed as being contradictory to the free radical oxidative theories of aging because redox-sensitive NF-κB, the main proinflammatory signaling pathway, is activated by different ROS and RNS, including proinflammatory peroxynitrite, which disturb redox balance. Thus, inflamm-aging and free radical theories of aging should rather be viewed as complementary.

The age-related "constitutive activation of NF-κB" has been verified in various tissues during aging [135–139]. In mice and rats, NF-κB activity has consistently been shown to be increased with age and in a variety of tissues, including heart, liver, kidney, and brain [140]. In humans, NF-κB protein concentrations were found to be fourfold higher in elderly human muscles compared with those of young people [141].

In addition, it was observed in studies performed in rodents that the levels of NF-κB components p52 and p65 were prominently increased in nuclear but not in cytoplasmic fractions in the tissues of old rodents [142]. Such observation may imply that during aging, the retention of NF-κB proteins into the nuclei is increased. It also implies that the efficiency of the NF-κB system is not solely based on the protein levels of the NF-κB components in cytoplasm but rather on the translocation of protein components to nuclei and the efficiency of transcriptional regulation [142]; as well, efficacy of the process of regulatory nuclear polyubiquitination of NF-κB subunits may be changed with aging (see later).

3.3 Nitration of Proteins Increases with Aging – Tyrosine Nitration

Increased nitration of proteins in aging is a well-documented phenomenon, such as observation of increased nitration of serum proteins with age [143] or peroxynitrite-induced senescence and apoptosis of human red blood cells [144]. Importantly, increased nitration is often linked to development of age-related diseases. It is argued that peroxynitrite excess will lead to the disruption of modulation of mitochondrial respiration providing a platform for development of prevalent neurodegenerative and metabolic diseases [145]. For example, it has been revealed that the Lewy bodies, a pathologic hallmark of Parkinson's disease, contain nitrated alpha-synuclein, which is prone to oligomerization. Studies have shown that oxidation and nitration of alpha-synuclein lead to the formation of stable dimers and oligomers through dityrosine cross-linking [146]. In addition, greater nitration of alpha-synuclein was shown to occur in the substantia nigra of 16-month-old rats versus 3-month-old rats, which is accompanied by a higher expression level of inducible nitric oxide synthase [147]. Also, elevated levels of 3-nitrotyrosine were found in brain from subjects with amnestic mild cognitive impairment (MCI), suggesting that nitrosative damage occurs early in the course of cognitive impairment

and that protein nitration may be important for conversion of MCI to AD [148]. Moreover, age-related changes in dopamine transporters and accumulation of 3-nitrotyrosine in rhesus monkey midbrain dopamine neurons were found; these findings are consistent with a role for age-related accumulation of nitrative damage and vulnerability of dopaminergic neurons in Parkinson's disease [149].

Also, there is the appearance of the 3-nitrotyrosine–modified isoform of the endoplasmic/sarcoplasmic reticulum $Ca(2^+)$-$Mg(2^+)$-adenosine triphosphatase (SERCA2) of the sarcoplasmic/endoplasmic reticulum $Ca(2^+)$-ATPase in aging and disease in both striated and smooth muscle of humans and rodent models [150]. Structure–function studies of nitrated SERCA2 in aging heart and skeletal muscle demonstrated stoichiometric nitration of vicinal tyrosines, Tyr-294 and Tyr-295, on the lumenal side of the membrane-spanning helix, M4, which correlates with partial inhibition of Ca(2+)-ATPase activity [150]. Nitration of protein tyrosine residues of Mn-SOD and SERCA is implicated in cardiovascular disease and aging; SOD and SERCA immunostaining for 3-NT were found positive in aging rat skeletal muscle as well as in atherosclerotic aorta and cardiac atrium from human diabetic patients [151]. Moreover, nitration of a critical tyrosine residue in the allosteric inhibitor site of muscle glycogen phosphorylase has been found to impair its catalytic activity, suggesting that glycogen phosphorylase functions may be regulated by tyrosine nitration [152]. Notably, significant levels of nitrotyrosine-modified proteins were present at an earlier age in the semimembranosus muscle in comparison with those in the soleus muscle [153].

3.4 NF-κB Signaling and Skeletal Muscle Atrophy

Aging is characterized by a gradual loss of muscle mass and strength (sarcopenia of old age) and presents a major health problem in elderly people. Age-related loss of muscle mass occurs through a reduction in the rate of protein synthesis, an increase in protein degradation, or a combination of both. However, the underlying mechanisms are still poorly understood, although muscle atrophy in many conditions shares a common mechanism in the upregulation of the muscle-specific ubiquitin E3-ligases atrophy gene-1/muscle atrophy F-box (Atrogin-1/MAFbx) and muscle ring-finger protein 1 (MuRF1) [154].

Sarcopenia is specifically restricted to age-related muscle wasting [155, 156]. Also, disuse of skeletal muscles (e.g., due to inactivity or denervation) also induces muscle atrophy [157]. Different approaches have provided evidence that the activation of NF-κB signaling may be the major signaling mechanism involved in inducing tissue atrophy [157–160]. It should be noted that most of the animal studies on skeletal muscle atrophy involved use of young and mature animals. Documented studies that investigate the exact mechanism by which NF-κB acts in the disuse atrophy of aging sarcopenic muscle are still lacking. Accordingly, the effects of nitrosative stress and the role for peroxynitrite in NF-κB signaling in skeletal muscle atrophy in old age have not yet been fully elucidated.

3.5 *Loss of Skeletal Muscle in Old Age Is Associated with Increased Nitration of Muscle Proteins and NF-κB Activation*

Aging is characterized by a gradual loss of skeletal muscle mass and strength (sarcopenia of old age) [161] and increased nitration of muscle proteins [162, 163]. Tyrosine nitration is particularly evident inside the fibers of aged muscle in disuse conditions (Fig. 1).

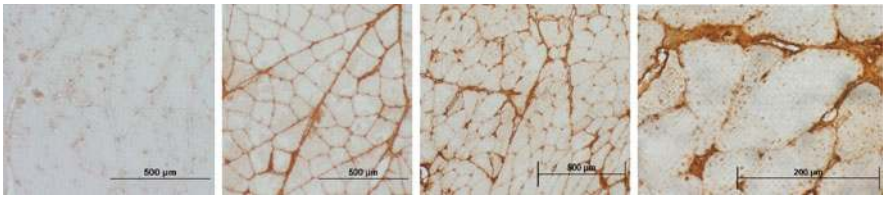

Fig. 1 Immunohistochemistry (using anti-nitrotyrosine antibody) showing level of tyrosine nitration. Cross section of gastrocnemius muscles of (**a**) 6-month-old unimmobilized rat, (**b**) 24-month-old rat, and (**c**, **d**) 24-month-old rat immobilized for 4 weeks. (**c**, **d**) In the immobilized old muscle, positive granulation of staining was observed inside the muscle fibers. Negative control studies, where primary anti-nitrotyrosine antibody was omitted, were also performed (figure not shown). Magnification: (**a, b, c**) 10×; (**d**) 40×

Peroxynitrite ($ONOO^-$) mediates nitration in vivo through free radical biochemistry, which results in formation of 3-nitrotyrosine [59, 86, 164, 165]. Tyrosine nitration represents an important posttranslational modification in vivo and could serve as a signaling pathway by itself [166] or by effecting phosphorylation [167, 168]. As seen in Fig. 2, with increasing concentration of peroxynitrite above 10 μM, there is an increase in nitro-Tyr IκBα with concomitant decrease of P-IκBα (Fig. 2). Accordingly, peroxynitrite molecule is considered a key mediator of the interplay between tyrosine nitration and phosphorylation [169, 170].

Loss of skeletal muscle is associated with activation of NF-κB signaling pathway [157–160, 171, 172] and all the NF-κB family members, c-Rel, p65 (Rel A), Rel B, p50, and p52, are expressed at different levels in mammal skeletal muscle [157]. Kandarian and co-workers found that association of p50 with a B-cell

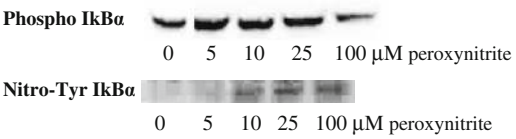

Fig. 2 Western immunoblot of IκBα in HT-29 cells after 20-min exposure to increasing concentrations of peroxynitrite. At 25 μM peroxynitrite, both P-IκBα and nitro-Tyr IκBα were presented. Consequently, it may be assumed that the pathways of NF-κB activation and posttranslational modifications may be triggered by peroxynitrite through nitration and/or phosphorylation

lymphoma-3 (Bcl-3) family member, which functions as a transcriptional coactivator, presented an alternative pathway of NF-κB activation during skeletal muscle atrophy in young healthy rodents [157, 159]. They also established that IκBα mediates the atrophy process because super-repressor IκBαΔN expression inhibited genes known to be upregulated with atrophy during muscle unloading [173]. By performing muscle atrophy studies on old animals, work from our laboratory showed that the classic p65/p50 pathway, which was actually downregulated in the first 2 weeks of atrophy due to hind-limb immobilization, becomes activated in weeks 3 and 4 [161]. In fact, macrophages penetration and acid phosphatase activity were noticeable in weeks 2 and 3 after onset of hind-limb immobilization, whereas in young animals acid phosphatase activity and ubiquitin degradation systems were activated mostly in weeks 3 and 4 [161]. Thus, it was suggested that NF-κB may be activated by different mechanisms in young and old muscles during the atrophy process [161, 174, 175].

3.6 Peroxynitrite-Induced Tyrosine Nitration of IκBα Causes NF-κB Activation

In the past decade, it was shown that RNS are inducers of NF-κB activation and expression of genes involved in inflammation [176–178]. In our recent in vitro studies, we have demonstrated the role of peroxynitrite in the activation of NF-κB–dependent protein degradation systems in skeletal muscle cells [179]. Also, we were first to show that peroxynitrite caused tyrosine nitration of IκBα and its dissociation from NF-κB, which accounted for the prolonged NF-κB activation and high levels of iNOS expression in cultured skeletal muscle cells in the absence of typical IκBα serine phosphorylation and proteosomal degradation [96]. In short, the NO donors 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA-NONOate) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) as well as the authentic peroxynitrite and its donor 3-morpholinosydnonimine (SIN-1) were able to activate NF-κB in cultured muscle cells as measured by p65 nuclear translocations and luciferase expression. However, NO donor-induced NF-κB activation was transient, dependent on IκBα degradation, and was decreased in the presence of IκBα super-repressor. Conversely, peroxynitrite donors induced NF-κB activation that was dependent on tyrosine nitration of IκBα but independent of its serine phosphorylation and degradation. This activation did not decrease in the presence of IκBα super-repressor. Moreover, prolonged exposure to peroxynitrite resulted in nontransient NF-κB activation and high iNOS expression. When applied, proteasome inhibitor N-[(Phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-mehylbutyl]-L-leucinamide (MG-132) did not diminish SIN-1–induced NF-κB activation, whereas tyrosine nitration inhibitor epigallocatechin gallate (EGCG) reestablished transient NF-κB activation through IκBα degradation after SIN-1 treatment. EGCG but not MG-132 decreased SIN-1–dependent iNOS expression. Accordingly, we have shown that peroxynitrite activates NF-κB in skeletal myocytes through an alternative mechanism, in which IκBα is nitrated on tyrosine and

dissociated from NF-κB, thus enabling its nontransient activation. This resulted in prolonged iNOS expression. Hence, we propose that peroxynitrite may exacerbate inflammatory responses mediated by NF-κB. Recently, Yakovlev and co-workers have demonstrated that peroxynitrite caused nitration of tyrosine 181 of IκBα and its dissociation from NF-κB [180]. In another study, peroxynitrite abrogated the classic pathway of NF-κB activation triggered by TNFα or lipopolysaccharide while concurrently inducing the processing of IKKα by NF-κB inducing kinase (NIK), the nonclassic NF-κB pathway [58]. Shuttling of these upstream kinases between the cytosol and nucleus is important because activated IKKα regulates many chromatin events in the nuclear phase of the NF-κB program such as phosphorylation of histone H3 and removal of corepressors from NF-κB–dependent promoters [181, 182]. Thus, the possibility that peroxynitrite may also activate IKKα signaling in muscle atrophy in old age cannot be excluded.

According to the classic model of NF-κB regulation, activation of NF-κB requires degradation of the inhibitory IκBs, and their re-synthesis is considered a master terminator of the NF-κB response. However, NF-κB activity was found terminated in IκBα$^{-/-}$ 3T3 cells by proteosomal degradation of p65 in the nucleus, suggesting that IκBs re-synthesis is not a sole terminator of the NF-κB response [183]. Actually, recent reports have found E3 ubiquitin ligase complexes COMMD1 and PDLIM2 to be responsible for the polyubiquitination targeting of NF-κB p65 and its subsequent degradation by the 26S proteasome in the nucleus [184, 185]. Currently, the effects of nitrosative stress on the process of NF-κB p65 nuclear ubiquitination and degradation in aging skeletal muscle under atrophy conditions are not known, although all three forms of NOS are present in skeletal muscles of mammals [186], and they may be transcriptionally regulated by the aging process [187]. Also, one has to take into account that during aging, the retention of NF-κB proteins in the nuclei is increased [142]. Thus, nitration by peroxynitrite may be a causative agent in modulation of the NF-κB network in skeletal muscle atrophy in old age by changing the feedback axis between IκBs re-synthesis and the level of proteosomal degradation of p65 in the nucleus.

3.7 Nitration by Peroxynitrite and NF-κB Activation Is Supported by Proinflammatory Conditions – Link to Inflamm-aging

One of the features of NF-κB signaling in pathologic conditions, including cancer, is NF-κB nontransient (continuous) activation. In addition to cytokines such as TNFα, the generation and the release of the ROS and RNS such as superoxide and nitric oxide, as well as the generation of peroxynitrite in reaction between these two radicals, also contribute to the activation of the inflammation-related pathway of IKKβ-mediated NF-κB activation. However, the possibility that nitrosative stress may cause prolonged NF-κB signaling to account for disease development has not yet been fully investigated, although simultaneous production of superoxide radical and NO can be increased by 1,000-fold under proinflammatory conditions, resulting in the increased formation of peroxynitrite by as much as 1,000,000-fold [59].

Although it has a short half-life at physiologic pH (~10 ms), peroxynitrite has an excellent ability to cross cell membranes [188, 189], which implies that it could influence target cells within one to two cell diameters (~5–20 μm) [190].

The importance of tyrosine nitration as a posttranslational modification in cell signaling came to light after the pioneering discovery of Kong et al. that peroxynitrite-mediated nitration of a single tyrosine residue in purified cdc2, a cell cycle kinase, prevented its phosphorylation on tyrosine [191]. Gow and co-workers have demonstrated that exposing bovine pulmonary artery endothelial cells to authentic peroxynitrite would result in a decrease in the levels of tyrosine-phosphorylated proteins with concomitant increase in nitrotyrosine-containing protein levels, a finding implying that tyrosine nitration interferes with the process of phosphorylation [89]. On the other hand, it has been shown also that nitration of tyrosine residues may simulate phosphorylation and, as a consequence, may result in the activation of T lymphocytes [192], pancreatic carcinoma cell tyrosine nitration of c-Src kinase resulting in increased tyrosine phosphorylation [167, 193], and tyrosine phosphorylation of the major intrinsic membrane protein, band 3, in erythrocytes [194].

Importantly, tyrosine phosphorylation and nitration are not mutually exclusive events of the cell signaling pathways. For example, Mourad and co-workers [195] have reported the existence of dynamic interplay between nitration and phosphorylation of tubulin cofactor B (TCoB) in the control of microtubule dynamics; p21-activated kinase 1 (Pak1) phosphorylates TCoB on Ser-65 and Ser-128 and plays an essential role in microtubule regrowth. However, TCoB is efficiently nitrated, mainly on Tyr-64 and Tyr-98, and nitrated TCoB attenuates the synthesis of new microtubules. Additionally, optimal nitration of TCoB requires the presence of functional Pak1 phosphorylation sites, thus providing a feedback mechanism to regulate phosphorylation-dependent microtubule regrowth [195]. Thus, the influence of tyrosine nitration on phosphorylation cascades may be more subtle and modulatory as it was initially postulated.

It should be noted that peroxynitrite not only nitrates but also potently oxidizes proteins such as oxidizing and inactivating regulating phosphotyrosine phosphatase (PTPase), which in turn would lead to enhanced tyrosine phosphorylation [196]. Recently, Levrand et al. [58] have shown that authentic peroxynitrite has inhibited NF-κB activation triggered by inflammatory stimuli (TNFα or lipopolysaccharide) in cardiac and endothelial cell lines. The inhibition of NF-κB–DNA binding was completely prevented with the SOD mimetics and antioxidant agent Mn(III)-tetrakis-(4-benzoic acid) porphyrin (MnTBAP), which leads to the conclusion that peroxynitrite-induced NF-κB inhibition may be dependent on peroxynitrite oxidative chemistry [58]. On the other hand, Levrand and co-workers have demonstrated that peroxynitrite, while inhibiting a classic NF-κB pathway, strongly activates phosphorylation of NIK and IKKα, the components of the alternative, noncanonical NF-κB activation pathway, even in the absence of stimulatory signal by LPS or TNFα, suggesting that peroxynitrite molecule alone is involved in the nonclassic pathway of NF-κB stimulation [58]. As mentioned before, we have also proved that total IKK activity has increased after exposure to peroxynitrite in vitro, although the

separate measurements of IKKα were not performed [96]. From our experiments, we have concluded that when tyrosine nitration is not blocked, the noncanonical activation of NF-κB would take place. On the other hand, when nitration is blocked with an agent such as EGCG, the canonical NF-κB pathway ensues. However, blocking nitration would not exclude the engagement of the oxidatively induced alternative pathway of NF-κB activation by peroxynitrite via NIK and IKKα phosphorylations. Thus, these two peroxynitrite chemistries, oxidative and nitrosative, should not be considered mutually exclusive events. In fact, it may be assumed that the alternative pathways of NF-κB activation and posttranslational modifications may be triggered by peroxynitrite through nitration and/or oxidation, which may be a matter of redox balance. It will be challenging to assess the relative contributions of either type of reaction of peroxynitrite, tyrosine nitration, or oxidation to the observed signaling effects.

3.8 Thus, Is NF-κB a Signaling Mediator of Aging?

Genetic studies in model organisms such as yeast, worms, flies, and mice leading to life-span extensions suggest that longevity is subject to regulation. In addition, various system-wide interventions in old animals can reverse features of aging. To better understand these processes, much effort has been put into the study of aging on a molecular level. In particular, genome-wide microarray analysis of differently aged individual organisms or tissues has been used to track the global expression changes that occur during normal aging. To circumvent this problem, Adler et al. [197, 198] have recently developed a novel computational approach to discover transcription factors that may be responsible for driving global expression changes with age. They develop a systematic approach to identify combinatorial *cis*-regulatory motifs that drive age-dependent gene expression across different tissues and organisms. Integrated analysis of 365 microarrays spanning nine tissue types predicted 14 motifs as major regulators of age-dependent gene expression in human and mouse. The motif most strongly associated with aging was that of the transcription factor NF-κB! Accordingly, the transcription factor NF-κB may be a candidate activator of age-related transcriptional changes in multiple human and mouse tissues. Inducible genetic blockade of NF-κB for 2 weeks in the epidermis of chronologically aged mice by adenovirus-mediated expression of dominantly active IκBα (Ad-IκBα) reverted the tissue characteristics and global gene expression programs to those of young mice [197]. Also, age-specific NF-κB blockade and orthogonal cell cycle interventions revealed that NF-κB controls cell cycle exit and gene expression signature of aging in parallel but not sequential pathways. These results identify a conserved network of regulatory pathways underlying mammalian aging and show that NF-κB is continually required to enforce many features of aging in a tissue-specific manner. In that way, genetic blockade of NF-κB in the skin of chronologically aged mice (ΔSP-p50-ER transgenic mice) reversed the global gene expression program and tissue characteristics to those of young mice, demonstrating for the first time that disruption of a single gene is sufficient to reverse features of

aging, at least for the short-term [197]. Age-associated genes whose expression was inhibited by NF-κB blockade included those related to chromatin/transcriptional regulation (*RAD50*, *SMC2L1*, *SMC6L1*, and *ATRX*), protein modification/signal transduction (*STK25*, *RAMP2*, and *HIP2*), cell cycle/growth control (*DNAJC2* and *IGFBP5*), and mitochondria (*ALAS2*, *GSTK1*, and *PTE1*).

It should also be noted that experiments have shown that activation of NF-κB can induce muscle atrophy [157], insulin resistance [199], and neurotoxicity in Alzheimer's disease [200], three prevalent age-associated morbidities. Our work in vitro has shown that NF-κB activity increases in cultured muscle cells exposed to proinflammatory RNS, which are increasingly generated in aging, and that RNS peroxynitrite may induce alternative activation of the NF-κB classic pathway of activation [96]. In addition, in our in vivo work we have observed a biphasic pattern of NF-κB activation in atrophic muscles of old rats [161, 175], which was somewhat different from the pattern of NF-κB activation described in atrophic muscles of young rodents by Kandarian and co-workers [157, 159].

From the evolutionary point of view, the NF-κB system can be viewed as a pleiotropic signaling mediator and that its output is most probably age dependent. The disposable soma theory, which was proposed by Kirkwood and Holliday [201], predicts that aging occurs due to the accumulation of damage during life and that failure of defensive or repair mechanisms contribute to aging. It postulated a special class of gene mutations with antagonistic pleiotropic effects in which hypothetical mutations save energy for reproduction (positive effect) by partially disabling molecular proofreading and other accuracy promoting devices in somatic cells (negative effect). In other words, aging evolves due to the pleiotropic effect of genes that are beneficial early in life and then harmful at later ages. Consequently, inflammation and the activation of the NF-κB system protect tissues and organisms against pathogen attacks and traumatic tissue damage. This is especially important in young organisms to protect reproduction. However, later in life, the pleiotropic functions of the NF-κB system may carry out the disposable soma program through its ROS/RNS oxidative stress–induced unfavorable activation, which may be described as inflamm-aging. Therefore, NF-κB may be considered a signaling mediator of aging from the evolutionary point of view.

4 Summary and Conclusions

The accumulated data indicate that major, chronic age-related diseases are inflammation-related and that the activation of redox-sensitive transcription factors under age-related nitrosative stress are likely to be an important cause. The extent to which observed changes in NF-κB activity drive aging and influence life span and health in humans and other mammals is not yet clear. However, concerning deregulation of NF-κB activity found in human disease, NF-κB activation by peroxynitrite includes the induction of alternative NF-κB pathways and the state of its continuous activation, which is observed in age-related pathologies, including cancer. Thus, understanding mechanisms of alternative pathways of NF-κB activation

would raise the possibility to induce therapeutic manipulations to achieve a desirable level of activation of different NF-κB subunits that change in aging in distinct manners. Moreover, systemic approaches are already able to identify major regulators of age-dependent gene expression in humans. Accordingly, these results show that NF-κB is incessantly required to enforce many features of aging, which suggests that NF-κB may be a major signaling mediator of aging.

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Intervention with Multiple Micronutrients Including Dietary and Endogenous Antioxidants for Healthy Aging

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Abstract Many studies suggest that increased oxidative stress and chronic inflammation are associated with aging in all species, including humans. Growing evidence suggests that these two biological events contribute directly to age-associated decline in organ function. The degenerative changes in the cells are initiated by oxidative damage to mitochondria, which produce more free radicals, which reduce proteasome activity, length of telomere, and immune function and improve lysosomal proteolytic activity. The plasma levels of proinflammatory cytokines (tumor necrosis factor-α and interleukin-6) also increase with aging and contribute to the loss of muscle mass and muscle strength. Limited data show that an adaptive response of antioxidant enzymes to increased oxidative stress occurs in rodents but not in humans. In rodents, plasma vitamin C and tissue coenzyme Q10 levels decline, but plasma glutathione and vitamin E levels are elevated as a function of aging. In contrast, in humans the plasma level of vitamin E decreases and retinol does not change. The fact that increased activity of certain antioxidant enzymes and certain antioxidants exists in the presence of elevated levels of oxidative stress and chronic inflammation suggests that the increased levels of antioxidant systems within the physiologic range may not be sufficient to downregulate the level of oxidative stress and chronic inflammation. Doses of antioxidants higher than those within the physiologic range are needed to reduce oxidative stress and chronic inflammation. This is supported by the fact that the individual antioxidants at high doses reduce the rate of aging. For example, supplementation with individual antioxidants such as vitamin E, coenzyme Q10, carotenoids, melatonin, flavonoids, glutathione-elevating agents (*N*-acetylcysteine), α-lipoic acid, and acetyl-L-carnitine decreased the rate of age-related decline in organ function by reducing oxidative stress and chronic inflammation in rodents. Additional studies on the activities of antioxidant enzymes and levels of dietary and endogenous antioxidants as a function of aging are needed. In the meantime, the author recommends consumption of multiple micronutrients including high doses of dietary and endogenous antioxidants together with changes in diet and lifestyle for

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maintaining healthy aging. Multiple antioxidants are recommended because many different types of free radicals are produced, and each antioxidant has a different affinity for each of these free radicals, depending upon the cellular environment. In addition, they are distributed differently in different organs and in subcellular compartments within the same cell and exhibit different mechanisms of action.

Keywords Oxidative stress · Inflammation · Cytokines · Antioxidants · Micronutrients · Free radicals · Reactive oxygen species · Reactive nitrogen species

1 Introduction

Aging in humans is the result of complex biological processes that are influenced by genetic, environmental, dietary, and lifestyle-related factors. Aging in general can be defined as a process in which the function of individual organs gradually declines. Most chronic diseases in humans are a reflection of deterioration of the function of individual organs each of which loses its function at a different rate, depending upon the genetic, environmental, dietary, and lifestyle-related stressors. The higher the levels of one or more of these stressors, the greater would be the rate of loss of organ function. Individual organs may exhibit differential sensitivity to these stressors; therefore, loss of function in the individual organs may appear at a different rate. The loss of function of cholinergic neurons in brain can lead to Alzheimer's disease and of dopaminergic neurons in brain to Parkinson's disease. In case of familial gene defects, the processes of loss of function in these neurons are accelerated; therefore, these neurologic diseases appear at an early age.

The loss of function in one organ may seriously affect other organs eventually causing death. For example, damage to the vascular system such as occlusion of major arteries can cause damage to heart muscle that can lead to death. Similarly, cancer in which cells gain function should not be considered as a part of the normal aging process. Such causes of death should not be taken into consideration while estimating the lifespan for a population.

Aging on the cellular level has been defined on the basis of programmed cell death, which presumes that all cells are genetically programmed to die within a specified time and that no intervention with pharmacological or physiologic agents can change this destiny. However, the concept of programmed cell death can be applicable to those dividing cells that are eliminated during embryogenesis and to those adult precursor cells that divide, differentiate, and die within a specified time interval. These precursor cells have a finite lifespan that varies depending upon the organ. The author suggests that the concept of programmed cell death should not apply to the nondividing cells such as liver cells, muscle cells, and neurons. In these cells, accumulation of epigenetic damage rather than genetic damage determines the rate of cell death. The epigenetic damage includes mitochondrial dysfunction, reduction of proteasome activity, and oxidation and nitration of proteins, which progress

gradually and lead eventually to cell death. The extent of epigenetic damage is dependent upon the levels of exposure to environmental, dietary, and lifestyle-related stressors.

Aging on the genetic level is difficult to define. This is due to the fact that complex regulatory mechanisms of gene expressions, phosphorylation of certain proteins, and translocation of some proteins from one compartment to another within the same cell are altered during aging.

At present, it is unknown whether the rate of normal aging can be reduced by intervening with pharmacological or physiologic agents. Despite decades of laboratory research, it has not been possible to develop a rational guideline for healthy aging (reduced rate of aging). Among various biochemical and genetic changes that can influence the rate of aging, oxidative stress and proinflammatory cytokines released during chronic inflammation appear to play a dominant role in determining the rate of aging in humans. If this is the case, supplementation with agents that can reduce oxidative stress and chronic inflammation would reduce the rate of aging. The linkage between increased oxidative stress and aging seems to be applicable also to lower organisms such as *Drosophila melanogaster*, nematodes, and birds. Therefore, this review describes the following: (a) the role of oxidative stress and chronic inflammation affecting the rate of aging; (b) the role of individual antioxidants in reducing oxidative stress and chronic inflammation; and (c) the rationale for recommending multiple micronutrients containing dietary and endogenous antioxidants together with modifications in the diet and lifestyle for healthy aging. Healthy aging is defined as a process during which the rate of loss of organ function is markedly reduced, thereby providing a good quality of life for a long period of time.

2 Oxidative Stress During Aging

Increased production of free radicals causes oxidative damage to cells. This damage is referred to as oxidative stress. Among various theories of aging, the free radical theory of aging has strong support from data obtained from different organisms including flies, birds, and mammals, including humans. This theory proposes that increased production of free radicals by mitochondria that contribute to the cellular damage occurs with age [1, 2]. The mitochondria are the major site where free radicals are generated during generation of ATP. During normal aerobic respiration, the mitochondria of one nerve cell of rat will process about 10^{12} oxygen molecules and reduce them to water. During this process, superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl ($OH^\bullet$) radicals are produced. It has been estimated that about 2% of partially reduced oxygen leaks out from the mitochondria to generate approximately 20 billion molecules of superoxide anion and hydrogen peroxide per cell [3, 4]. The mitochondria are very susceptible to oxidative damage, because mitochondrial DNA (mtDNA) is not protected by histones or DNA-binding proteins. Therefore, it is not surprising that they are easily damaged with age. The

damaged mitochondria can produce more free radicals. The role of oxidative stress in aging has been reviewed [5, 6]. Based on the studies published, the author proposes that free radical–induced damage to mitochondria is the initial damage that initiates a cascade of events such as greater production of free radicals, shortening of telomeres, and reduced proteasome activity and immune function that are associated with aging. These cellular abnormalities contribute to age-related decline in organ function and chronic diseases.

Sources of oxidative stress: Mitochondria remain the major sites where free radicals derived from oxygen and nitrogen are generated [4, 7, 8]; these free radicals include superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$), and nitric oxide ($NO^\bullet$). The oxidation of $NO^\bullet$ forms another type of free radical called peroxynitrite, which is very damaging to the cells, especially nerve cells.

Phagocytes also produce free radicals that include superoxide, hydrogen peroxide, nitric oxide, and hypochlorous (HOCl) [7, 9, 10]. Hypochlorous is a strong inducer of inflammation and also acts as a strong oxidizing and chlorinating agent and can form nitryl chloride (NO_2Cl) and nitrogen dioxide ($NO_2^\bullet$) in the presence of nitrite [11], all of which are toxic to cells. In addition to phagocytes, activated human polymorphonuclear neutrophils convert nitrite into NO_2Cl and $NO_2^\bullet$ [12]. Thus, increased production of reactive oxygen, reactive nitrogen, and chlorinated species that can increase oxidative stress could increase the rate of aging.

Environmental, dietary/metabolic, and lifestyle-related stressors influencing oxidative stress: Environmental stressors include extreme heat and cold, high levels of ozone in air, excessive amounts of dust particles in air, and intense sound or vibration; pathogenic bacteria or viruses can increase oxidative stress in humans. Dietary factors include high caloric intake, increased daily consumption of iron or copper, and ingestion of xenobiotic substances. Free radicals are produced during normal metabolism of certain compounds. For example, during degradation of fatty acids and oxidative metabolism of ingested xenobiotic substances, free radicals are produced. Some enzymes during the normal degradation of their substrates generate free radicals. For example, monoamine oxidase, tyrosine hydroxylase, and L-amino acid oxidases produce hydrogen peroxide as a normal by-product of their activity [13]. Other enzymes such as xanthine oxidase and aldehyde oxidase also form superoxide anions during metabolism of their substrates. Auto-oxidation of ascorbate and catecholamines generate free radicals [14]. In addition, calcium-dependent activation of phospholipase A2 releases arachidonic acid, which liberates $O_2^{-\bullet}$ during the synthesis of eicosanoids [15]. Stimulation of glutamate receptor NMDA (*N*-methyl-D-aspartate) produces excessive amounts of $O_2^{-\bullet}$ and $OH^\bullet$ [16]. Certain lifestyle-related factors such as cigarette smoking can increase the level of nitric oxide ($NO^\bullet$) [17, 18], and oxidation of $NO^\bullet$ produces peroxynitrite, a highly reactive species of radicals. Smoking also depletes antioxidants [19, 20], thereby further increasing the oxidative stress in smokers. In addition, free iron and copper also can increase oxidative stress by combining with molecules such as vitamin C [21].

In addition to reactive oxygen and nitrogen species, there are other damaging molecules produced by lipid peroxidation. For example, peroxidation of membrane phospholipid acyl chains generates reactive carbonyl species (α- and β-unsaturated

aldehydes, dialdehydes, and ketoaldehydes), which are relatively stable. These carbonyl species can diffuse from one subcellular compartment to another within the same cell or they can escape from the cells and damage targets far away from the site of formation. These carbonyl species react with cellular constituents and form advanced lipid peroxidation end products (ALEs), and they play an important role in accelerating the aging process [22]. This is supported by the fact hat the level of ALEs in several tissues and species increases with age, and dietary restriction that increases the lifespan decreases the levels of ALEs.

The oxidation and nitration of intracellular proteins play an important role in aging because oxidation and nitration can induce loss of function [23]. The oxidized proteins can easily form aggregates that also contribute to the loss of cell function. Normally, damaged proteins are removed by the proteasome pathway; however, if this pathway is impaired, these damaged proteins will accumulate to cause progressive loss in cell function and eventually cell death. For example, progressive increase in oxidative stress in the brain is strongly implicated in the gradual decline of cognitive function with aging.

Heavy metals at high concentrations are toxic to humans and animals. Metal homeostasis is regulated by a metal-responsive transcriptional factor (MTF-1). Mutation in MTF-1 reduced the life span of *Drosophila melanogaster*, suggesting that the wild-type MTF-1 is an essential component for maintaining the normal life span. The overexpression of MTF-1 in neurons protects against oxidative damage and prolongs the lifespan of CuZn superoxide dismutase (CuZn-SOD) deficient flies [24].

3 Oxidative Stress Influencing Mitochondria, Lysosome, and Proteosome Function During Aging

Among various organelles in the cells, mitochondria exhibit unique functions. They are present in all cells, but most abundantly in nondividing cells such as liver cells, muscle cells, and neurons. Their main function is to generate energy. However, they are the major source of free radicals derived from oxygen and nitrogen, and at the same time they are very vulnerable to free radicals. Damaged mitochondria produce more free radicals; this creates a vicious cycle of damage by free radicals and production of more free radicals.

Mitochondrial dysfunction: Various organs may age at different rates depending upon the levels of oxidative damage to the mitochondria in the individual cells of that organ. Several studies suggest that damage to mitochondria may initiate degenerative changes in the cells during aging of at least nondividing organs such as liver, brain, muscle, and bone. Several animal studies have shown that age-associated increase in the generation of free radicals by mitochondria occurs. For example, the levels of $O_2^{\bullet -}$ and H_2O_2 increased with aging in mitochondria isolated from aged heart of gerbils [25]. The generation of H_2O_2 by mitochondria from older hepatocyte of rat increases by 23% [26]. In the house fly, the rate of the production of H_2O_2 progressively increases with age [27].

The increased production of free radicals by mitochondria with age may cause increased rate of mutation in mtDNA and oxidative damage to proteins. Indeed, it has been proposed that mutations in mitochondria may contribute to accelerate aging [28]. The mtDNA is especially sensitive to oxidative damage, because it is not protected by histones or DNA-binding proteins. Also, it either has no repair mechanisms or repair mechanisms are less efficient in comparison with those of nuclear DNA [29]. The progressive damage to mitochondria may reduce production of energy and thus can cause progressive degenerative changes in the cells during aging and eventually loss of function of organs involved. Mitochondrial dysfunction leads to reduced lifespan in yeast [30]. In mammals, aged tissue produces reduced amounts of ATP by oxidative phosphorylation [31]. Indeed, aging of the mammalian brain exhibits a gradual but continuous decrease in production of ATP by oxidative phosphorylation in the mitochondria [32].

Mitochondrial dysfunction also plays a major role in vascular aging [33, 34]. Vascular aging is primarily characterized by an impaired endothelium-dependent vasodilation that is regulated by nitric oxide (NO). The expression of endothelial nitric oxide synthase (eNOS) is markedly upregulated in the endothelial cells with increasing age, resulting in production of excessive amounts of $NO^{\bullet}$. The levels of superoxide ($O_2^{-\bullet}$) are also produced. The combination of superoxide and nitric oxide can form peroxynitrite ($ONOO^-$). Peroxynitrite causes oxidative modification of proteins that contribute to vascular aging.

The plasma cysteine/acid soluble thiol ratio, an indicator of redox state, is increased in old age, and this increase may account for the loss of body cell mass that is associated with aging in humans. This is further supported by the fact that supplementation with *N*-acetylcysteine caused an increase in body cell mass of healthy subjects with high plasma cysteine/thiol ratio [35, 36].

Increased oxidative stress and mitochondrial dysfunction are also associated with some age-related neurologic diseases such as Alzheimer's disease and Parkinson's disease [37–39]. Increased oxidative stress and mitochondrial dysfunction are also associated with the genetic basis of these neurologic diseases.

Impairment of proteasome and lysosomal-mediated proteolytic activities: Progressive loss of muscle mass (sarcopenia) during aging occurs in both humans and rodents. There could be several reasons for this. It has been proposed that reduced degradation of oxidized proteins may be one of the factors that contribute to sarcopenia [40]. The cells can respond successfully to oxidative damage of proteins only when the ability of proteasome and lysosome to degrade altered proteins remains intact. Increased oxidative stress can impair proteasome activity as well as lysosomal-mediated proteolytic activity. Indeed, during aging these two biological processes are impaired [40–43].

4 Oxidative Stress Influencing the Length of Telomere During Aging

The data on the role of telomere in human aging come primarily from the normal human fibroblasts or other normal cells in culture. These studies suggest that

telomere shortening is associated with aging and that increased oxidative stress accelerates the rate of telomere shortening [44, 45]. Increased oxidative stress induced translocation of nuclear telomerase reverse transcriptase (TERT) from the nucleus to the cytoplasm [46]. Treatment with *N*-acetylcysteine (NAC) reduced the translocation of nuclear TERT from the nucleus to the cytoplasm [46]. This increase of oxidative stress caused premature aging of normal endothelial cells in culture. The role of oxidative stress in telomere shortening is further supported by the fact that dietary antioxidants vitamin C [47] and vitamin E [48] reduced the rate of telomere shortening. These studies suggest that increased oxidative stress causes shortening of telomere and that it is possible to prevent this shortening of telomeres by protecting them from oxidative damage. Therefore, daily supplementation with antioxidants is likely to maintain the length of telomeres and thereby reduce the rate of aging.

5 Chronic Inflammation During Aging

During chronic inflammation, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are released. In addition, prostaglandins, adhesion molecules, and complement proteins are also released all of which are toxic to the cells. The increased levels of these products of chronic inflammation can increase the levels of degenerative changes in the cells and thereby enhance the rate of aging and the risk of chronic diseases. The microglia in the aged brain had increased levels of the proinflammatory cytokine TNF-α [49]. Treatment with TNF-α plus β-amyloid (Aβ-42), a fragment of amyloid precursor protein (APP), caused more toxicity in the older neurons than in the younger neurons [49]. In addition, it has been suggested [50] that TNF-α treatment caused nuclear translocation of nuclear factor kappa B (NF-κB) in neurons. This phenomenon together with the lower level of Bcl-2 promoted cell death in older neurons [50]. It has been reported that treatment of neuron obtained from middle-aged rats with TNF-α and Aβ-42 increased TNF receptors (TNFR1 and TNFR2); however, similarly treated older neurons do not increase these surface receptors of TNF-α [51]. Chronic inflammation has been linked with several age-related neurologic disorders, such as Alzheimer's disease, cardiovascular disease, as well as type 2 diabetes [38, 52].

Increased mitochondrial oxidative stress activates NF-κB in the endothelial cells of blood vessels, which enhances the expression of inflammatory genes [53]. Age-related increase in oxidative stress may promote vascular inflammation [54]. Thus, aged blood vessels exhibit increased NF-κB activity. This was attributed to increased oxidative stress that occurs during aging [53].

Higher plasma concentrations of IL-6 and TNF-α are associated with lower muscle mass and lower muscle strength in well-functioning older men and women [55]. This suggests that proinflammatory cytokines contribute to the loss of muscle mass and muscle strength that are associated with aging. In another study, higher plasma levels of IL-6 and C-reactive proteins increased the risk of loss of muscle strength in older men and women, whereas higher levels of α1-antichymotrypsin decreased the risk of muscle strength loss [56]. Higher plasma levels of IL-6 predict onset of

disability in older individuals [57]. This may be due to the fact that increased levels of IL-6 contribute to muscle atrophy and may increase the risk of certain chronic diseases.

6 Aging Influencing Immune Function

Phagocytes are one of the major sources of free radicals and represent one of the functions of the immune system. Normally, phagocytes attack and eliminate invading pathogenic organisms by generating excessive amounts of oxidants such as superoxide ($O_2^{-\bullet}$), nitric oxide ($NO^{\bullet}$), H_2O_2, and HOCl and engulfing through phagocytosis. The engulfed microorganisms are killed and eliminated by a combination of oxidants and lysosomal digestive enzymes [58].

It has been reported that oxidant production is a function of aging. For example, older macrophages produce reduced levels of oxidants [59–61], suggesting the phagocytic activity of macrophages may be reduced in older individuals. Others have reported that production of reactive oxygen species appears to increase in older peritoneal macrophages [62].

In mice, the antitumor activity of macrophages is reduced in older animals [63, 64]. Furthermore, macrophages from old mice were less responsive to the activation signals of lipopolysaccharide (LPS) plus interferon-γ (IFN-γ) for macrophage-mediated tumoricidal activity [65]. It has been shown that natural killer (NK) activity decreases as a function of age [66], and this activity can be suppressed by adherent cells from spleen and peritoneal cavity. The decline in NK activity found in older mice is due to an increase in suppressor function of adherent cells [66]. These studies suggest that the immune functions are impaired in older individuals, possibly because of increased oxidative damage caused by free radicals and proinflammatory cytokines.

7 Aging Influencing Antioxidant Defense Systems

Antioxidant enzymes: Antioxidant enzymes represented by glutathione peroxidase, catalase, and cytosolic CuZn-SOD and mitochondrial Mn-SOD play an important role in protecting cells against oxidative damage. Glutathione peroxidase is one of the major antioxidant enzymes that neutralize hydrogen peroxide and lipid peroxide and is present in both cytosol and mitochondria. Catalase found in cytosol and mitochondria also metabolized H_2O_2 into water and oxygen. There are two forms of SOD distributed differently within the cell. CuZn-SOD is present in the cytosol, whereas Mn-SOD is present in the mitochondria. Some of these enzymes respond to increased oxidative stress by elevating their activities. This response is referred to as an adaptive response and is an indicator of the presence of high oxidative stress in the cells. However, the activities of some of these antioxidant enzymes may not change or may even decline as a function of aging.

Changes in antioxidant enzymes activities in animals: The changes in the activities of antioxidant enzymes in animals (primarily rodents) as a function of aging are variable, depending upon the organs. It has been reported that antioxidant enzymes activities gradually increase as a function of aging in the skeletal muscle of rats [67]. The increase in the activity of catalase in heart and skeletal muscle and brain and of CuZn-SOD in the skeletal muscle was observed in rats [67, 68]. The increase in antioxidant enzymes activities are considered as an adaptive response to the increased oxidative stress, because markers of oxidative damage and chronic inflammation are elevated in spite of an increase in activities of antioxidant enzymes. These studies suggest that the increased levels of certain antioxidant enzymes are not sufficient to downregulate the level of oxidative stress and chronic inflammation.

The activity of glutathione peroxidase in the brain and CuZn-SOD in the heart showed no significant changes in enzyme activity in rats as a function of aging [68]. However, in other organs such as liver, brain, and heart, decrease in catalase activity was reported [68].

Prostaglandin E2 (PGE2) is one of the toxic products released during chronic inflammation. Prostaglandin A1 (PGA1), which is formed during extraction of PGE2, is stable and therefore is used in experimental systems. We have reported that PGA1-induced degeneration of murine differentiated neurons increased the expression of catalase gene and decreased the expression of glutathione peroxidase and Mn-SOD genes without changing the expression of CuZn-SOD as determined by gene array and confirmed by real-time PCR [69]. The protein levels of glutathione peroxidase increased, whereas the protein level of Mn-SOD decreased, and the levels of catalase and CuZn-SOD did not change as determined by Western blot [69].

The activities of antioxidant enzymes as a function of aging varied in different organs. The phenomenon of adaptive response to increased oxidative stress was observed for only certain antioxidant enzymes and in certain organs. The markers of oxidative damage and chronic inflammation were elevated, and immune functions were impaired in older animals irrespective of changes in the activities of antioxidant enzymes. Thus, increased activities of certain antioxidant enzymes are not sufficient to downregulate increased oxidative stress and chronic inflammation. Additional studies in which antioxidant enzyme activity markers of oxidative damage and chronic inflammation are measured at the same time in the same animals are needed.

Changes in antioxidant enzymes activities in humans: Very limited data on changes in antioxidant enzymes activities as a function of aging in humans exist. However, based on a few studies, it appears that changes in enzyme activities as a function of aging are in part different from those observed in rodents. One major difference is that an adaptive response of certain antioxidant enzymes to increased oxidative stress that is found in rodents is not observed in humans to date. In older humans, the serum concentrations of SOD, glutathione peroxidase, and albumin were lower than those found in younger individuals, but the activity of catalase did not change [70]. The total antioxidant capacity decreased and lipid peroxides level increased in older subjects in comparison with the younger subjects [70]. In another

study, the activities of catalase and SOD in serum did not change as a function of aging; however, the activity of glutathione peroxidase declined in older subjects [71]. Furthermore age-related increase in lipid peroxidation and protein oxidation were observed [71]. In human skeletal muscle, total SOD decreased as a function of aging, although Mn-SOD increased in older individuals [72]. The activities of catalase and glutathione peroxidase did not change [72]. These observations are opposite to those observed in the skeletal muscle of rats [67]. Thus, the results on changes in the antioxidant enzymes activities in rodents cannot be extrapolated to humans, in whom no increase in enzyme activity as an adaptive response to increased oxidative stress was observed. This may be due to the fact that the adaptive response of enzymes to increased oxidative stress in humans is very sensitive to damage or that the increased levels of oxidative damage in humans overwhelms the adaptive response of antioxidant enzymes.

In older individuals with a disease, the changes in antioxidant enzyme activity were different from those in older individuals without the disease. It is established that age-related macular degeneration is the leading cause of irreversible blindness in developed countries. It appears that increased oxidative stress and decrease in certain antioxidant enzymes play an important role in the pathophysiology of age-related macular degeneration. It has been reported that SOD and glutathione peroxidase were lower in both plasma and red blood cells (RBCs) of patients with maculopathy in comparison with those found in age- and sex-matched control subjects; however, the catalase activity in RBCs remained unchanged [73]. The activity of α-ketoglutarate dehydrogenase complex (KGDHC), a mitochondrial enzyme, is decreased in the brains of Alzheimer's disease (AD) patients in comparison with that in matched control subjects [74]. It is interesting to observe that in AD patients who carry the ApoE4 allele of the ApoE gene, the Clinical Dementia Rating (CDR) correlated better with KGDHC activity than with the densities of extracellular neuritic plaques and intracellular neurofibrillary tangles. However, in patients with AD who do not carry the ApoE4 allele, the CDR correlated better with the densities of extracellular neuritic plaques and intracellular neurofibrillary tangles. The activity of glutamine synthetase decreased in the autopsied samples of brains of AD patients [75], and the levels of glutathione peroxidase in ventricular cerebral spinal fluid (CSF) also decreased in AD patients in comparison with those in age-matched control subjects [76]. Substantia nigra samples from autopsied brains of Parkinson's disease patients showed reduced levels of antioxidant enzymes [77, 78]. These studies suggest that the activities of certain antioxidant enzymes in the brains of patients with neurodegenerative diseases decline more than those found in age-matched control subjects.

Dietary and endogenous antioxidants levels: In addition to antioxidant enzymes, dietary (vitamin A, β-carotene, vitamin C, vitamin E, and selenium) and endogenous (glutathione, coenzyme Q10, L-carnitine, and α-lipoic acid) antioxidants play an important role in reducing oxidative stress and chronic inflammation. The organ, cellular, and subcellular distributions of these antioxidants appear to be highly variable for the same antioxidant. The levels of different dietary and endogenous antioxidants also differ in their distributions. Therefore, measurements of the

plasma, whole tissue, or cell levels may not be a true reflection of changes in the levels of antioxidants in the subcellular fractions that may be critical in determining the rate of progression of aging or the risk of age-related chronic diseases. In addition, the levels of different antioxidants distributed in the subcellular fractions of most mammals (primarily rodents) may be different from those found in humans, because most mammals except humans and guinea pigs synthesize their own vitamin C, which could affect the level of other antioxidants in the body. Therefore, it is impossible to extrapolate the value of antioxidant level obtained from animal studies to humans. Most studies in animals or humans have measured antioxidant levels primarily in plasma. Only the values for a few antioxidants as function of aging are available primarily in rodents, and these values are highly variable.

Vitamin C: Vitamin C is a water-soluble antioxidant that has multiple biological functions. One of them includes regeneration of oxidized glutathione and vitamin E to reduced form in order to maintain their antioxidant function. The plasma level of vitamin C declined as a function of age in various species [33, 79–81]. This result cannot be extrapolated to humans, as humans do not synthesize their own vitamin C. Humans primarily rely on dietary sources for vitamin C, and consumption of this vitamin from the diet is highly variable among the U.S. population. Therefore, changes in the vitamin C levels as a function of aging is meaningless with respect to its role in aging. This is further confounded by the fact that the plasma half-life of vitamin C is rather short (a few hours).

Glutathione: Glutathione is also a water-soluble antioxidant present in the cell in millimolar concentrations. It maintains glutathione peroxidase activity and prevents oxidation of vitamin E and vitamin C. In addition to acting as an antioxidant, it has several other functions. It has been shown that glutathione level increases in the brains of mice as a function of aging [82, 83], but others have reported no significant change in the brains of old rats [84]. The glutathione level also increased in the plasma, heart, and liver [83, 84]. Some have reported that the level of glutathione in the skeletal muscle increases as a function of aging [67, 83]. Other studies have reported no change in the level of glutathione in the livers of rodents [83, 85, 86]. In contrast, some studies have reported a decline in the glutathione level of brain [85, 86] and eye lens [81] as a function of aging. These studies suggest that the level of glutathione in most tissues of rodents increases or shows no significant change as a function of aging. In contrast with the level of glutathione found in whole tissues of rodents, the levels in the subcellular fractions consistently showed a decline as a function of aging. For example, the level of glutathione in the cerebral cortex synaptosomes [87] and mitochondria of the liver, kidney, and brain [88] declined as a function of aging. In a model of prematurely aging mice, the level of glutathione decreased and the level of MDA increased in comparison with normal mice [89].

Changes in the level of glutathione in humans as a function of aging are not available; however, in age-related diseases such as Parkinson's disease, the autopsied brain tissues consistently showed decline in the levels of glutathione [90–92]. The serum levels of vitamins A and E and β-carotene were lower in patients with AD (who were well nourished) than in control subjects [93].

Vitamin E: Like the levels of water-soluble antioxidants, the lipid-soluble antioxidant vitamin E level also revealed variable changes in rodents as a function of aging. For example, vitamin E levels increased in certain regions of brain, lung, and liver [94], aortic wall of the blood vessel [33], and in serum [95], with no change in heart [96], liver [79], blood [97], and membranes [95], and a decline in plasma [79] and the substantia nigra region of the brain [98] as a function of aging. These studies suggest that the level of vitamin E either increased or showed no change as function of aging in most studies in rodents.

In elderly humans, the plasma level of vitamin E declined in 70% of the younger subjects, whereas plasma level of retinol did not significantly change. However, on the basis of the ratio of lipid-adjusted vitamin E to plasma lipids, only 12% showed decline in vitamin E level [99]. No data on changes in tissue vitamin E levels as a function of aging are available in humans.

Coenzyme Q10: Coenzyme Q10 recycles vitamin C and vitamin E. Tissue levels of coenzyme Q10 decreased with age [98].

Many studies with rodents revealed that the activities of antioxidant enzymes and the levels of certain antioxidants (vitamin E and glutathione) increased as a function of aging, although no changes or decline in their levels were reported in some studies. How can one explain the existence of increased oxidative stress and increased levels of certain markers of proinflammatory cytokines (TNF-α and IL-6) in the presence of elevated levels or no change in antioxidant enzymes and antioxidants? The author proposes that increased physiologic activity of antioxidant enzymes or antioxidant level is not sufficient to decrease oxidative stress or chronic inflammation. In order to reduce these two biological processes that play a crucial role in aging and age-related chronic diseases, pharmacological doses of dietary and endogenous antioxidants are needed. Indeed, supplementation with high doses of one or more dietary or endogenous antioxidants decreased oxidative stress and chronic inflammation in rodents. This is described in the next section.

8 Antioxidant Supplementation Influencing Age-Related Functional Deficits

Vitamin E: Supplementation with vitamin E restores mitochondrial dysfunction (to produce ATP by oxidative phosphorylation) in aged brain and liver [31]. In healthy elderly humans, supplementation with vitamin E and fish oil was more effective in reducing the levels of proinflammatory cytokines (IL-6 and TNF-α) than fish oil alone [100]. Age-dependent loss of T helper1 (Th1) cytokines occurs, especially of IFN-γ, which plays an important role in defending against influenza infection [101]. In addition, the production of PGE2, which suppresses Th1 cytokines, increases with age in mice. Vitamin E supplementation reduced influenza titer in old mice, and this antiviral effect of vitamin E is mediated through reduced production of PGE2 and enhanced production of Th1 cytokines [101]. Other antioxidants such

as glutathione, melatonin, or strawberry extract, which reduce oxidative stress, did not affect the level of influenza titer [102].

Vitamin E supplementation increased the activity of SOD in old trainee rats; however, exercise alone failed to increase SOD activity in these older animals and reduce oxidative damage [103]. It has been shown that vitamin supplementation plus exercise improved age-related deficit in antioxidant enzymes in the cerebral cortex and hippocampus regions of rat brain [104]. Age-related increase in lipid peroxidation and protein oxidation is reduced in the brain of older rats by vitamin E supplementation [105]. Reduced level of SOD was found in cerebral cortex of old rat, whereas it was highest in the hippocampus region of the brain; however, vitamin E supplementation increased it [105]. Vitamin E in combination with fish oil reduced proinflammatory cytokines more than that produced by fish oil alone in healthy elderly subjects [100]. In contrast, the immune-enhancing effect of vitamin E alone was reduced in healthy elderly men and women when fish oil was taken concomitantly. This may be due to the fact that plasma level of vitamin E increased by smaller amounts in the presence of fish oil [106]. From this one study, it appears that supplementation with fish oil interferes with the absorption of vitamin E. Additional studies are needed to substantiate this observation.

Coenzyme Q10: Supplementation with coenzyme Q10 prolonged lifespan of animals fed on polyunsaturated fatty acids-6-enriched. It decreases oxidative stress and cardiovascular risk and regulates inflammation during aging [107, 108]. Combination of vitamin E and coenzyme Q10 improved age-related learning deficits in mice, but the individual agents failed to do so [109]. Administration of coenzyme Q10 increased the level of vitamin E in the mitochondria, which was related to the regenerating effect of coenzyme Q10 [110]. In mice and rats, ingestion of coenzyme Q10 elevated coenzyme Q9, the predominant homologue in mitochondria. It has been reported that the rate of mitochondrial superoxide anion radical generation is directly proportional to mitochondrial coenzyme Q9 and inversely related to coenzyme Q10 [111]. This suggests that supplementation with coenzyme Q10 in rodents may prevent mitochondrial dysfunction.

Carotenoids: Intake of dietary lutein and zeaxanthin or zinc reduced risk of age-related macular degeneration (AMD); however, higher consumption of β-carotene was associated with increased risk of AMD [112]. Another study showed no effect of vitamin E or β-carotene supplementation on the risk of AMD [113].

Melatonin: Supplementation with melatonin for 30 days reversed age-related retention deficits in mice [114]. Melatonin regulates circadian rhythms through hypothalamic suprachiasmatic nucleus (SCN). Age-related loss of loss of sensitivity to melatonin occurs in the SCN of mice [115].

Flavonoids: Quercetin is a bioflavonoid that exhibits a strong antioxidant property. Supplementation with quercetin for 30 days reversed age-related retention deficits in mice [116]. Others have reported that supplementation with flavonoids (apigenin-7-glucoside and quercetin) reversed age-related and lipopolysaccharide-induced retention deficits in mice [117].

Glutathione and N-acetylcysteine (NAC): In a model of prematurely aging mice, treatment with NAC plus thioproline increased chemotaxis, phagocytosis, and IL-β release and decreased superoxide levels and TNF-α production [118]. This suggests that antioxidant supplementation can protect against early decline in immune function in prematurely aging mice. These antioxidants also reversed age-related behavioral dysfunction in prematurely aging mice [119].

Alpha-lipoic acid: A combination of α-lipoic acid and acetyl-L-carnitine reduced mitochondrial decay and oxidative damage in rat brain resulting from aging [120]. These results support many other studies discussed previously that oxidative damage of mitochondria plays a central role in increasing the risk of age-related chronic diseases and functional deficits. Alpha-lipoic acid and acetyl-L-carnitine are substrates for mitochondrial enzyme carnitine acetyltransferase (CAT). In the brains of older rats, the activity of CAT and its binding affinity with substrates declined in comparison with younger rats [121]. Feeding old rats with high doses of acetyl-L-carnitine and α-lipoic acid can ameliorate oxidative damage, CAT activity and its binding affinity with substrates, and mitochondrial dysfunction [121].

Multiple dietary antioxidants: The effects of supplementation with antioxidants (vitamin C, 500 mg; vitamin E, 400 IU, and β-carotene,15 mg); zinc, 80 mg as zinc oxide and copper, 2 mg as cupric oxide; or antioxidant plus zinc on AMD were investigated [122]. Results showed that antioxidants or zinc reduced the risk of developing advanced AMD in higher-risk groups; however, the combination was more effective than the individual agents. Diet supplementation with antioxidants protected against early decline in immune function and behavior in prematurely aging mice [89]. It has been shown that the macrophages of prematurely aging mice exhibit depressed chemotaxis and phagocytosis activity. However, supplementation with dietary antioxidants (vitamin C, vitamin E, β-carotene, zinc, and selenium) decreased the levels of proinflammatory cytokines and improved natural killing cell activity, lymphocyte chemotaxis activity, and proliferation response of lymphocytes to concanavalin A [123], as well as oxidative stress [124]. Dietary antioxidant supplementation protects immune function against oxidative damage and improves lifespan [125]. Psychomotor performance is decreased with aging, and this could be related to increased oxidative damage and increased levels of proinflammatory cytokine IL-6. Supplementation with dietary antioxidants improved psychomotor performance in old mice and decreased the level of oxidative damage and IL-6 [126].

9 Rationale for Using Multiple Dietary and Endogenous Antioxidants in Age-Related Functional Deficits

Animal studies with antioxidants have produced expected beneficial results in reducing the risk of diseases. However, this experimental paradigm of using single antioxidants in humans with high risk of developing chronic diseases has produced inconsistent results varying from beneficial effect, to no effect to harmful effects and

therefore should not be used in any future investigations in humans. The most widely quoted studies are those that were performed with β-carotene in heavy tobacco smokers [127–129] and those with vitamin E in high-risk heart disease [130–132]. In these studies, β-carotene increased the risk of lung cancer [127–129], and vitamin E increased the risk factors of heart disease, enhanced mortality in patients with heart disease, and increased the risk of secondary cancer in cancer survivors [130–132]. Based on the biology of individual antioxidants and the internal oxidative environment of the host, these results could have been predicted. It is known that an individual antioxidant, when oxidized, may act as a pro-oxidant. It is also known that heavy tobacco smokers, patients with heart disease, or cancer survivors have an increased internal oxidative environment. Therefore, administration of a single antioxidant in these high-risk populations will result in oxidation of this antioxidant, thereby increasing the risk of chronic diseases. Therefore, conclusions drawn from these studies are inaccurate; nevertheless, they have remained the major sources on which the recommendations for not taking supplements and vitamins are made.

The author recommends multiple micronutrients including dietary and endogenous antioxidants for additional reasons. These include the fact that many different types of free radicals are produced in the body, and each antioxidant has a different affinity for each of these free radicals, depending upon the cellular environment. In addition, they exhibit different mechanisms of action. For example, β-carotene (BC) was more effective in quenching oxygen radicals than most other antioxidants [133]. BC can perform certain biological functions that cannot be produced by its metabolite, vitamin A, and vice versa [134, 135]. BC treatment enhanced the expression of the connexin gene, a gap junction protein gene, whereas vitamin A treatment did not produce such an effect [135]. Vitamin A induced cell differentiation in certain normal and cancer cells, whereas BC did not [136, 137]. The gradient of oxygen pressure varies within the cell and tissues. Vitamin E was more effective as a quencher of free radicals in reduced oxygen pressure, whereas BC and vitamin A were more effective in higher atmospheric pressure [138]. Vitamin C is necessary to protect cellular components in aqueous environments, whereas carotenoids and vitamins A and E protect cellular components in nonaqueous environments. Vitamin C also plays an important role in maintaining cellular levels of vitamin E by recycling the vitamin E radical (oxidized) to the reduced (antioxidant) form [139]. Also, the DNA damage produced by oxidized vitamin C can be ameliorated by vitamin E. The form and type of vitamin E used are also important to improve beneficial effects of vitamin E. It is known that various organs of rats selectively absorb the natural form of vitamin E [140]. It has been established that α-tocopherol succinate (α-TS) is the most effective form of vitamin E [141, 142]. We have reported that oral ingestion of α-TS (800 IU/day) for over 6 months in humans increased plasma levels of not only α-tocopherol but also α-TS, suggesting that α-TS can be absorbed from the intestinal tract without hydrolysis to α-tocopherol, provided the pool of α-tocopherol in the body has become saturated [142]. Selenium, a cofactor of glutathione peroxidase, acts as an antioxidant. Therefore, selenium supplementation together with other dietary and endogenous antioxidants is also essential for healthy aging.

Glutathione, one of the endogenously made compounds, represents a potent intracellular protective agent against oxidative damage. It catabolizes H_2O_2 and anions and is very effective (in the presence of glutathione peroxidase) in quenching peroxynitrite [143]. Therefore, increasing the intracellular levels of glutathione may be important for maintaining healthy aging. Oral supplementation with glutathione failed to significantly increase plasma levels of glutathione in human subjects [144], suggesting that this tripeptide is completely hydrolyzed in the gastrointestinal tract. *N*-Acetylcysteine and α-lipoic acid increase the intracellular levels of glutathione, and therefore they can also be used in combination with dietary antioxidants. Coenzyme Q10 is required for generating ATP by mitochondria. Mitochondrial dysfunction that is associated with aging may not have adequate amounts of coenzyme Q10; therefore, supplementation with coenzyme Q10 may be necessary for maintaining healthy aging. In addition, it also scavenges peroxy radicals faster than α-tocopherol [145] and like vitamin C can regenerate vitamin E in a redox cycle [146]. The inclusion of B-vitamins into a multiple antioxidant preparation is needed, because B6, B12, and folic acid are essential for maintaining healthy aging.

10 Changes in Diet and Lifestyle

Dietary recommendations include daily consumption of a low-fat and high-fiber diet with plenty of fresh fruits and vegetables, avoiding excessive amounts of protein, carbohydrate, or calories, restricting intake of nitrite-rich cured meat, charcoal-broiled or smoked meat or fish, caffeine-containing beverages (cold or hot), and pickled fruits and vegetables.

Lifestyle-related recommendations include stopping smoking and chewing tobacco, avoiding secondhand smoke and overexposure to sun, UV light for tanning, and hyperbaric therapy for energy, restricting intake of alcohol, reducing stress by vacation, yoga, or meditation, and performing moderate exercise 4–5 times a week.

11 Summary and Conclusions

Increased oxidation caused by generation of excessive amounts of free radicals and proinflammatory cytokines produced during chronic inflammation contribute to the age-related decline in organ function and chronic diseases. Mitochondria are not only the first target to be damaged by free radicals but also the most sensitive target to oxidative stress. Damaged mitochondria produce more free radicals that initiate a cascade of events that reduce proteasome activity, length of telomere, and immune function and improve lysosomal proteolytic activity.

Changes in the antioxidant enzymes during aging have been investigated more in rodents than in humans, and they are highly variable. In rodents, an increase, no change, or decline in antioxidant enzymes activities in the presence of elevated levels of markers of oxidative damage and inflammation have been observed; however,

in humans, the activities of antioxidant enzymes consistently showed either decrease or no change as a function of aging. An increase in the activities of certain antioxidant enzymes in the presence of elevated levels of markers of oxidative stress and inflammation suggests that they were not sufficient to downregulate oxidative stress and chronic inflammation. An adaptive response of certain antioxidant enzymes to increased oxidative stress is found in rodents but is not observed in humans.

Very limited data exist with respect to changes in the levels of dietary and endogenous antioxidants as a function of aging in rodents or in humans. In rodents, plasma vitamin C and tissue coenzyme levels Q10 decline, but plasma glutathione and vitamin E levels are elevated as a function of aging. In contrast, the plasma level of vitamin E decreased, and retinol did not change in elderly humans.

As increases of oxidative stress and chronic inflammation occur in the presence of certain elevated antioxidant enzymes and antioxidants [69], doses of antioxidants higher than those within the physiologic range are needed to reduce oxidative stress and inflammation and age-related decline in organ function. This is supported by the fact that the individual antioxidants at high doses reduce the rate of aging. For example, supplementation with individual antioxidants such as vitamin E, coenzyme Q10, carotenoids, melatonin, flavonoids, glutathione-elevating agents (*N*-acetylcysteine) α-lipoic acid, and acetyl-L-carnitine decreased the rate of aging-related decline in organ function by reducing oxidative stress and chronic inflammation in rodents.

Animal studies with antioxidants have produced expected beneficial results in reducing the risk of diseases. However, this experimental paradigm of using single antioxidants in humans with high risk of developing chronic diseases has produced inconsistent results varying from beneficial effect, to no effect, to harmful effects and therefore should not be used in any future investigations in humans. Multiple micronutrients including dietary and endogenous antioxidants are recommended for additional reasons. They include the fact that many different types of free radicals are produced in the body, and each antioxidant has a different affinity for each of these free radicals, depending upon the cellular environment. In addition, they exhibit different mechanisms of action. Additional studies on the activities of antioxidant enzymes and levels of dietary and endogenous antioxidants as a function of aging are needed. In the meantime, the author recommends consumption of multiple micronutrients including high doses of dietary and endogenous antioxidants together with changes in diet and lifestyle for maintaining healthy aging.

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Advanced Glycation End Products, RAGE, and Aging

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Abstract The products of nonenzymatic glycation and oxidation of proteins and lipids, the advanced glycation end products (AGEs), accumulate in diverse biological settings including aging. AGEs may be generated rather quickly or over long duration as a consequence of distinct triggering mechanisms, thereby accounting for their roles in multiple settings and disease states. The potency of the effect of AGEs on tissues and cells is, in part, due to the ability of AGEs to activate and interact with receptor for advanced glycation end products (RAGE). In this chapter, we discuss multiple mechanisms by which AGE interactions cause perturbation in aging and propose that targeting this AGE–RAGE pathway may represent a logical step in the prevention and treatment of aging and age-associated disorders.

Keywords RAGE · AGEs · Aging · Aldose reductase · Polyol pathway

1 Introduction

Advanced glycation end products (AGEs), the products of nonenzymatic glycation and oxidation of proteins and lipids, accumulate in diverse biological settings, such as diabetes, inflammation, renal failure, and aging. AGEs have been shown to impart multiple potential effects on vessels and tissues. Many "receptors" for AGEs that have been identified include lactoferrin, scavenger receptors types I and II, oligosaccharyl transferase-48 (OST-48), 80 K-H phosphoprotein, galectin-3, and CD36 [1–4]. This chapter will focus on the role of AGEs and their interaction with receptor for advanced glycation end products (RAGE), a central signal transduction receptor for these species.

RAGE, a multiligand receptor of the immunoglobulin superfamily, was first described as a receptor for AGEs [5, 6]. Subsequent studies have identified RAGE

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as a receptor for amyloid-beta peptide (Aβ) and β-sheet fibrils; 100/calgranulins; amphoterin; and Mac-1 [7–13]. RAGE function is integrally linked to its ability to activate an array of signal transduction cascades, thus suggesting that RAGE transduces the effects of AGEs by its capacity to trigger signaling cascades, rather than mediating AGE removal. In this context, we have been hypothesizing that the engagement of RAGE by AGEs in a variety of settings triggers rapid generation of reactive oxygen species (ROS) and the upregulation of inflammatory pathways, mechanisms dependent on RAGE signal transduction. Once triggered, this ligand–RAGE axis markedly perturbs cellular properties and sets the stage for the sequelae of AGE generation and accumulation [12]. Here, we will discuss classic settings that promote the generation of AGEs in aging. We also present evidence that interventions that block AGE–RAGE or RAGE–ligand interaction may represent a targeted strategy to suppress the long-term maladaptive consequences of AGEing.

2 AGEs, Oxidant Stress, and Inflammation

Glycation adducts of proteins are formed when proteins react with glucose-reactive α-oxoaldehydes such as glyoxal, methylglyoxal (MG), and 3-deoxyglucosone (3-DG) [14]. Initially, Schiff base adducts formed from glucose and lysine and N-terminal amino acid residues may rearrange to form the key intermediate fructosamine. Fructosamine degradation and the reaction of α-oxoaldehydes with proteins lead to formation of many AGEs. Bis(lysyl)imidazolium cross-links, hydroimidazolones, and monolysyl adducts are some of the diverse array of in vivo AGE products that have been detected and characterized [15]. Experimental evidence has been presented that both carboxymethyl lysine (CML) adducts of proteins and lipids, as well as AGEs derived through the generation of hydroimidazolone, species that accumulate in diabetes, are specific ligands for RAGE [16, 17]. Indeed, evidence indicates that CML-AGEs are highly prevalent in diabetes, as well as in aging and renal failure [18–21].

MG is formed in cells primarily from the triose phosphate intermediates of glycolysis, dihydroxyacetone phosphate, and glyceraldehyde. MG-derived AGEs have been demonstrated in diabetic and aging tissues [22]. MG is detoxified by glyoxalase I in tissue [23]. Depletion of glutathione, due to oxidative stress, attenuates the activity of glyoxalase I and leads to buildup of MG and MG-derived AGEs. Modification of antioxidant proteins by MG may diminish their function and further magnify generation of reactive oxygen species. Thus, conditions favoring oxidant stress may synergize, in conditions such as hyperglycemia and aging, to propel constant MG generation.

3-DG is generated in cells and tissue as a consequence of flux via the polyol pathway. By the polyol pathway, glucose is reduced to sorbitol by aldose reductase (AR); fructose generated by this pathway is converted into fructose-3-phosphate by the action of 3-phosphokinase (3-PK). One product of this reaction is 3-DG, a major precursor in the generation of an array of AGEs, in particular, CML adducts

and others [24, 25]. In patients with renal failure, levels of plasma 3-DG increase with increased AR activity in erythrocytes [25]. Consistent with a key role for AR in AGE generation, the administration of the AR inhibitor epalrestat to diabetic subjects reduced levels of CML adducts and their precursors in erythrocytes [26]. In other studies, epalrestat reduced plasma levels of AGEs (CML adducts) in patients with type 2 diabetes [27]. Furthermore, the tangled web of the polyol pathway and its products provides a further direct stimulus to the generation of AGEs; it has been shown that fructose may induce protein oxidation and AGE formation, as well [28, 29]. Mutual impact of AGEs in fuelling the polyol pathway was demonstrated by studies showing that AGEs may induce AR, a key enzyme of the polyol pathway [30].

A plethora of evidence suggests that AGEs are involved in a vicious cycle of inflammation, generation of reactive oxygen species (ROS), and amplified production of AGEs. AGEs have been linked to increased generation of ROS by multiple mechanisms, such as by decreasing activities of superoxide dismutase (SOD) and catalase, diminishing glutathione stores, and activation of protein kinase C [31–33]. Importantly, activation of myeloperoxidase (MPO) pathways was shown to directly generate CML-AGEs, thus linking inflammation to AGE formation [34]. Furthermore, oxidative stress begetting AGE and back again was indicated by studies in which lipid peroxidation product malondialdehyde caused secondary oxidative damage to proteins [35]. In addition, non-AGE ligands for RAGE may accumulate in oxidative and inflammatory settings as well. Thus, several findings have led to the hypothesis that a key consequence of AGEs generated by glucose, ROS, or other acute inflammatory stimuli and subsequent interaction with its receptor is the migration of inflammatory cells into the initial area of stress.

Consequences of AGE interactions with its receptor have been shown to have distinct cell specific effects. In endothelial cells, AGE–RAGE interaction modulates the expression of adhesion molecules and the expression of proinflammatory and prothrombotic molecules such as vascular cell adhesion molecule-1 (VCAM-1); in fibroblasts, AGE–RAGE interaction modulates the production of collagen; in smooth muscle cells (SMCs), AGE–RAGE interaction modulates the migration, proliferation, and expression of matrix modifying molecules; in mononuclear phagocytes (MPs), AGE–RAGE interaction modulates chemotaxis and haptotaxis and the expression of proinflammatory and prothrombotic molecules; and in lymphocytes, AGE–RAGE interaction stimulates the proliferation and generation of interleukin-2 (IL-2) [36–40]. A large body of evidence suggests that one consequence of AGE–RAGE interaction is the generation of ROS, at least in part via the activation of NADPH oxidase [41–43], although other sources of ROS dependent upon RAGE have not been ruled out.

3 AGE and AGEing Hypothesis

The AGEing hypothesis of aging was proposed based on multiple studies that demonstrated accumulation of a variety of types of AGEs in aging tissues, from

CML adducts; to carboxyethyl (CEL) lysine adducts; methylglyoxal; pentosidine; and others [20, 44, 45]. In addition to proteins and lipids, evidence exists that DNA may also undergo AGE modification. Potential consequences of modification of DNA by AGEs include implications for both regulatory and epigenetic components of the aging process [46]. The issue of the role of AGEs as a cause and/or biomarker in the maladaptive aspects of the aging process is yet to be resolved [47, 48]. Evidence suggests that AGEs form during natural aging, especially as a consequence of exposure of long-lived proteins to even euglycemic levels of glucose. Progression of insulin resistance with aging is likely to further propel AGE generation in aging tissues. In addition to increased AGE formation, aging appears to be associated in part with reduced AGE defenses. For example, glyoxalase I, a cytosolic enzyme that functions to decrease glycation reactions, displays decreased activity with aging [23]. Kil and colleagues have shown that aging-associated glycation-induced inactivation of NADP(+)-dependent isocitrate dehydrogenase results in decreased activity of this enzyme and, in parallel, the perturbation of cellular antioxidant defense mechanisms [49]. These findings support the premise that in aging, injury-provoking mechanisms may be enhanced, in the face of slowly deteriorating anti-injury defense mechanisms.

3.1 AGEs in Brain

AGEs increase in the brain during normal aging. Experimental evidence supports the premise that AGEs are further increased in the brain in the presence of vascular or Alzheimer's dementia [50–54]. Presence of diabetes and Alzheimer's disease further increases AGE accumulation above and beyond that seen in Alzheimer's disease alone [54]. The intimate link between vascular and Alzheimer's dementia is underscored by the observation that stroke is increased in subjects with Alzheimer's disease, especially in the presence of known vascular risk factors [55]. Further investigations may, ultimately, highlight unifying roles for AGEs in neuronal stress thus exacerbating aging and neurodegenerative processes in the brain. Indeed, central roles for altered inflammatory mechanisms in the diabetic-aged brain, in part secondary to AGEs, may amplify neuronal stress. In this context, hyperglycemic db/db mice subjected to transient cerebral hypoxia/ischemia demonstrated the decreased expression of antiapoptotic molecules such as bfl-1 in microglia, decreased glial activation upon reperfusion, and increased tissue injury [56]. Further, multiple studies suggest that AGEs are directly neurotoxic to cultured neurons [50, 57]. AGEs and their precursors (methylglyoxal and glyoxal) may increase the aggregation and cytotoxicity of intracellular amyloid-beta carboxy-terminal fragments [58]. Taken together, these considerations strongly indicate that AGEs may be central to the exacerbation of dementia and enhanced predilection of stroke in aging subjects. In this context, both AGEs and Aβ are signal transduction ligands of RAGE. The link between Aβ–RAGE interaction and Alzheimer pathology has been studied in experimental systems. The overexpression of mutant amyloid precursor protein (APP)

and neuronal RAGE in transgenic mice accelerates hippocampal neuronal loss and decline in long-term potentiation and behavior [59]. RAGE interacts with Aβ as well as other beta-sheet fibrils, such as prion peptide [8]. The settings of diabetes and Alzheimer's disease are further complicated by the premise that in Alzheimer's disease subjects, the pancreas (islets), too, may be affected by amyloid deposition. Taken together, such data support the hypothesis that vulnerability to diabetes and Alzheimer's disease may share a key common link.

3.2 AGE–RAGE, Aging, and the Heart

The aging heart is susceptible to glycation processes, consequences of which include ventricular dysfunction, such as increased ventricular and vascular stiffness, and altered regulation of vascular tone [60]. Potential roles for RAGE in the cardiac dysfunction of AGEing are suggested by the studies of Simm and colleagues. These authors reported that in human subjects undergoing cardiac surgery, an age-dependent increase in RAGE protein in the atria was observed, with the highest levels observed in the senescent population and the lowest levels observed in the youngest children [61]. Correlation analyses revealed that the degree of RAGE expression was associated with reduced heart function in these subjects. Although the potential mechanistic link between these observations remains to be elucidated, these experiments, nonetheless, underscore the observation that RAGE is upregulated at sites of tissue stress in an array of disorders, including the aging human heart.

In the isolated perfused heart, AR inhibition protects hearts from ischemic injury [62–66]. The apparent interdependence of AGE–RAGE and AR pathways in the generation of AGEs and ROS led us to test the concept that RAGE mediates, at least in part, cardiac dysfunction in the diabetic heart. Recent studies from our laboratory have shown that CML-AGEs are increased in the diabetic mouse and diabetic rat hearts; in parallel, RAGE expression was enhanced particularly in endothelial cells and infiltrating macrophages [67]. Furthermore, exposure to ischemia/reperfusion increased expression of AGEs and RAGE and was linked to injury, increased oxidant stress, and impaired contractile function in diabetic and nondiabetic hearts. Blockade of RAGE attenuated oxidant stress, improved contractile function recovery, and reduced injury in hearts [67, 68]. These findings underscore the premise that AR- and RAGE-dependent pathways likely synergize in the diabetic heart to drive exaggerated inflammation and metabolic dysfunction. The relevance of these findings to the treatment of human aging heart disease has yet to be tested. Preliminary studies from our laboratory have shown increases in AGEs, RAGE, and AR in hearts from aging Fischer 344 rats and humans [69–72]. Clinically, in the context of AR inhibition alone, it has recently been shown that the administration of the specific inhibitor of AR (zopolrestat) for 1 year in human diabetic subjects resulted in increased resting left ventricle ejection fraction, cardiac output, left ventricular (LV) stroke volume, and exercise ejection fraction [73].

3.3 Nutritional and Therapeutic Approaches to Limit AGEs

Vlassara and colleagues have suggested that diets high in AGEs may accelerate the consequences of natural aging and diabetes [74, 75]. When tested in human subjects, the reduction of inflammatory mediators was observed in human diabetic subjects consuming low-AGE food [76]. Further, the restriction of glycotoxins was found to reduce excess levels of AGEs in human subjects with renal failure [77]. Taken together, these observations strongly suggest that lifestyle modification, including diet, may exert potent influences on inflammation and aging.

To break the cycle of AGE formation and protein cross-linking, many strategies are in various stages of development to either block AGE formation or break down established AGE cross-links in the tissues. The first agent studied in the context of agents to prevent AGE formation was aminoguanidine (or pimagedine). Based on extensive preclinical efficacy in animal models in which AGEs are prevalent [78, 79], pimagedine was administered to 690 subjects with type 1 diabetes, nephropathy, and retinopathy. The estimated glomerular filtration rate progressed more slowly in the pimagedine-treated group, and the degree of proteinuria was significantly lower in the pimagedine-treated group [80]. However, the trial failed to meet significance in the primary end point (doubling of serum creatinine); yet, notwithstanding this finding, this trial was the first to support the AGE hypothesis and its role in diabetic complications in human subjects based on changes in secondary end points. The next level of anti-AGE strategies involves the AGE cross-link breakers. The prototypic agent, ALT-711, has already been tested in human clinical trials. After 56 days of treatment, ALT-711 resulted in improved total arterial compliance in aged humans with vascular stiffening [81, 82]. In addition, newer agents, such as pyridoxamine, an inhibitor of advanced glycation reactions, are on the horizon. This target has shown efficacy in preclinical models to date [83]. It is our hypothesis that even fleeting AGE deposition imparts a first and early signature to the tissues that may be, if not checked naturally or pharmacologically, transduced into chronic cellular perturbation. However, it is highly likely that AGEs serve homeostatic purposes, perhaps in clearance of microbes or quenchers of biochemical species that are likely to cause injury. Although the balance of the evidence suggests that AGE species may trigger maladaptive responses in the organism, there is also a reason to suspect that exclusive roles for AGEs in pathology may be too simplistic.

4 Conclusions

In addition to natural aging, acute stresses may trigger rapid AGE generation. As illustrated in Fig. 1, we hypothesize that stimulated by oxidative stress and ROS generation, inflammatory stimuli, stress such as hypoxia/ischemia, and polyol pathway activity, AGE formation is likely a key *first* step in a broad array of injury settings. Once formed, such AGEs are central to amplification of stress pathways. In aging, reduced anti-AGE defenses likely contribute to increased accumulation of

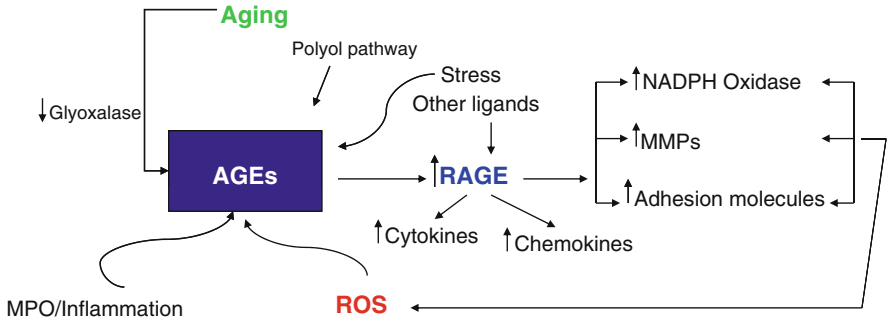

Fig. 1 Scheme illustrating the potential AGE-driven mechanisms that are central to amplification of stress pathways leading to cellular perturbation and tissue injury in aging

these species, thus amplifying AGE effects. Specific consequences of AGE accumulation are (a) the upregulation of RAGE itself, (b) the attraction of inflammatory cells, such as polymorphonuclear leukocytes, MP, and lymphocytes, and (c) further generation of ROS; due to AGE/ligand–RAGE interaction, such ROS may beget further AGE generation, inflammation, and ROS production. Such feedback mechanisms may sustain the cycle of cellular stress, and, thus, eventually cause tissue dysfunction and irreparable damage in aging. The testing of these premises will require agents to target AGEs and/or their signal transduction receptor RAGE, first in preclinical models, and then in clinical trials.

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Sirtuins and Mammalian Aging

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Abstract Aging is characterized by a time-dependent loss of function, generally agreed to be the result of an increased incidence of damage produced by free radicals and other reactive oxygen species and a decreased ability of the animal to mount sufficient protective defenses to maintain adequate function. In many species, imposing food calorie restriction is effective at bolstering these defenses and extending life span. At the same time, caloric restriction induces increased activation of one or more regulatory enzymes, the sirtuins. Sirtuins are NAD^+-dependent deacetylases that act on a wide array of protein substrates to connect metabolic state – as reflected in the NAD^+ concentration – with regulation of many key cellular and physiologic processes including apoptosis, DNA repair, inflammation, immunity, transformation, and autophagy. Although increasing the expression of a single sirtuin in a few simpler and shorter-lived animals can significantly extend life span, the effects on mammalian life span of any of the seven mammalian sirtuins are much less robust. Whereas overexpression of sirtuins in mammals typically improves physiology without increasing life span, reduction often results in progeria, tissue degeneration and dysfunction, shortened life span, or outright lethality. Changes in sirtuin expression or activity are often found to play key roles in a variety of age-associated degenerative diseases in both humans and in animal models; treatments that appropriately modulate sirtuin activity often ameliorate these conditions. Thus there is accelerating interest in advancing understanding not only of the roles sirtuins normally play in regulating and maintaining homeostasis but also in how to modulate their activities so as to treat age-related degenerative diseases more successfully.

Keywords Sirtuins · Aging · Mammals

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1 Introduction

Aging has been defined as "the loss of functional reserve over time, leading to a decreased capacity to maintain homeostasis under stress and increased risk of morbidity and mortality" [1]. The rate of aging may be roughly gauged by mean life span, a function of mortality. Mean life span is largely determined by species, indicating that genetics exerts significant control over the aging process. But within a species, individual life span is highly variable. For animal species at least, the shape of the mortality curve that determines life span does not follow Gaussian or normal statistics. This indicates that the underlying aging process is nonrandom and highly complex; that is to say, it is controlled in a nonlinear fashion by many independent but interacting variables. Indeed, the one modality – caloric restriction – that extends life span in a wide variety of animal species accomplishes this not by affecting just one variable but by modulating a large number of interconnected physiologic and molecular parameters. Unsurprisingly, no single substance has been identified to date as an "elixir of life" or "fountain of youth."

It light of this, it is remarkable that modulation of one class of endogenous enzymes, the sirtuins, can effect measurable control over life span and of overall health during an organism's lifetime. As deacetylases that depend on nicotinamide adenine dinucleotide (NAD^+), sirtuins are uniquely able to couple metabolic state (as encapsulated by the NAD^+ concentration) with modulation of the activity of a wide variety of transcription factors, gene-silencing histones, and regulatory and repair proteins. The importance of sirtuin proteins is underscored by their occurrence in a very wide range of organisms that includes archaeans, bacteria, yeasts, plants, and metazoans [2]. In all those species in which sirtuins have been studied, invariably they have been found to play key roles in regulating essential processes such as metabolism, DNA repair, cell cycle control, and stress response.

1.1 Aging and Longevity

Thermodynamics dictates that biological systems, just as for the universe as a whole, are subject to entropic disorder over time [3], manifested, for example, by oxidative damage to biomolecules, genetic instability, and protein glycosylation, misfolding, denaturation, and aberrant aggregation. By the disposable soma theory [4], organisms have developed systems to hold these dysfunctional occurrences at bay until reproductive maturity, after which the accumulating stochastic damage causes a gradual decline of function finally resulting in death [5]. That some species enjoy much longer mean life spans than others is an indication that the basic power to cope with entropic damage is genetically determined. That there is a high variability in life span among individuals of the same species indicates that there are stochastic environmental and developmental forces that powerfully modulate the fundamental genetic program. This has been succinctly summarized as "Entropy explains aging, genetic determinism explains longevity" [3].

Efforts to increase longevity experimentally have met with some success. In relatively simple and short-lived animals, a number of nutrient supplements and single-gene mutations – notably sirtuin orthologs – have been demonstrated to increase life span of organisms such as yeast, worms, and flies. In mammals, such single-element interventions have not had comparable success. In contrast with the life-span extension produced by sir2 in yeast, the cellular replicative life span of human fibroblasts is not extended by individual overexpression of any of the seven human sirtuins [6]. Increased life span in mammals has been demonstrated only for more complex, physiologic interventions such as caloric restriction [7], exercise [8], or heterochronic parabiosis (old/young shared circulatory systems [9]). Indeed, even in the case of caloric restriction, a physiologic stressor generally acknowledged as demonstrating the most robust extension of life span in the widest range of species, no increase has been shown to date in either monkeys [10] or humans.

Yet a number of biomarkers are related to life span and the rate of aging. A more oxidative state accompanied by increased oxidative damage is nearly universally observed as mammals age and succumb to degenerative diseases [11]. Paralleling this is an age-related dysregulation of the immune system – a source of chronic, low-level inflammation and increased production of reactive oxygen species (ROS) – a condition that has been termed *inflamm-aging* [12–14].

As the age-associated decline in function progresses, the incidence of a set of degenerative diseases increases roughly exponentially. These diseases include cancer, type 2 diabetes, Alzheimer's disease, Parkinson's disease, cardiovascular disease, macular degeneration, and osteoarthritis. These conditions almost invariably are accompanied by a common set of degenerative indices, including (1) increased generation of ROS (2), consequent accumulation of oxidative damage to DNA, RNA, proteins, and lipids (3), irresolvable protein aggregates (4), increased protein nitrosylation and glycosylation (5), genetic instability, and (6) chronic activation of inflammatory pathways [12]. To a remarkable degree, expression and/or activation of sirtuins appears to prevent or correct these indices, so that sirtuin modulation holds great promise for the amelioration of age-associated degenerative diseases and perhaps for the slowing of the aging process itself.

1.2 Sirtuin Function and Longevity

Silent mating type information regulation 2 homologs (sirtuins) are, in mammals, a class of seven enzymes denoted Sirt1–Sirt7 that are predominately deacetylases but may act weakly as protein mono-ADP-ribosylases (Table 1; for recent reviews see [15–19] and [20]). In either case, they utilize NAD^+ as cofactor-substrate. During cellular metabolism, NADH, the reduced form of NAD^+, is produced by glycolysis, by β-oxidation, or by the tricarboxylic acid cycle; it is then reoxidized to NAD^+ by mitochondrial oxidative phosphorylation. Dependent as the sirtuins are on NAD^+, their activity is thus highly responsive to the metabolic state of the cell [21]. Histones were the first class of proteins to be identified as sirtuin substrates; currently, sirtuins

Table 1 Effects of sirtuin deacetylation, ADP-ribosylation, and binding on longevity and degenerative disease.

Sirtuin	Substrate	Function	Effect on longevity or disease	References
Sirt1, Sirt3	AceCS (ACSS2)	Metabolism	Caloric restriction response	[141]
Sirt1	Acaca	Fat metabolism		[142]
Sirt1	BCL6	Apoptosis, fat metabolism	B-cell transformation ↑	[134]
Sirt1	β-Catenin		Colon cancer suppression	[130]
Sirt1	Cortactin	Cell migration ↑	Cancer metastasis (↑?)	[137]
Sirt1	CTIP2 (Bcl11b)			[143]
Sirt1	[b]E2F1	Apoptosis	DNA damage modulation	[144]
Sirt1	Histone H1	Transcription		[145]
Sirt1, Sirt6	Histone H3	Transcription	Inflammation suppression	[85, 89, 145]
Sirt1, Sirt2	Histone H4	Transcription		[72, 145]
Sirt1	HSP70	Protein folding		[142]
Sirt1, Sirt7	p53 activity ↓	Apoptosis ↓; cell survival ⇧	Vascular health	[42, 146, 147]
Sirt1	P73	Apoptosis		[148]
Sirt1	P300	Transcription		[149]
Sirt1	PARP1	Stress response	Cardiomyocyte cell survival ⇧	[150]
Sirt1	[b]DNA	Repair		[69]
Sirt1	eNOS		Vascular health	[151]
Sirt1, Sirt2	FoxO1	Transcription, apoptosis	Vascular health	[152, 153]
Sirt1, Sirt2	FoxO3a activity ↓	Apoptosis ↓		[74, 100, 154]
Sirt1	FoxO4	Stress response		[155]
Sirt1	HIV tat	Transcription		[156]
Sirt1	IRS2		Oxidative stress sensitivity ↑	[43]
Sirt1	LKB1		Lipid metabolism	[157]
Sirt1	LXR	Cholesterol regulation	Atherosclerosis	[158]
Sirt1	Ku70	DNA repair ⇧ apoptosis ⇩		[159, 160]
Sirt1	NBS1	DNA damage response		[161]
Sirt1	NF-κB (RelA)	Apoptosis	Inflammation ⇩	[98, 162]
Sirt1	PGC-1α	Transcription	Mitochondrial biogenesis	[67, 163]
Sirt1	STAT3	Gluconeogenesis		[164]

Table 1 (continued)

Sirtuin	Substrate	Function	Effect on longevity or disease	References
Sirt1	SUV39H1	Trimethylation of H3K9 → heterochromatin modulation	Connects glucose levels to ribosomal RNA synthesis	[165, 166]
Sirt1	TAF_I68	Polymerase I transcription	Connects glucose levels to ribosomal RNA synthesis	[165, 167]
Sirt1	Wrn	Stabilize, activate and move Wrn to nucleoli	Maintain genome stability ⇧	[142]
Sirt1	Zyxin	Actin filament formation	CNS development	[168]
Sirt2	α-Tubulin	Cell cycle		[169]
Sirt2	[a]HDAC6	Cell cycle		[170]
Sirt3	ACSS2 P	Metabolism		[171]
Sirt3	IDH2 P	Metabolism		[81]
Sirt3	[b]NDUFA9 (complex I)	Regulates basal ATP levels	Complex I inhibited in $Sirt3^{-/-}$ mice	[172]
Sirt3, Sirt4	[a]GDH ↓	Metabolism; insulin secretion		[25, 80]
Sirt5	CPS1	Urea cycle regulation	Ammonia detoxification ⇧	[84]
Sirt5	Cytochrome c	Oxidative metabolism, apoptosis		[81]
Sirt6	[a]DNA polymerase β	BER, DNA repair		[86]
Sirt7	[b]RNA polymerase I	rRNA transcription ⇧		[93]

Sirtuins deacetylate substrates, except [a]ADP-ribosylation, [b]binding. ⇧, ⇩ beneficial, and ↑, ↓ detrimental changes

are known to deacetylate a wide variety of proteins. Thus they act to couple the cellular metabolic state not only with histone-mediated gene silencing but also with a wide variety of other processes critical to an organism's longevity including DNA repair, chromatin regulation, cell cycle control, and stress response [22]. Sirtuin activity is localized in several key parts of the cell – in the nucleus (Sirts 1, 2, 6, and possibly Sirt3), in the nucleolus (Sirt7), in mitochondria (Sirts 3, 4, and 5), and in the cytoplasm (Sirts 1 and 2).

As deacetylases, sirtuins induce the displacement of nicotinamide from NAD^+ by an acetyl moiety; in the process, the acetyl moiety is detached from the ε-amino group of a protein-bound lysine residue to form *O*-acetyl-ADP and an unsubstituted lysyl ε-amine on the substrate protein [23] (Fig. 1). This reaction converts an uncharged lysyl acetamide to a positively charged lysyl ammonium ion; the

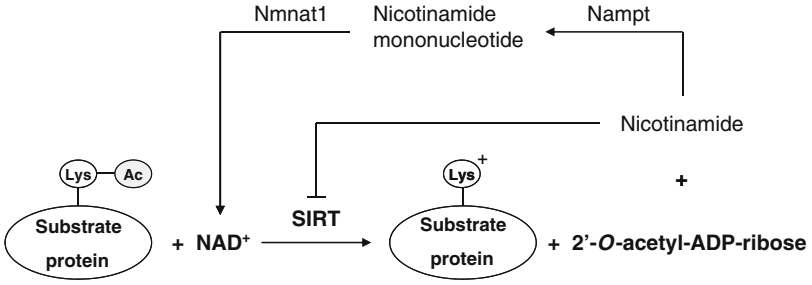

Fig. 1 Sirtuin NAD^+-dependent deacetylase activity. Sirtuins remove acetyl moieties from the ε-amino groups of protein substrate lysine residues by cleaving NAD^+, attaching the acetyl moiety to the resulting ADP-ribose, and releasing nicotinamide. The product nicotinamide inhibits the reaction but can be recycled to NAD^+. Nampt = nicotinamide phosphoribosyltransferase; Nmnat1 = nicotinamide nucleotide adenylyltransferase

change in charge and residue size often substantially modifies the activity and/or conformation of the substrate protein.

In ADP-ribosylation, nicotinamide is displaced by a cysteine thiol or lysine amino group to form the mono-ADP-ribosylated protein (Fig. 2). Although ADP-ribosylation activity for many sirtuins can be demonstrated in vitro, this activity may be weak [24], and there is little indication of any physiologic, in vivo role. The sole currently known exception is the inhibition of glutamate dehydrogenase (GDH) by Sirt4 in pancreatic β-cell mitochondria via ADP-ribosylation [25].

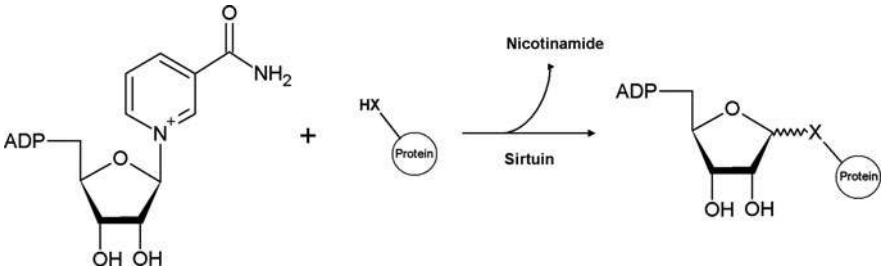

Fig. 2 Sirtuin-catalyzed ADP-ribosylation. ADP ribose is transferred from NAD^+ to a protein substrate, accompanied by release of nicotinamide. ADP = adenosine diphosphate, X = NH or S. ADP-ribosylation is most important for Sirt4 and Sirt6

Sirtuin activity is in turn subject to regulation. Sirtuin activity is typically subject to product inhibition by nicotinamide, and the nicotinamide concentration is in turn modulated by a nicotinamide-recycling loop (Fig. 1). This loop converts nicotinamide back into NAD^+ under control of the rate-limiting phosphoribosyl transferase Nampt and of the transferase Nmnat. This regulatory network is quite intricate, as Sirt1 histone deacetylase activity is also regulated directly by the presence of Nampt or the binding of Nmnat to Sirt1 at the promoters of Sirt1-targeted genes [26]. Thus sirtuin activity is controlled not only by reactant availability and

product inhibition but additionally by direct interaction with the key enzymes that modulate reactant and product concentrations. This multilevel regulatory network that both modulates Sirt1 activity and tightly controls reactant and product concentrations has been termed the "NAD World"; dysregulation of this mechanism has been linked to diabetes and other age-related degenerative conditions [27].

Sirt1 activity also can be modulated by phosphorylation [28], sumoylation [29], or by a variety of small molecules. In addition to the reaction product nicotinamide, sirtinol is among Sirt1's inhibitors, and the stilbene resveratrol is probably the best known of its activators. Care must be taken, however, in interpreting the physiologic effects of small molecules as having sirtuin-specific pathways because they may act via other routes. For example, dietary resveratrol reduces amyloid-β (Aβ) plaque levels in an Alzheimer's disease mouse model independent of Sirt1 activation [30]. Regardless, substantial efforts are being put forth to identify potent small-molecule sirtuin modulators [31–35].

The connection between sirtuin action and longevity is direct for relatively simple organisms with comparatively short life spans. Increased expression of the yeast sirtuin sir2p leads to longer life span, and mutation or deletion leads to shortened life span in yeast [36]; its ortholog in worms [37, 38] has a similar effect on life span. In *Drosophila melanogaster*, an extra copy of the ortholog dSir2 increases longevity in both males (by 18%) and females (by 29%) [39].

The connection between longevity and sirtuin function in mammals is by no means as clear-cut, but connections between mammalian sirtuins, aging, and degenerative diseases have been intensively reviewed [40, 41]. Knockout of *Sirt7* leads to shortened mean and maximum life span of mice [42]. Although deletion of *Sirt1*, the closest mammalian ortholog to *Sir2p*, produces generally deleterious effects on longevity [43] and health, its overexpression has variable, and not always beneficial, effects. For example, whereas Sirt1 expression decreases with age in brain cortex of the SAMP8 senescence-accelerated mouse but not in cortex of the normal SAMR1 mouse [44], Sirt1 overexpression is associated with microsatellite instability and high tumor grade in human colorectal cancer tissue [45].

Several studies have sought to find a link between sirtuin genetic variations and human longevity. The strongest linkage is with *Sirt3* variations. Variable number tandem repeat (VNTR) enhancer polymorphisms in *Sirt3* intron 5 differ significantly between centenarians and younger subjects [46]. This study of 945 subjects aged from 20 to 106 years determined that one of these alleles lacks enhancer activity that resulted in lower Sirt3 levels in a reporter gene assay. This nonfunctional allele was absent in all males over 90 years old, suggesting that increased Sirt3 levels are important for human longevity. A second study [47] of 710 subjects aged 18–108 years found a significant age-dependent variation in the regulatory region of *Sirt3*. Interestingly, this region coregulates a proteasome component gene, suggesting that this age-associated variation is capable of benefiting not only Sirt3 activity but also the age-related decline in proteasomal function as well.

The linkage between *Sirt1* variations and human longevity is considerably weaker. These include a case-control comparison of allelic variations in younger (aged 60–75 years) and 1,026 elderly (aged 95–109 years) German subjects [48],

and a comparison of prevalence of five single nucleotide polymorphisms (SNPs) in 1,245 nonagenarians and centenarians versus that in young control subjects [49]. Taken together, these studies indicate that Sirt1 genetic variations do not significantly influence human longevity or cause of mortality.

Given the direct relation between sirtuin levels and life span in relatively simpler and shorter-lived species, it might be anticipated that sirtuin expression could change with age in longer-lived animals. However, no changes in sirtuin gene expression levels have been reported in a number of comprehensive microarray-based surveys of age-related gene expression changes in either mice [50, 51] or humans [52, 53]. It might also be anticipated that the number and/or structure of sirtuins might differ substantially between shorter-lived mammals such as mice and longer-lived mammals such as humans, given that there is roughly a 30-times difference in life span between these two species. However, mice and humans and, so far as is known, other mammals all use the same set of seven, highly homologous sirtuins.

Currently, sirtuins do not appear to interact with or directly affect antioxidant genes such as the dismutases, or cysteine lygases that synthesize glutathione or antioxidant repair genes such as thioredoxins, or methionine sulforeductases, or lipid peroxide repair proteins. Of the cellular repair and restoration processes, sirtuins primarily seem to affect directly (1) the DNA repair that could be produced by free radical damage, and (2) autophagy. Even so, whereas three of the seven are in mitochondria, none are known to be found in peroxisomes or to interact with proteosomes. Thus, sirtuins seem to act primarily as regulators of metabolic function that act to minimize oxidative and other stress levels in the first place, and less as modulators of repair machinery that improve the cell's response to increased stress. This is in accord with the observation that longer-lived organisms generally have lower rates of mitochondrial oxygen radical production, rather than higher levels of antioxidant enzyme activities (which are in fact lower than those in shorter-lived organisms) [54].

2 Sirtuins: Localization, Substrates, and Functions

2.1 Sirt1

Sirt1 is the closest mammalian ortholog to yeast Sir2 and the most thoroughly studied of the seven mammalian sirtuins (reviewed in [55–58]). Sirt1 is found in either the nucleus or the cytoplasm, and a shuttling mechanism appears to enable its dynamic transport between these two regions [59]. Where it is located, then, can depend on cell type, tissue, developmental stage, and treatment conditions.

In contrast with Sirt1's yeast ortholog Sir2, mammalian Sirt1 does not seem to extend life span. While Sirt1-null mice die soon after birth [60], the life span of Sirt1 heterozygous knockout mice is the same as that of wild-type mice of the same strain [61]. Rather than straightforward life-span extension, Sirt1 overexpression

experiments show variable effects that depend on how the overexpression was produced, and in which cell type(s) [62]. Sirt1 modulates key aging-impaired processes such as stress resistance and adaptation to metabolic imbalances (due to such conditions as fasting, exercise, nutritional deficiencies, and caloric restriction) [63]. For example, mice with Sirt1 knocked into the β-actin locus widely overexpress Sirt1 and resemble those on caloric restriction [64].

As aging is a pleiotropic, multifactorial process, it is to be expected that the regulatory mechanisms should be complex. Thus, beneficial effects can be found both by activating and by antagonizing Sirt1 activity [65]. Sirt1 agonists have a demonstrated role in aging and in modulating age-related diseases. Resveratrol, the best known of these, can extend life span in lower organisms and ameliorate risk factors for age-related degenerative diseases in mammals [66]. However, resveratrol acts via other additional pathways, so, as previously pointed out, its anti-aging effects may utilize in part mechanisms not mediated by Sirt1.

The Sirt1 protein acts on a large number of substrates that regulate cellular oxidative, genotoxic, and environmental stresses (Table 1). Related to metabolic regulation, the Sirt1 deacetylation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is required for activation of mitochondrial fatty acid oxidation genes [67]. Demonstrating its involvement in immunity and inflammation, Sirt1 deacetylates the RelA (p65) subunit of NF-κB. An indication of its involvement with DNA maintenance is Sirt1's ability to deacetylate the progeroid Werner's syndrome protein (Wrn) and to regulate the location of this DNA helicase [68]. Increased Sirt1 expression promotes survival in a mouse model of genomic instability and suppresses age-dependent transcriptional changes [69].

Many human cancers exhibit reduced levels of Sirt1 compared with those in normal controls; this may be a consequence of Sirt1 acting as a tumor suppressor through its role in DNA damage response and genome integrity [70]. Conversely, H_2O_2 accelerates cellular senescence by accumulation of the acetylated, activated form of the proapoptotic, tumor suppressor protein p53 via a decrease in the function of Sirt1 by NAD^+ depletion [71].

Sirt1 expression and activity are regulated at many levels, ranging from transcription to posttranslational modifications, and each mode of regulation has implications for degenerative diseases and response to age-related damage [57].

2.2 Sirt2

The deacetylase Sirt2 is located in both cytoplasm and nucleus. In the nucleus, it acts to preserve the integrity of the genome during mitosis by blocking entry into chromosome condensation in response to mitotic stress caused by microtubule inhibitors [72]. Sirt2 may affect progression through mitosis in response to stress [73]. It can also promote cell death in cells under severe stress by inducing increased expression of the proapoptotic factor Bim. In doing so, Sirt2 acts by binding to and deacetylating FoxO3a; this has the consequence of elevating Bim and the antioxidant Mn-SOD, both FoxO target genes [74].

Sirt2 influences a wide variety of other processes, including interference with cell adhesion in tumor cells, cell migration in fibroblasts, and neurite outgrowth and growth cone motility in neurons, and is itself subject to inhibitory regulation by phosphorylation at a single phosphorylation site [73].

Sirt2 is enriched in oligodendroglia, where it participates in normal oligodendrocyte differentiation and myelin formation by deacetylating α-tubulin; acetylation of α-tubulin appears to increase differentiation whereas deacetylation decreases it, suggesting that Sirt2 may act to prevent these morphologic changes from getting out of control [75]. Acetylation of tubulin stabilizes it, so tubulin deacetylation by Sirt2 accelerates axonal degeneration in WLD^s neurons [76].

Tubulin plays a role in control of mitochondrial respiration by regulating voltage-dependent closure of the voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane [77]. Dysregulation of mitochondrial respiration increases with age, but the possible contribution of impaired extra-mitochondrial regulation of this process is little explored. If tubulin's control of VDAC permeability were regulated by tubulin acetylation, it would provide a means by which Sirt2 could influence mitochondrial respiratory homeostasis.

2.3 Sirt3

Although its location has been controversial, most evidence shows that Sirt3 is found predominately, perhaps exclusively, in mitochondria [78].

The crystal structure of Sirt3 has been determined, both unbound and bound with a substrate peptide [79]. This study showed that the substrate protein first binds and induces a conformational change that then allows the NAD^+ cofactor to bind and react.

Sirt3 deacetylates and activates a number of key metabolic enzymes, including ACSS2 [79], GDH [80], and IDH2 [81]; despite its ability to modulate these critical metabolic enzymes, Sirt3-deficient mice show no metabolic irregularities, even when subjected to fasting or cold [80].

2.4 Sirt4

Sirt4 is resident in the mitochondrial matrix, where it acts not as a deacetylase but as a catalyst for mono-ADP-ribosylation of proteins [82]. It affects the rate of insulin secretion in pancreatic β-cells by interacting with two important metabolic enzymes; that is, by repressing the activity of glutamate dehydrogenase [25] and by modifying the ANT2/3 subunit of the ATP/ADP translocase [82].

Caloric restriction generally seems to increase sirtuin activity. However, Haigis et al. [25] propose that caloric restriction downregulates Sirt4 activity and activates a switch to increase amino acid–stimulated insulin secretion.

2.5 Sirt5

Sirt5 is located in mitochondria [6], probably in the intermembrane space [81], where it acts as a deacetylase. The structure of Sirt5 has been reported either bound to the reaction intermediate ADP-ribose or to the inhibitor suramin [83]. In contrast with the numerous proteins deacetylated by Sirt1, Sirt5 currently is known to deacetylate only a few, including the urea cycle–regulating enzyme CPS1 [84]. Fasting (and perhaps caloric restriction) activates Sirt5, resulting in increased deacetylation of CPS1 and upregulation of ammonia detoxification via the urea cycle. During fasting, protein catabolism accelerates, generating increased amounts of urea. Thus Sirt5 provides the appropriate metabolic adjustments required so the liver can process the increased amounts of urea produced during fasting [84]. Given Sirt5's pivotal role as modulator of the urea cycle in response to fasting, future research may profitably investigate its ability to correct possible aging-related dysregulation of urea metabolism.

Sirt5 also deacetylates cytochrome c, but the physiologic effects of this are not yet known [81]. Understanding how deacetylation may affect cytochrome c activity is a potentially important area of investigation, as cytochrome c plays essential roles in both oxidative metabolism, in which it shuttles electrons between complexes III and IV of the mitochondrial electron transport system (ETS), and in apoptosis, when its release into the cytosol triggers apoptosome formation and caspase activation. Loss of function and dysregulation of the ETS and dysregulation or inappropriate triggering of apoptosis have long been identified as important hallmarks of the aging process.

2.6 Sirt6

Sirt6 is located in the nucleus associated with heterochromatin [6] and telomeres, where it deacetylates histone H3 [85]. Consistent with its location, Sirt6 is involved in the DNA repair process of base excision repair and helps to preserve cells from genomic instability; Sirt6 deficiency in mice results in shortened life span and a progeroid phenotype [86]. Sirt6 depletion also leads to premature cellular senescence and to telomeric dysfunction associated with abnormal telomeric structures that resemble defects observed in the premature aging disorder known as Werner's syndrome [85]. These are indications that Sirt6 plays a key role linking DNA repair, phenotypic aging, and life span [87].

An indication that Sirt6 may play a role in the regulation of metabolism is the observation that its levels are increased by caloric restriction [88].

Sirt6 is intimately involved in the inflammatory response. Sirt6 interacts with NF-κB and suppresses expression of NF-κB–regulated genes by deacetylating lysine 9 of histone H3 (H3K9) proteins that are bound to a subset of NF-κB–regulated promoters (Fig. 3); Sirt6$^{-/-}$ mice die at 28 days of multiorgan failure, and this defect is reversed by NF-κB depletion [89]. In lipopolysaccharide-stimulated

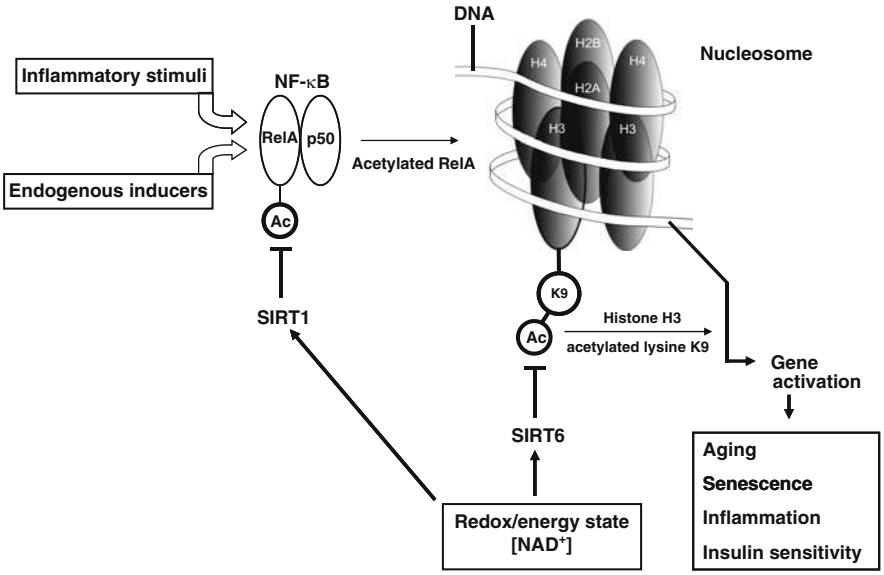

Fig. 3 Sirtuins can suppress excessive inflammatory gene activation mediated by NF-κB at two different points. Sirt1 can bind to, and deacetylate, the RelA component of NF-κB, resulting in suppression of NF-κB transactivation of genes otherwise upregulated with age. Sirt6, like Sirt1, can also associate with chromatin-bound NF-κB. Under these conditions (1), Sirt6 is induced to deacetylate the nucleosome component histone H3 and (2) the binding of NF-κB to chromatin is destabilized; the result is again suppression of age-associated gene expression. Ac = acetyl moiety; K9 = lysine 9 of histone H3. Adapted from [91]

macrophages, adequate levels of NAD^+ are required for translation of the proinflammatory cytokine TNF. Sirt6 connects this requirement to TNF translation while having no effect on TNF mRNA levels [90]. This modulation of translation suggests a wider functional role for Sirt6 beyond its ability to deacetylate histones and its involvement with NF-κB–regulated promoters. Thus, as an NAD^+-sensitive enzyme, Sirt6 connects metabolic state with inflammatory activity and the immune response [91] by multiple mechanisms.

2.7 Sirt7

Unique among sirtuins, Sirt7 is localized to the nucleolus where it regulates ribosomal DNA transcription [92]. Sirt7 interacts with RNA polymerase I, the enzyme responsible for producing ribosomal RNA, as well as with histones. As its depletion stops cell proliferation, Sirt7 is required for cell viability [93]. Sirt7 also interacts with and inactivates the proapoptotic factor p53, and its depletion results in increased apoptosis, cardiomyopathy, and inflammation in mouse heart [42].

3 Sirtuins in Aging-Related Processes

3.1 Immunity, Inflammation, and Aging

Higher organisms utilize both innate and adaptive immunity as major defenses against endogenous and environmental threats; aging is associated not only with a decline in adaptive immunity but also with an aberrant, chronic activation of the innate immune system. A key component of the innate immune response is the transcription factor NF-κB. In addition to immune response, this dimeric factor is key to the response to a number of other environmental and cellular stressors, including oxidative stress, hypoxia, and genotoxic stress [13]. The patterns of gene expression induced by activated NF-κB are essential to the normal and healthy response to environmental and cellular stresses, and the pathways regulating the appropriate activation of NF-κB to a number of these stressors, such as infection and oxidative stress, have been determined [94]. However, many genes induced by NF-κB are proinflammatory, so that their aberrant chronic or excessively high expression can induce damage. It is these aberrant or atypical patterns of NF-κB–induced gene expression, perhaps related to the normal response to viral attack, that seem to be associated with age and may provide a mechanism for the functional decline associated with it [95, 96].

NF-κB is normally sequestered in the cytoplasm but migrates to the nucleus upon activation. Whereas cytoplasmic levels remain constant with age, nuclear (activated) levels of NF-κB, particularly its p65 (RelA) and p52 subunits, increase with age in a variety of different tissues of both rats and mice [96]. Sirtuins may be able to mitigate the increased nuclear presence of NF-κB in aged animals, and therefore reduce age-associated proinflammatory damage, by at least two mechanisms. First, Sirt1 is able to bind and deacetylate RelA, resulting in a reduction in NF-κB gene activation (Fig. 3) [97, 98]. This Sirt1-induced repression of NF-κB activation also requires the presence of the transcriptional corepressor TLE1; it is possible that RelA deacetylation is not necessary for NF-κB repression, but that the mere association of both Sirt1 and TLE1 with RelA is sufficient [99]. A second mechanism, mediated by the FoxO transcription factors, has been described [97]. Sirt1 can deacetylate FoxO1 and FoxO3, most commonly resulting in their activation. In this way, Sirt1 can increase the ability of FoxO3 to protect cells against oxidative stress (as heightened in aged tissue), reduce its ability to induce apoptosis [100], and thus help maintain tissue integrity. These effects could reasonably be ascribed to countering the increased levels of activated NF-κB in aged animals, as ultimately an often-observed consequence of FoxO activation is repression of NF-κB.

3.2 Autophagy

Life span is related to the efficiency of autophagy, the process of cleaning up and removing cellular debris, and the integrity of the autophagosomal–lysosomal

network appears to be critical in the progression of aging [101]. Moreover, conditional deletion of autophagy genes can generate accelerated age-related neuropathologies in mice [102]. Sirt1 activity is necessary to induce autophagy, at least when initiated by starvation, as autophagy is not activated by deacetylase-inactive Sirt1; the mechanism likely involves deacetylation of a set of autophagy-related (Agt) proteins required for autophagosome formation, because the acetylation of these proteins is dramatically increased in the absence of Sirt1 [103, 104].

4 Sirtuins in Aging-Associated Degenerative Diseases

4.1 Type 2 Diabetes and Metabolic Syndrome

Type 2 diabetes (NIDDM) is characterized by the declining ability of peripheral cells to absorb glucose in response to insulin stimulation; initially the pancreatic β-cells compensate by secreting additional insulin, but they eventually undergo apoptosis and lose their ability to secrete sufficient insulin, resulting in hyperglycemia and its sequelae [105]. Age is a highly important risk factor for NIDDM [106]. A number of studies have demonstrated generally beneficial effects of Sirt1 overexpression in treating diabetes in animal models. Transgenic mice with Sirt1 knocked into the β-actin locus exhibited improved glucose tolerance [64]. Use of a bacterial artificial chromosome (BAC) insertion technique to induce universal Sirt1 overexpression produced ("SirBACO") mice with improved glucose tolerance when subjected to a high-fat, diabetes-inducing diet, or when crossed with a diabetes model strain. Rather than any dramatic improvement in peripheral insulin resistance or increase in β-cell insulin secretion (which was unchanged), the improved tolerance was a result of decreased hepatic glucose production and higher plasma adiponectin levels [62].

In contrast, 3- or 8-month-old mice with 12-times to 18-times Sirt1 overexpression limited to pancreatic β-cells ("BESTO" mice) also demonstrated improved glucose tolerance, but the mechanism differed [107]. In the case of the *BESTO* mice, the improvement was due to enhanced β-cell insulin secretion that was accompanied by reduced β-cell uncoupling protein 2 (UCP2) levels and, consequently, increased amounts of ATP. Unfortunately, these beneficial improvements were blunted but not eliminated by the time the *BESTO* mice reached 24 months of age [108]. The loss of improved tolerance in the older animals was due not to lowered levels or activity of Sirt1, but to lowered levels of the NAD precursor nicotinamide mononucleotide (NMN), possibly consequent to age-related lowered levels or activity of Nampt, an enzyme required for synthesis of NMN and NAD. In β-cells, Nampt-mediated NAD biosynthesis promotes glucose-stimulated insulin secretion at least in part by activating Sirt1. Nampt-mediated systemic NAD biosynthesis also declines with advanced age, resulting in reduced Sirt1 activity and insulin secretion in aged β-cells [108]. The importance of this mechanism and of the involvement of UCP2, however, is underscored by the discovery that reduced Sirt1 levels elevate UCP2, reduce

ATP, and blunt insulin secretion in a UCP2-dependent manner [109]. Clearly, Sirt1 has heavy involvement in the physiologic and endocrinologic changes occurring in NIDDM, but to take proper advantage, treatment strategies may well have to take into account where (i.e., in what cell types) and the age and physiologic state of the intended patient group.

Sirt1 plays a role in regulating NIDDM-affected respiration efficiency. Maximal oxygen uptake (Vo_{2max}) is the highest oxygen uptake achievable by an individual for a given amount of exercise and is commonly used as a measure of physical fitness and mitochondrial function. Not only do untrained individuals with NIDDM have reduced Vo_{2max} compared with that of healthy control individuals, but also a low Vo_{2max} also predicts future NIDDM. Bcl6 is a transcription factor that is associated with lowered Vo_{2max}. It negatively regulates peroxisome proliferator activated receptor delta (PPARδ) expression; increased levels of Bcl6 would then result in lowered rates of fat oxidation [110]. Sirt1 in turn negatively regulates Bcl6 expression and so should tend to maintain the optimal levels of fat oxidative metabolism related to proper regulation of respiration.

4.2 Alzheimer's Disease

Alzheimer's disease (AD) is an age-related neurodegenerative disease that results in a progressive loss of cognition, particularly of short-term memory and use of language. Its incidence rapidly increases after middle age, so that 13% of people over 71 years of age are affected. Whereas the cerebellum is largely spared, the cortex is affected by progressive loss of cholinergic neurons, development of extracellular neuritic plaques containing aggregated Aβ peptides, and intracellular neurofibrillary tangles containing hyperphosphorylated tau protein [111].

Sirt1 gene expression and protein levels are both reduced in parietal cortex of AD subjects; these reductions parallel a decline in global cognition scores and increases in symptom duration and tau filament accumulation [112]. Variations in single nucleotide polymorphisms in a human population is related to cognitive functioning, although no connection between AD or life span and genetic variation of sirtuins has been found [49]. On the other hand, Sirt1 activity is increased, and amyloid neuropathology decreased, by caloric restriction in an AD mouse model [113].

The role of sirtuins in neurodegenerative diseases such as Alzheimer's is complex, as in general, Sirt1 protects against neuronal death, whereas Sirt2 promotes neurodegeneration [114].

Sleep disturbances are common in AD patients [115] and in the elderly generally [116]. Sirt1 is an integral component of the circadian clock circuit, both (1) by acting to couple the body's light–dark response to circadian variations in metabolic state via high-magnitude expression of core clock genes and Per2 deacetylation [117] and (2) by binding to the promoters of *dbp*, *per2,* and the NAD^+-salvaging enzyme Nampt gene [118, 119]. Thus investigation of possible connections between AD and/or age-associated changes in sirtuin levels and sleep disturbance may be valuable.

4.3 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of severe vision loss in the elderly. AMD pathology is associated with aberrant activation of complement as facilitated by reduced activity of the anti-inflammatory complementary factor (CF) H [120]. Oxidative stress may play a major role in the development of AMD, as suggested by the observations that smoking increases the incidence and severity of AMD, and antioxidants slow its progression. Results from one study [121] in retinal pigmented epithelial (RPE) cells suggest that increasing the activation of Sirt1 may have potential to protect against AMD. This study used H_2O_2 as a source of oxidative stress in RPE cells to show that peroxide-induced acetylation of FoxO3 enhanced its binding to the CFH promoter and that this increased binding resulted in reduced CFH expression. Further, expression of Sirt1, by decreasing acetylation of FoxO3, reversed CFH gene repression; this ability of Sirt1 to restore CFH and its anti-inflammatory effects from the depressed levels found in AMD thus offers the potential to ameliorate AMD pathology [121].

4.4 Cardiovascular Disease

Cardiovascular disease is the most important cause of death worldwide, and age is its most powerful risk factor [122]. Sirt1 and Sirt7 can resist stress- and aging-associated myocardial dysfunction through the deacetylation of p53 and forkhead box O1 (FoxO1). Sirt1 modulates the activity of endothelial nitric oxide synthase (eNOS), FoxO1, and p53, and the expression of angiotensin II type 1 receptor; these actions promote vasodilatory and regenerative functions in vascular wall muscle cells [122].

Sirt7-deficient mice have reduced mean and maximum life spans and develop cardiac hypertrophy, inflammatory cardiomyopathy, and extensive fibrosis. Sirt7 interacts with the proapoptotic factor p53 and efficiently deacetylates it in vitro, two observations that are consonant with the hyperacetylation and activation of p53 that leads to extensive apoptosis in the hearts of Sirt7 deficient mice [42].

4.5 Stroke

Stroke, the sudden loss of consciousness caused by rupture or obstruction of a cerebral artery and one of the most important causes of mortality and disability worldwide, has a number of risk factors potentially related to sirtuin action including hypertension, diabetes, dyslipidemia, and inflammation [123]. Treatments that induce Sirt1 activation may help prevent stroke incidence or lessen its damage. In mice, resveratrol pretreatment afforded significant hippocampal neuroprotection after either global cerebral ischemia or cardiac asphyxia. Two results indicate that

the resveratrol-induced neuroprotection was mediated by Sirt1 – the neuroprotection was accompanied by increased Sirt1 expression, and Sirt1 inhibition abolished it [124]. Sirt1 activation may help prevent stroke by reducing the severity of some of its pathologic risk factors. In mice, angiotensin II–induced hypertension mediated by a reduction in angiotensin II type 1 receptor (AT1R) can be reversed by dietary administration of resveratrol; overexpression of Sirt1 amplified the effect on AT1R while resveratrol's effect was abrogated by the Sirt1 inhibitor nicotinamide, showing that the antihypertensive effect was due to Sirt1 activation [125]. Also in mice, resveratrol reversed the dyslipidemia induced by a high-fat atherogenic diet, which effect was again accompanied by increased Sirt1 levels [126]. Sirtuins are involved in producing beneficial effects against other stroke risk factors, including diabetes and inflammation (see earlier).

4.6 Cancer

Cancer incidence increases exponentially with age; invasive cancers strike 33% of men and 22% of women between the ages of 60 and 79 years while afflicting fewer than 2% of either sex under 40 years of age [127]. The relationship between sirtuin activity and cancer incidence is complex. On the one hand, the propensity of sirtuins to promote cell survival and suppress apoptosis would seem to permit increased tumorigenesis, but on the other hand, these same properties can suppress other degenerative processes associated with aging, thus extending an organism's life span. Remarkably, both activators and inhibitors of sirtuins have potential as anticancer agents, and an understanding of the mechanisms involved sheds light on the remarkable intricacy and subtlety of sirtuin cellular regulation.

A number of studies show sirtuins and their activators to be protective against cancer. Resveratrol, a Sirt1 activator, has been found to inhibit cancer at all three stages: initiation, promotion, and progression [128]. Sirt1 deacetylates p53, a proapoptotic factor that activates the apoptosis-inducing protein bax. Acetylation of histone H3 at lysine 56 (H3K56ac) occurs at increased amounts in many cancers, and Sirt1 and Sirt2 deacetylate H3K56ac [129]. Increased levels of Sirt1 itself may be capable of suppressing tumor cell proliferation in a form of colon cancer driven by β-catenin. In the mouse model, Sirt1 deacetylated β-catenin and suppressed the ability of oncogenic β-catenin to drive cell proliferation, while in human colon cancer specimens, there was an inverse relation between Sirt1 levels and oncogenic β-catenin [130]. There is the possibility, then, that Sirt1 may be able to suppress colon cancer [130].

Sirt1 and its activator resveratrol can also suppress BRCA1-mutant breast cancers [131]. Mutant BRCA1 lowers Sirt1 levels in tumor cells, while Sirt1 overexpression suppressed tumorigenesis in nude mice expressing mutant but not wild-type BRCA1. Tumor suppression was the consequence of lowered gene expression of the tumor growth promoter survivin, resulting from binding of Sirt1 to the *survivin* promoter and the deacetylation there of histone H3 by Sirt1.

Levels of Sirt2 are downregulated in gliomas [72], an indication that it may possess cancer-inhibitory activity.

Indicative of the complexity of sirtuin involvement in cancer etiology, not only can sirtuin activators act against cancer, but also the sirtuin inhibitors sirtinol, salermide [32] and cambinol-related molecules [132] also show anticancer activity.

Counterbalancing these findings of tumor suppression and the generally beneficial contributions of sirtuins to homeostasis are other indications that high expression levels and activity of sirtuins are associated with cancer incidence and aggressiveness. Poor prognosis in one type of B-cell lymphoma is associated with increased biopsy levels of Sirt1 [133]. A possible contributing mechanism for this relationship involves Sirt1 regulation of Bcl6 activity. Bcl6 is a transcriptional repressor that is normally acetylated and inactive but that can induce B-cell transformation when activated; Bcl6 is a Sirt1 deacetylation target, and Sirt1 inhibition results in accumulation of inactive Bcl6 and cell cycle arrest of B-cell lymphoma cells [134].

Levels of Sirt7 expression are significantly increased in human breast cancer biopsy tissue, and increased levels of both Sirt3 and Sirt7 transcription are associated with node-positive breast cancer [135]. In these instances, sirtuin activity is linked to increased cancer transformation.

DNA repair efficiency declines with age, whereas microsatellite instability (MSI), a consequence of suboptimal repair, increases [136]. High levels of MSI are also present in some cancers. In human colon cancer, in particular, increased Sirt1 levels correlate with the excessive quantities of MSI present. Metastasizing cancer cells require facile migratory activity, a capability promoted by the F1-actin-binding protein cortactin. Deacetylation of cortactin by Sirt1 increases the migratory capacity of mouse embryonic fibroblasts, and increased levels of Sirt1 are found in human breast, ovarian, and prostate cancer tissues [137], although the question of a more direct correlation between Sirt1 levels and in vivo tumor metastatic potential should be more thoroughly explored.

4.7 Sarcopenia

Sarcopenia is the gradual, degenerative loss of muscle mass and strength associated with aging. Increased expression and activation of sirtuins may actually exacerbate sarcopenia, in contrast with their generally beneficial effects on many of the age-related degenerative conditions discussed above. In one study [138], Sirt1 levels were increased in the nuclei of muscle satellite cells in aged rats. FoxO1 levels were also increased, contributing to increased $p27^{kip1}$ levels and consequent suppression of cell proliferation. Reduced ability of satellite cells to proliferate may limit maintenance of muscle mass and contribute to development of sarcopenia. Similarly, increased expression of FoxO3 has been shown to suppress proliferation of aged rat muscle precursor cells [139]. Although not directly demonstrated, the increased FoxO expression reasonably can be ascribed to higher Sirt1 levels, as a

consequence of Sirt1's ability to deacetylate and activate both of these FoxOs. Thus increased Sirt1 expression and activation may contribute to sarcopenia.

5 Conclusions

An allegory for maximizing longevity, a poem by Oliver Wendell Holmes about a one-horse shay [140] describes how the horse and each component of the shay must wear or age at exactly the same rate, and catastrophically fail at exactly the same time, in order for the entire carriage to function properly for the longest possible time. The analogous real-life observation is that the very aged tend to enjoy an extended period of good health after which they succumb to a fatal disease quickly. In contrast, those stricken in their middle years by a degenerative disease may experience many years of declining health. A number of such degenerative diseases have been identified, the incidence of all of which increases roughly exponentially with age. Delaying the incidence of such diseases until as late in life as possible is an attractive goal for improving human health, often described as "rectangularizing the mortality curve." This is a goal already achieved to some degree by any organism in proportion to how long-lived it is. In order for an organism to better "rectangularize the curve" and maintain an extended life span, it would greatly benefit from possessing an active control mechanism for regulating maintenance of both operational (viz., metabolic) and repair functions in response to both the occurrence of environmental challenges and the passage of time. The goal of this review has been to summarize what is known of the sirtuins and how their contribution to such regulation may affect incidence of degenerative disease and present opportunities to extend longevity.

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Glossary

Acaca	acetyl-Coenzyme A carboxylase alpha
ACSS2	acyl-CoA synthetase short-chain family member 2
AT1R	angiotensin II type 1 receptorAtg*x*
Atg*x*	autophagy related *x* homolog
BAC	bacterial artificial chromosome
Bcl11b	B-cell CLL/lymphoma 11B
Bcl6	B-cell CLL/lymphoma 6
CPS1	carbamoyl phosphate synthetase 1
CTIP2	= Bcl11b
E2F1	E2F transcription factor 1
eNOS	endothelial nitric oxide synthase
FoxO*x*	forkhead box O*x*

GDH	glutamate dehydrogenase
HDAC6	histone deacetylase 6
HSP70	heat shock protein 70
IDH2	isocitrate dehydrogenase 2
IRS2	insulin receptor substrate 2
Ku70	= XRCC6 = X-ray repair cross-complementing protein 6
LKB1	= STK11
LXR	liver X receptor (LXR-α = NR1H3; LXR-β = NR1H2)
Nampt	nicotinamide phosphoribosyltransferase
NBS1	Nijmegen breakage syndrome protein 1; nibrin
NIDDM	non-insulin-dependent (type II) diabetes
NMN	nicotinamide mononucleotide
Nmnat	nicotinamide mononucleotide adenylyltransferase
PARP1	poly (ADP-ribose) polymerase 1
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPARδ	peroxisome proliferator activated receptor delta
Sirt	sirtuin (silent mating type information regulation 2 homolog)
SNP	single nucleotide polymorphism
STAT3	signal transducer and activator of transcription 3
STK11	serine/threonine kinase 11
SUV39H1	suppressor of variegation 3-9 homolog 1; histone H3-K9 methyltransferase 1
TAF_I68	RNA polymerase I-specific TATA box-binding protein-associated factor 68 kDa
TLE1	transducin-like enhancer of split-1
UCP2	uncoupling protein 2
VNTR	variable number tandem repeat
Wrn	Werner syndrome, RecQ helicase-like protein

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Estrogenic Modulation of Longevity by Induction of Antioxidant Enzymes

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Abstract In many species including humans, females live longer than males. We and others have observed that mitochondria from females of Wistar rats and of OF1 mice produce half the amount of peroxide produced by males. We attributed this to a change in the expression of antioxidant, longevity-related genes. We have found that in those species in which females live longer than males, estrogens activate longevity-related genes, particularly antioxidant ones. It should be emphasized that estrogens do not act as antioxidants because of their phenolic ring but rather they act indirectly; that is, they behave as hormones and bind to estrogen receptors, which eventually leads to the upregulation of the expression of antioxidant genes. The pathway by which estrogens activate the expression of these genes has been elucidated, and we have traced it to the activation of the mitogen activated proteins (MAP) kinase pathway. It is remarkable that estrogens activate proliferation genes (related to their feminizing function and also to their cancer-promoting effects) by binding to estrogen receptor alpha whereas the longevity-related genes, in particular the antioxidant ones, are mediated by binding to estrogen receptor beta. Phytoestrogens, which in their vast majority bind to estrogen receptor beta, promote longevity-related genes without increasing the rate of cell division or promoting feminization. Thus, a practical approach discussed here is that administration of phytoestrogens may be very beneficial for longevity because they bind very preferentially to estrogen receptor beta and promote the upregulation of longevity-related genes.

Keywords Aging · Mitochondria · Estrogens · Phytoestrogens · Longevity genes

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1 Mitochondria as Sources and Targets of Age-Associated Damage

The free radical theory of aging originally proposed by Harman is one of the many theories that have been proposed to explain the phenomenon of aging [1]. However, this theory has received particular attention because it suggests a number of testable experiments to determine if free radicals are indeed involved in damage caused by aging. One of the most critical tests of this theory was to overexpress antioxidant genes in animals and determine their effects on longevity. Pioneer work by Orr and Sohal [2] showed that overexpressing catalase and superoxide dismutase increased the life span of *Drosophila melanogaster*. Much more recently, it has been shown that superoxide dismutase mimics can prolong life span [3, 4]. Moreover, we have recently shown that overexpression of p53 and p16 in mice results in an increased longevity. This may be explained by the fact that p53 behaves as an antioxidant because it increases the expression of a new type of glutaredoxins, namely sestrins [5].

Very relevant to the development of this chapter, the free radical theory of aging was defined by Jaime Miquel when he proposed that mitochondria are the major source of radicals in the aging cell and that they are also the major target for damage of these radicals [6]. Indeed, Jaime Miquel proposed in the early 1980s that mitochondrial DNA could be a major molecule that is damaged by free radicals [7]. Experiments performed in our laboratory in the 1990s showed that mitochondria were damaged within cells and that the observed impairment in mitochondrial function was not due to increased frailty of mitochondria, but to real damage that occurs within the cell in the aging animal [8]. Moreover, we found that peroxide production by mitochondria was much more marked in postmitotic cells (i.e., neurons) than in cells that still have a marked regenerative capacity such as hepatic or glial cells [9] (see Table 1). Damage to mitochondrial DNA was indeed much more marked in the old than in the young animals and again, it was higher in brain than in liver. Thus, the mitochondrial theory of aging provided us with a framework to understand the basic phenomena of aging, and it also gave us room for intervention (i.e., administer exogenous antioxidants such as vitamin C or E or increase the expression of endogenous ones like the antioxidant enzymes). With this framework in mind, we studied the differential rate of aging in males and females of different species.

Table 1 Tissue differences in mitochondrial H_2O_2 production

Tissue	Mitochondrial H_2O_2 production (nmol min^{-1} mg $protein^{-1}$)
Liver	$0.10 \pm 0.03^*$
Brain (nonsynaptic)	$0.08 \pm 0.02^*$
Brain (synaptic)	0.29 ± 0.04

The statistical difference is indicated as follows: $^*p < 0.05$ versus synaptic mitochondria

2 Difference in Oxidative Stress in Aging Expressed a Differential Longevity Between Genders

A critical test of the free radical theory of aging as outlined above is the fact that in many species in which females live longer than males, the former produce approximately half the amount of mitochondrial peroxide as that produced by the latter [10]. It is very important to state that not all species behave the same in terms of gender-related longevity, and that there are some species in which males live longer than females (see later). But in the Wistar rat, our experimental rat model, as well as in humans, females do indeed live longer than males by approximately a 10% increase in the average life span.

In early 2000, we measured peroxide production by mitochondria from female and from male Wistar rats and found that females produce approximately half the amount of peroxides as that produced by males. This was completely reversed when rats were ovariectomized, thus tracing the differential gender effect on radical production to ovarian hormones. This was confirmed when we measured the free radical production of mitochondria from ovariectomized females that had been treated with estrogens. In this case, estradiol was able to reverse the effect of ovariectomy, and the rate of peroxide production was similar to that of females (see Fig. 1). The increase in radical production resulted in a significantly lower damage to mitochondrial DNA in females than in males. In fact, the level of 8-oxo-deoxyguanosine was fourfold higher in males than in females [10]. Estrogens are antioxidants because they contain a phenolic structure in the A-ring. However, the level of estrogens is so low that it does not make sense that they act as antioxidants because of its phenolic structure. A simple calculation indicates that estrogens cannot act as chemical antioxidants. The recommended dose for supplementation of vitamin E to humans is approximately 400 mg/day. However, the recommended

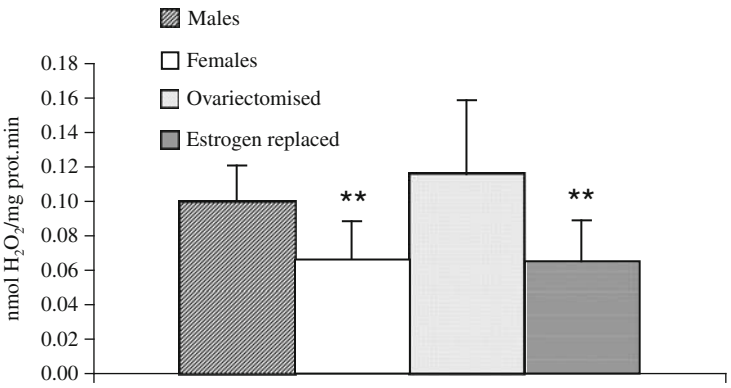

Fig. 1 Oxidant production by hepatic mitochondria from males and females: effect of ovariectomy and of estrogen replacement therapy. The statistical difference is indicated as follows: $^{*}p < 0.01$ versus male rats

dose for estrogen replacement therapy in postmenopausal women is 50 μg/day (i.e., 8,000 times less than the recommended dose for vitamin E). Thus, estrogens could not be chemical antioxidants. Rather, they could act binding to estrogen receptors and stimulating the synthesis of antioxidant enzymes. This was indeed the case, and we observed that females expressed much more superoxide dismutase (SOD2) as well as glutathione peroxidase [10]. The fact that glutathione peroxidase activity is higher in females than in males has been known since the 1960s [11], but this had not been related to longevity at all. Thus, we came to the conclusion that estrogens could activate antioxidant and perhaps other longevity-related genes.

We found this effect both in vivo and in vitro. In vivo, we found that expression of SOD2 and glutathione peroxidase was higher in females than in males, as stated earlier. We developed this concept in in vitro studies and measured the expression of superoxide dismutase and of glutathione peroxidase in MCF7 cells; these are cells that derive from a mammary carcinoma and are rich in estrogen receptors. We found that estrogens act by binding to estrogen receptors and by eventually activating the MAP kinase–nuclear factor kappa B (NF-κB) pathway, which in turn upregulates the expression of antioxidant enzymes, see Fig. 2 [12, 13].

From the practical viewpoint, estrogen replacement therapy is of course impossible in men and presents serious drawbacks in postmenopausal women [14].

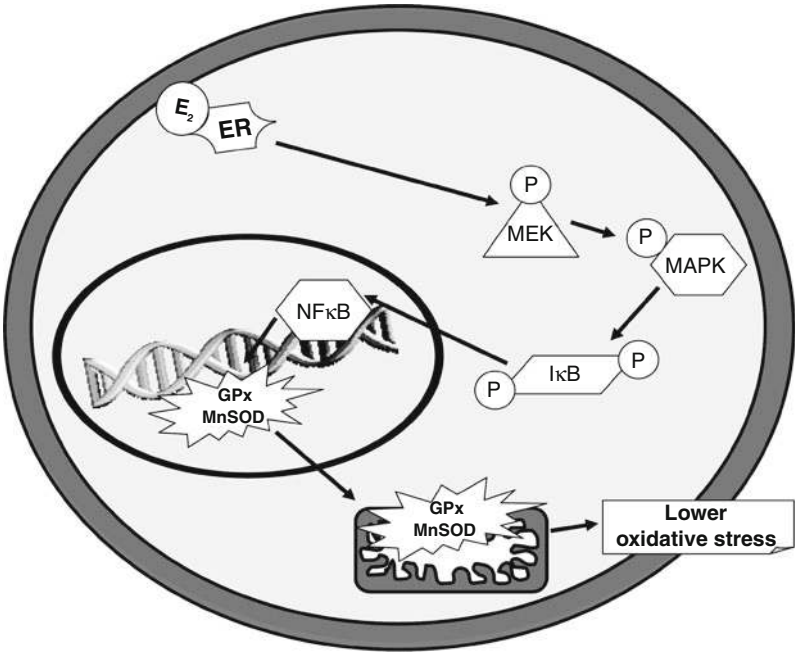

Fig. 2 Proposed mechanism of estradiol antioxidant action. Estradiol binds to estrogen receptors, activates MAP kinase and NF-κB, which in turn activates the expression of antioxidant gene expression

However, a possible alternative was to treat animals or persons with genistein (i.e., a phytoestrogen that preferentially binds to estrogen receptor beta). We tested the effect of genistein in MCF7 cells and found that indeed it acts as an antioxidant because it binds to estrogen receptors, its effects being inhibitable by tamoxifen, and that downstream it follows a similar pathway as that of estradiol activating MAP kinases and NF-κB and increases the expression of superoxide dismutase [15]. Thus, in our hands, not only does estrogen activate longevity-related genes, but so does phytoestrogen, and this is a fact of importance because there are very few, if any, reports of undesirable effects of phytoestrogens at the concentrations that we used, which are nutritionally relevant (i.e., those that are found in blood plasma of normal people who live in the Far East and who have a soya-rich diet) [16]. Our conclusion is that phytoestrogens may provide an interesting approach to activate longevity-related genes without the many undesirable side effects that may occur when estrogens are used.

3 Telomerase Is a Longevity-Associated Gene Regulated by Estrogens

Telomerase is a very interesting enzyme whose activity consists of increasing the length of telomeres, which is normally shortened after each cell division. This explains the "Hayflick limit" (i.e., that the cells can duplicate only a limited number of times, which has thus attracted much attention). A critical idea was the finding that the vast majority of neoplastic tumors contain an active telomerase. This enzyme is also active in cell lines and in normal cells that have to maintain a high rate of division during the lifetime, such as spermatogonic cells. Normal nondividing cells have a very low activity of telomerase [17]. The regulation of telomerase activity has been the subject of numerous reviews (for instance, see [18]).

Telomerase was not considered as a longevity-associated gene because its overexpression did not lead to increases in life span, probably due to the fact that the number of animals that died by tumors was higher in telomerase overexpressing animals than in controls. Recently, in a collaborative work with Drs. María Blasco and Manuel Serrano of the Spanish National Center for Oncological Research (CNIO), we found that telomerase can be considered as a longevity-associated gene when overexpressed in mice that are already protected against cancer by overexpressing p53/p16 [19]. In fact, mice overexpressing p53 and p16 do show an increased longevity [5]. When those animals are crossed with mice overexpressing telomerase, triple transgenics are obtained that overexpress p53/p16 telomerase. In these animals, a vast amount of average life span, approximately 50%, is obtained. And probably, an increase in maximal life span may also occur (see [19]). Thus, under these conditions, we have found that telomerase can be considered a longevity-associated gene.

As stated above, telomerase must be the subject of tight regulation. A few years ago, we observed that glutathione, a critical antioxidant in the cell [20], regulates

telomerase. We found that reduced glutathione increases telomerase activity [21]. Moreover, nuclear glutathione is increased in the S-M phase of the cells, that is, when the cells enter a replication cycle, and then telomerase apparently may be increased in cells in these conditions [22]. This is just an example of a metabolic regulation of telomerase activity. But telomerase can be also activated by estradiol. In fact, the telomerase gene contains an estrogen-responding element in its promoter region [23]. This in fact constitutes a hormonal regulation of telomerase activity.

The fact that estrogen regulates tissue development has been consistently confirmed. This led us to think that estrogen might delay aging by increasing the rate of tissue regeneration. This contrasts with a mechanism by which estrogen also promotes longevity by protecting against damage to postmitotic cells. The latter might be mediated, for instance, by antioxidant enzymes as mentioned above. The former, in contrast, depends on promoting the capacity for cell duplications and thus regeneration. Estrogens bind to two different receptors, namely estrogen-receptor (ER)-α and ER-β. ER-β promotes genes that in fact protect postmitotic cells against damage due to agents such as radicals. ER-α activation delays aging by increasing cell renewal [24]. However, it has a serious drawback. If the cell does not keep strict control of all the checkpoints in the cell cycle, neoplastic transformation may occur. But in any case, increasing the application of cells is a way of delaying aging, at least at the cellular level. Telomerase is critical for this purpose. In fact, it has a major responsibility for protecting cells against loss of vitality due to shortened telomeres. Thus, targeting estrogen-telomerase access via estrogen agonists or antagonists may be a means of protecting animals against tissue damage associated with aging. The fact that the human telomerase reverse transcriptase (hTERT) promoter is stimulated by estrogen and its receptor complex is supported. The lesions in the estrogen-response elements dramatically lower estrogen-induced stimulation of telomerase activity [23, 25]. Moreover, there are two c-myc in the hTERT promoter region. The lesion of these two c-myc completely reverted estrogen upregulation of telomerase activity [26]. Thus, estrogen activates ER-α and regulates hTERT gene expression in coordination with c-myc. In fact, it has recently been shown by gene expression analysis that in the absence of estrogen telomerase, gene expression is reduced, and this leads to a compromised cell proliferation [27].

The picture that emerges is that estrogens may act in two different ways to protect against impairment in cell function: one is to protect postmitotic cells against damage caused by agents such as free radicals. This could be imitated by ER-β. But estrogens also act by promoting cell division and thus cell renewal. This would be mediated by activation of ER-α, which in turn activates the expression of telomerase. It is now very important to find agonists that may activate ER-β such as genistein but also others that may activate ER-α to promote cell division but also protect cells against the loss of division that leads to the neoplastic transformation. That this is possible is illustrated by the fact that experimentally when we have introduced extra copies of p53 and p16 (to protect against cancer) and then of telomerase, animals have shown a marked increase in longevity [19].

4 Differential Longevity Between Genders: A Methodological Approach

Not all species – and even within a species, not all strains – show the same differential longevity between genders. For most readers, it will be apparent that females tend to live longer than males. This is because, in our species, women live longer than men. This is also true for the Wistar rat and for the white OF1 mouse, but it is not the case for the much-used black C57BL6 strain of mice. In this strain, males live longer than females. In some substrains derived from the black C57BL6 mouse, females and males have the same longevity. Thus, the statement "females produce fewer radicals than males" cannot be pronounced without a special mention of which species and strain.

Difference in longevity between some strains and in others allows us to subject the free radical theory of aging to a stringent test. In those animals in which females live longer than males, the former should produce fewer radicals than the latter. However, in those animals in which males live longer than females, then males ought to produce fewer radicals than females.

This is a clear example of the August Krogh principle, which states that for any biological problem, there is an animal that is ideal to study it. This concept was put forward by the great Danish physiologist August Krogh in 1929 [28]. However, the term "the August Krogh principle" was coined by the great German-British biochemist Sir Hans Krebs in 1975. There have been many examples of this principle. Perhaps the most prominent is the finding that *Thermus aquaticus* contains a thermostable polymerase that allows the polymerase chain reaction (PCR) to occur [29]. In most animals, polymerase is thermolabile, and therefore PCR cannot be used [30]. In the case of aging, the fact that we have different longevity in different species and strains again allows us to study this most interesting problem in comparative physiology (i.e., that different longevity between genders is determined by a different rate of radical production).

So we have studied radical production in Wistar rats, which are a strain in which longevity is higher in females than in males. We have also studied the white OF1 mouse in which again females live longer than males. We found that in these two species, females produce approximately half the amount of radicals as that produced by males [10]. And we attributed this not to the presence of estradiol but to the fact that it affects the rate of radical production by binding to receptors and activating this specific pathway, which leads to the upregulation of antioxidant enzymes [12]. Thus, it is not the presence of estradiol, but rather the fact that estradiol by binding to estrogen receptors activates the expression of antioxidant and other longevity-associated genes.

On the contrary, in strains in which longevity is the same in males and females or even in which males live longer than females, authors show that the rate of oxidant production in males is the same [31] or even lower that that in females [32]. This apparent contradiction is in fact a confirmation of our findings because in the paper by Ali et al., the authors found that in fact their males lived longer than females

(because they used C57BL6 mice), and they did observe an increased oxidative stress in females over that in males. In the case of Sanz et al., the authors did not find any significant difference in indicators of oxidative stress such as protein oxidation or DNA oxidation. Moreover, the authors also studied apoptosis, which in fact we found is related to radical production [33]. Again, no differences in apoptotic index were found between genders. But then, Sanz et al. used mixed-background mice that were derived from a strain "129S/ICR/B6." These animals were back-crossed into C57BL6-6 J. Longevity of these animals did not differ between males and females.

So, in those animals in which females live longer than males (studied by us), females produce fewer reactive oxygen species and had less oxidative stress; in those in which males live longer than females, males produce fewer radicals (see the paper by Ali et al.); and in animals in which females and males have the same life span, the same rate of radicals occurs [31]. So this is another illustration of the Krogh principle: finding the right kind of strain, one can in fact solve a biological problem. Longevity is related to the rate of radical production, and in those animals like humans in which females live longer than males, females produce fewer radicals, but if males live longer than females, then it is the males that produce fewer radicals.

Finally, this brought into consideration the fact that it is not the presence of estrogens that activates a cascade of signals that tend to lower the rate of radical production. It is rather the fact that the interaction between estradiol and estrogen receptors is causing radical production to be affected. This was discussed by Sanz et al. [31] under the perspective of thinking that estrogens could not be responsible for changes in longevity because estrogens are present in animals in which radicals may be lower in males than in females, and these animals of course have estradiol. The critical point is to find out why in some strains estradiol does not activate longevity-associated genes and in some of those like the Wistar rats or the OF1 mouse they do indeed activate the expression of these genes. And of course, this is strong evidence that it is not the chemical fact that estradiol behaves as an antioxidant in vitro because of its phenolic structure. If this were the case, animals would be more reduced always in the presence of estradiol (i.e., in all cases females would be under less oxidative stress than males). The fact remains that the rate of radical production determines the different longevity between genders and that the most interesting case (because it is the same as what happens in humans) is that in some species (rat) or even strains within a species (OF1 mouse), females are protected by the interaction of estrogens with estrogen receptors.

5 Concluding Remarks

In this chapter, we have evaluated the role of estrogens in modulating longevity-related genes. We have concentrated more attention on the expression of antioxidant longevity-related genes, but this by no means is the only case. Other important enzymes such as telomerase are also regulated by estrogenic compounds.

The practical application of these studies lies in the fact that we can treat animals with phytoestrogens, which lack the feminizing effects of estradiol because their binding to ER-α is very weak, and they can be used to prolong the life span of animals and, eventually, humans. We are performing studies along these lines by studying longevity (a longevity curve) in animals that are fed a diet rich in phytoestrogens and that are otherwise kept under control conditions.

Thus, studying the estrogenic control of longevity-associated genes may be of both theoretical and practical importance.

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Mitochondrial Respiratory Function Decline in Aging and Life-Span Extension by Caloric Restriction

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Abstract Extensive studies have established that progressive accumulation of somatic mutations in mitochondrial DNA (mtDNA) leads to a decline in mitochondrial bioenergetic function and contributes to human aging. Mitochondrial respiratory chain dysfunction not only causes inefficient ATP production but also increases mitochondrial generation of reactive oxygen species (ROS), which can induce further oxidative damage and mutation to mtDNA during the aging process. Indeed, a wide spectrum of mtDNA mutations has been found to occur in somatic tissues of elderly subjects. Besides, mitochondrial abnormalities have been associated with age-related diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). On the other hand, age-associated reduction in the efficiency of oxidative phosphorylation (OXPHOS) may cause alterations of gene expression in affected tissues for the sake of compensation or adaptation. Recent studies have revealed that the expression levels of several clusters of genes and posttranslational modifications of proteins are changed in various tissues of old animals and cultured cells from elderly subjects. In addition, the changes in age-associated gene expression profiles were tissue-specific, which suggests that different tissues are subject to different degree of oxidative stress during the aging process. Surprisingly, the majority of the age-related alterations in gene expression can be reversed by caloric restriction (CR) through the elevation of sirtuin 1 (Sirt1) expression, which promotes survival in organisms ranging from yeast to rodents and primates. The anti-aging effects of Sirt1 mediated by CR could be conferred by the activation of mitochondrial biogenesis and respiratory function. On the other hand, the mitochondrial sirtuins (Sirt3, Sirt4, and Sirt5) also play a critical role not only in the upregulation of mitochondrial function but also in the protection against oxidative stress and culminate in the extension of the life span of animals. Taking these findings together, we suggest that mitochondrial dysfunction, accumulation of mtDNA mutation, enhanced oxidative stress, and alteration of gene

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expression are important contributors to human aging. On the other hand, Sirt1 and mitochondrial sirtuins could be the therapeutic targets for the treatment of various age-related diseases such as mitochondrial disorders, neurodegenerative diseases, and metabolic syndrome.

Keywords Aging · Mitochondrial dysfunction · Oxidative stress · mtDNA mutation · Vicious cycle · Caloric restriction · Sirt1

1 Introduction

The mitochondrial theory of aging, a refined version of the free radical theory of aging, was first proposed by Dr. Harman about three decades ago and later expanded based on the fact that accumulation of somatic mutations in mitochondrial DNA (mtDNA) during the lifetime of an individual leads to mitochondrial function decline [1]. In human cells, mitochondrial respiratory chain is not only the major source of ATP but also a major site of reactive oxygen species (ROS) production through electron leakage from Complex I (NADH:ubiquinone oxidoreductase) and Complex III (ubiquinol:cytochrome *c* oxidoreductase) of the respiratory chain [2, 3] under normal physiologic condition. It has been shown that the age-related increase in the rate of ROS production from mitochondria led to the enhancement of the ROS-elicited oxidative damage to mtDNA [4–6]. In addition, abundant evidence indicates that the somatic mutations of mtDNA in aged tissues may impair mitochondrial respiration and oxidative phosphorylation (OXPHOS) after reaching a threshold [7–9]. The defect in the mitochondrial OXPHOS system could lead to the overproduction of ROS, which may further enhance the oxidative damage to various biomolecules in mitochondria. The age-associated accumulation of oxidative damage and mutation to mtDNA ultimately leads to a progressive decline in the bioenergetic function of tissue cells in aged individuals [10, 11]. Therefore, the mitochondrial theory of aging predicts that a "vicious cycle" contributes to the aging process, and a large amount of data from morphologic, bioenergetic, biochemical, and genetic studies of mammalian tissues has lent support to this theory [12–14].

To understand fully the molecular events associated with aging, a number of investigators have examined the genome-wide changes in the gene expression profile in somatic tissues or cultured cells from subjects of different ages. It is concluded that induction of stress response genes is a result of the increased oxidative stress and damage during aging. Besides, the modification of mitochondrial proteins, such as carbonylation, nitration, and phophorylation on targeted proteins, is highly prevalent in aged skeletal muscle and heart of mammals, possibly contributing to the functional decline with age. On the other hand, the majority of the age-related changes in the gene expression profiles in the tissues of animals can be reversed, although to different extents, by caloric restriction (CR), which can prolong the life span of a variety of laboratory animals. The response of CR seems to have been conserved during animal evolution, and there are intensive ongoing studies to elucidate the underlying molecular mechanisms [15, 16]. In addition, it has been

demonstrated that the diet-restricted regimen exerts its life-span–extending effects by downregulation of glycolytic metabolism and decrease of ROS-induced damage. These findings suggest that CR can boost mitochondrial function, which may underscore the salutary effects of CR.

2 Mitochondrial Function Decline During the Aging Process

2.1 Production of ATP and ROS in Human and Animal Cells

Every cell in human and animal tissues contains several hundreds to 1,000 mitochondria, and each mitochondrion harbors 2–10 copies of mtDNA. Human mtDNA is a 16,569-bp, circular, double-strand DNA molecule that encodes 2 rRNAs, 22 tRNAs, and 13 polypeptides that constitute four respiratory enzyme complexes essential for the assembly of the OXPHOS system. The mitochondrial respiratory chain is composed of four multi-subunit enzyme complexes: NADH-coenzyme Q oxidoreductase (Complex I), succinate-coenzyme Q oxidoreductase (Complex II), ubiquinol-cytochrome c reductase (complex III), and cytochrome c oxidase (Complex IV). The primary function of mitochondria is to produce energy in the form of ATP through the coupling of the respiration-generated proton gradient with the proton-driven phosphorylation of ADP by the F_1F_o-ATPase (Complex V). In addition to ATP production, there are many nuclear DNA–encoded proteins that reside in the mitochondria and play various roles in the biosynthesis of heme and steroid hormones, substrate oxidation through the tricarboxylic acid cycle, β-oxidation of fatty acids, and in part of the urea cycle. Moreover, the regulation of mitochondrial biogenesis is important for cells to adjust their energy production to meet energy demand under different physiologic conditions or environmental stress [17]. Under normal physiologic conditions, about 1–5% of the oxygen consumed by mitochondria is converted to ROS including superoxide anions, hydrogen peroxide (H_2O_2), and hydroxyl radicals [3]. Superoxide anions are generated by electron leakage from complex I and complex III [2] and are rapidly metabolized into H_2O_2 by manganese-dependent superoxide dismutase (Mn-SOD) in mitochondria [18]. If H_2O_2 is not efficiently removed, highly reactive hydroxyl radicals may be formed via Fenton reaction in the presence of Fe^{2+} or Cu^{+}. It has been shown that the rate of production of ROS from mitochondria increases with age in mammalian tissues [19, 20], which is related to the decline of mitochondrial function [9, 21]. In fact, it was demonstrated by several investigators that the rate of ROS production in mitochondria is an important determinant of the life span of mammals [22].

2.2 Accumulation of Mitochondrial DNA Mutation in Aged Tissues

After Dr. Harman proposed the mitochondrial theory of aging, evidence grew to support the suggestion that a large number of somatic mutations in mtDNA

progressively accumulate with time in a variety of tissues in the human and in animals [23]. The mutant mtDNA usually coexists with the wild-type mtDNA within a cell, which is termed *heteroplasmy*, and the degree of heteroplasmy often varies in different tissues of an individual [24]. In the past 20 years, a large number of studies revealed that a broad spectrum of mtDNA deletions, point mutations, and insertions are accumulated with age in different tissues of the human and of animals. The age-associated mtDNA mutations identified in different human tissues are summarized in Table 1. It is generally accepted that large-scale deletions of mtDNA are early events of the aging process and may be considered as a biomarker of aging in the human and in animals [33]. In addition, a large amount of point mutations in the D-loop region, which contains the replication origin and transcription promoters of mtDNA, was detected in cultured skin fibroblasts from elderly subjects [9]. It is noteworthy that there is a high degree of heteroplasmy in the D-loop region of mtDNA in human brain, and the sequence variations are increased in aged individuals [34]. Moreover, in vitro studies showed that the mutation rate in the D-loop region of mtDNA was very high because of its susceptibility to oxidative damage [35], which was confirmed by several in vivo studies [36–38]. Although it is poorly understood as to how ROS cause mtDNA mutations, ROS-induced oxidative damage is correlated with the mtDNA mutations in human heart and lung during aging [39–41]. On the other hand, it has been reported that treatment of human diploid fibroblasts with a sublethal dose of H_2O_2 or ultraviolet (UV) irradiation can induce cellular senescence accompanied with the accumulation of 4,977-bp deletion of mtDNA, which is the most frequently observed mtDNA deletion [42–44]. These studies provide evidence to support the notion that ROS are involved in

Table 1 Age-associated mitochondrial DNA deletions and mutations in human tissues

Biopsies	Deletions	Point mutation	Insertion	References
Cochlea	4,977 bp; 9,682 bp; 5,142 bp			[25]
Skeletal muscle	4,559 bp; 7,664 bp; 8,511 bp; 4,977 bp (78%)	A189G (54%); T408A (73%)		[26–29]
Heart muscle	4,977 bp (50%)			[27, 28]
Caudate nucleus	4,977 bp (85%)			[27]
Cerebellum	4,977 bp (80%)			[27, 28]
Substantia nigra	4,977 bp (95%)			[28, 30]
Putamen	4,977 bp (95%)			[28]
Frontal lobe	4,977 bp (90%)			[28]
Colonic crypt		G8557A; G15804A; G9985A; T10020C	C9537ins; C309ins	[31]
Leukocytes		C150T (17.3%)		[32]
Skin fibroblasts		T414G (57%)		[9]

The percentage refers to the frequency of occurrence of mtDNA deletions or mutations in elderly human subjects (more than 60 years old).

the mechanisms underlying age-associated somatic mutations of mtDNA, which is a link between the free radical theory of aging and the mitochondrial theory of aging.

Recently, Larsson and colleagues established a mouse model to provide solid evidence for a direct link between mtDNA mutation and mammalian aging [45]. They created transgenic mice carrying a homozygous mutation in the α subunit of DNA polymerase γ that expressed a proofreading-deficient version of DNA polymerase in mice [45–47]. The knock-in homozygous mutant mice developed an mtDNA mutator phenotype with an increase in the proportions of point mutation and large-scale deletion of mtDNA. It was found that the increase in somatic mtDNA mutations and defects in mitochondrial respiratory function were associated with reduced life span and premature onset of age-related phenotypes such as alopecia, kyphosis, osteoporosis, anemia, heart enlargement. and reduced fertility. In addition, Vermulst and colleagues reported that the rates at which mtDNA mutations reached age-related phenotypic changes were markedly different among the tissues examined [47]. The brain and heart tissues accumulated mtDNA mutations much more quickly than did the other tissues. This may explain, at least partly, why age-related declines in the biochemical and physiologic functions are usually much more pronounced in the tissues with higher energy demand [48]. Therefore, these findings further support the causal role of the loss of mtDNA integrity and the accumulation of mtDNA mutations in the aging process [49–51]. However, it is still unclear how such large accumulation of mutations in mtDNA led to the aging phenotype in the transgenic mice. It was a surprise to find no increase in mitochondrial ROS production in such mtDNA mutator mice [52, 53]. Some may argue that the major fault of this experimental design is that it did not represent natural aging. We may consider it a genetic disease model for premature aging. Lastly, mitochondria may play different roles in the aging process of the wild-type animals, in which somatic mtDNA mutations accumulated much more slowly than that in the mutant mice [32, 54].

2.3 Bioenergetic Function Decline of Mitochondria During Aging

As mtDNA mutations are accumulated in somatic tissues during aging, the mitochondrial function would be affected in the lifetime of an individual. It is generally accepted that mtDNA mutations cannot cause mitochondrial dysfunction until they reach a threshold. Besides, the mutant mtDNA molecules are distributed unevenly among the cells of affected tissues, which usually result in a mosaic pattern of mtDNA mutations. Moreover, it is conceivable that accumulation of somatic mtDNA mutations may aggravate the preexisting defects in mitochondria until the combined defects reach a threshold and result in bioenergetic failure of the affected tissues of the elderly subjects [55]. Several lines of evidence led to the suggestion that the accumulation of mtDNA mutations and deletions results in defects in respiratory enzyme complexes in somatic tissues of aged individuals [56, 57]. Recently,

biochemical analyses of electron transport chain (ETC) activities in homogenate of various tissues from the human and the mouse have revealed an age-dependent decrease of bioenergetic function of mitochondria (Table 2). In the past two decades, a number of studies demonstrated that point mutations and large-scale deletions of mtDNA are associated with Complex I and IV deficiencies in aging tissues [61–64]. In addition, by immunohistochemical (IHC) studies of the subunits of the mitochondrial respiratory chain, Dr. Müller-Höcker first observed cytochrome *c* oxidase deficiency in the heart, limb, diaphragm, and extraocular muscle of normal elderly subjects, and that the extent of deficiency of cytochrome *c* oxidase in muscle fiber was increased with age in the human and in animals [65, 66]. In the following studies, Dr. Desai found that the activity of Complex I was decreased with age in the gastrocnemius muscle of mice [67] and that various defects in Complex III, IV, and V also occurred in skeletal muscles from elderly subjects [68]. Similar changes of mitochondrial respiratory function during the aging process have been observed in various tissues of aged laboratory animals [69–71]. It should be noted that the above-mentioned defects of respiratory enzyme complexes are randomly distributed in aging tissues and not associated with the loss of the activity of Complex II, whose constituent subunits are all encoded by nuclear DNA [67].

In addition to the decline in the activities of respiratory enzyme complexes during the aging process, the respiratory control ratio (RCR), which is the ratio between the rates of ADP-stimulated (State 3) and resting (State 4) states of respiration, and the ADP/O ratio were found to decrease with age in various human tissues [72, 73]. Functional decline in the OXPHOS system was also reported in primary culture of human skin fibroblasts with replicative senescence [74]. These changes are closely related to the decrease in energy coupling and the efficiency of mitochondrial ATP synthesis. On the other hand, Attardi's group demonstrated that the mtDNA/nuclear DNA ratio and oxygen consumption rate in cybrids, established from skin fibroblasts of elderly subjects, were significantly lower compared with those from young donors [75]. This finding provided a direct link of the quality and quantity of mtDNA to the mitochondrial respiratory function in human cells [76]. Besides, the age-related reduction in the mtDNA copy number and increase in oxidative damage to DNA are associated with a general decrease in mitochondrial gene transcription and protein synthesis in mitochondria of human skeletal muscle and cultured skin fibroblasts from elderly subjects [77]. Moreover, it was recently shown that the capacity of mitochondria to produce H_2O_2 was significantly increased in aged hearts of rats with a significant increase in the level of oxidized cardiolipin in mitochondria [78]. It may be concluded that the rate of ROS production of mitochondria is increased with age in human and animal tissues [79] and is also elevated in human skin fibroblasts undergoing replicative senescence [80].

More recently, many studies demonstrated that inhibition of mitochondrial respiratory function in human cells could lead to the acceleration of cellular senescence. The stress-induced cellular premature senescence (SIPS) model has been used to show that the stress-induced senescent cells are accompanied with the accumulation

Table 2 Changes of the respiratory function of mitochondria in aged tissues of mammals

	Activities of mitochondrial respiratory enzymes					Oxygen consumption rate				
Tissue	I	II	III	IV	COX staining	State 3	State 4	RCR	ATP production	References
Human										
Skeletal muscle		↑ 69%	↓ 13%	↓ 34%	Deficiency				↓ 10%	[26, 56, 58, 71]
Skin fibroblasts	↓ 50% (I+III)	↓ 70% (II+III)		↓ 54%						[21]
Colonic crypt					Deficiency					[31]
Substantia nigra					Deficiency					[30]
Liver						↓ 82%	↓ 86%	↓ 89%		[69]
Myocardium						↓ 73%	↓ 84%	↓ 82%		[79]
Mice										
Skeletal muscle				–		↓ 80%	↓ 70%	↓ 40%		[59]
Kidney				↓ 20%						[60]
Oculus				↓ 20%						[60]
Heart					Deficiency					[63]

The percentages indicate the amplitude of changes of mitochondrial function in aged tissues compared with that of the corresponding young tissues.

of large-scale deletions of mtDNA and decline of mitochondrial function [81–83]. In addition, with partial depletion of mtDNA by ethidium bromide (EtBr) treatment, the morphology of the skin fibroblasts appeared like UV-induced photoaged skin [84, 85]. Furthermore, the downregulation of mitochondrial transcription factor A (mtTFA) by using RNA interference (RNAi) technique, which caused partial depletion of mtDNA, resulted in impairment of mitochondrial function and retarded growth of cells [86]. These findings suggest that the decrease in the rates of mitochondrial transcription and protein synthesis may contribute to the age-related decline in the capacity of the respiratory function. Furthermore, it was demonstrated that the reduction in the respiratory function by specific mitochondrial inhibitors was causally related to reduced cell proliferation and induction of cellular senescence [87, 88].

2.4 Mitochondrial Dysfunction in Age-Related Diseases

In the past decade, mtDNA mutations have been proposed to associate with some degenerative diseases [89]. Mitochondrial abnormalities were reported in patients with Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and mitochondrial diseases (MD). The mitochondrial Complex I defect was detected in the affected tissues of PD patients [90], and the association of mtDNA polymorphisms with PD has also been reported [91]. Besides, the defects in cytochrome *c* oxidase and ATP synthase have been observed in the affected tissues of patients with sporadic AD [92]. In patients with ALS syndrome, there was a reduction in the activity of respiratory enzyme complexes and decrease of mtDNA-encoded subunits [93]. Besides, mitochondrial respiratory chain defects together with the decline of energy metabolism and overexpression of the uncoupling protein (UCP) were also found in skeletal muscle tissues of ALS patients [93, 94]. It is a general observation that most of the patients with mitochondrial diseases exhibit a delayed onset and a progressive clinical course, which frequently results in premature aging and early death [95]. Recent studies also have provided additional evidence to substantiate the notion that oxidative stress elicited by the respiratory chain defects in the affected tissues of the patients plays a role in the pathogenesis and progression of some mitochondrial diseases [96–98].

3 Age-Associated Alterations in Gene Expression and Protein Modification

It has been shown that age-associated decline in respiratory function can lead to the increase of intracellular ROS, which not only damage cellular constituents but also increase stress responses and oxidant-mediated signaling pathways. To identify the

molecular events associated with aging, a number of studies using cDNA microarray have revealed age-related, genome-wide profile changes in skeletal muscle, brain, heart, and liver of animals [99–103]. Based on the analysis of the transcriptional alterations in aging tissues, it was suggested that induction of stress response genes is a result of the increased oxidative stress and damage during aging. In addition, the genes involved in inflammatory response, energy metabolism, and biosynthetic pathways were also changed in aging mammalian tissues [104]. Recently, the expression levels of genes involved in mitochondrial biogenesis-related transcription were found to be significantly decreased in aged heart, skeletal muscle, and adipose tissues of rats [105–107]. These changes were associated with reduction in the activities of Complexes I and V, which diminished the capacity of cardiac mitochondria to produce ATP. It is noteworthy that most of the age-associated changes in gene expression profiles were tissue-specific as revealed by comparative analysis of the gene expression profiles in various tissues of the mouse, rat, and human [101]. These results suggest that different tissues are subject to different degrees of oxidative stress during the aging process.

On the other hand, proteomic studies on the proteins in tissue cells revealed that the profiles of several clusters of proteins were consistently altered in aged individuals [108, 109]. Carbonylation was found to be a highly prevalent protein modification in skeletal muscle mitochondria, possibly contributing to the functional decline with age [110]. In addition, it was reported that nitrated proteins are accumulated during the aging process. Succinyl-CoA:3-ketoacid coenzyme A transferase (SCOT), the mitochondrial enzyme involved in the breakdown of ketone bodies in the extrahepatic tissues, was found to display an age-dependent increase in tryptophan nitration in rat heart [111, 112]. Recently, Dencher and co-workers [113] demonstrated that in brain mitochondria of rats, the age-related oligomerization of the F_1Fo-ATPase might be one of the clues to understand the link between the OXPHOS system and longevity. In addition, Gannon et al. [114] used phosphoproteomic technique to analyze skeletal muscle of old rats. The results showed that the alterations in the levels of phosphoproteins are involved in the complex metabolic pathways in the cytosol and mitochondria during the aging process. One of the important phosphorylated proteins was aconitase, which contains iron-sulfur clusters [12]. The decline of aconitase activity was found to be involved in age-associated diseases. It was also found that the phosphorylation of aconitase is regulated by members of the protein kinase C (PKC) family, which in turn affects the activity of the tricarboxylic acid cycle and contributes to mitochondrial dysfunction and bioenergetic defects in the hearts of diabetic rats [115].

It was recently demonstrated that the majority of the age-related changes in the gene expression profile in the aged tissues could be reversed by caloric restriction (CR) [116, 117]. Compared with transcriptional patterns of tissues from calorie-restricted animals, it was suggested that CR can retard the aging process by inducing metabolic shifts with specific transcriptional profiles [118]. Moreover, several studies have revealed that mitochondrial biogenesis and respiratory function were increased and ROS production repressed in the human and in animals under CR [119–121].

4 The Role of Sirt1 in Life-Span Extension by Caloric Restriction

4.1 Sirtuins in the Life-Span–Extending Effect of Caloric Restriction

The sirtuins are a conserved family of proteins possessing NAD^+-dependent protein deacetylase and are involved in the control of a variety of cellular processes such as aging, metabolism, and gene silencing [122, 123]. They were first linked to longevity and stress tolerance in the yeast, and the yeast gene silent information regulator 2 (Sir2) has been shown to mediate life-span extension conferred by CR and mild stresses [124, 125]. Recent studies demonstrated that Sirt1 mediates salutary physiologic effects of CR in mammals [126]. Sirt1, the well-studied mammalian Sir2 homolog, can deacetylate histone proteins and many key transcription factors, such as p53, peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1), and forkhead transcription factors (FOXOs), thereby regulating several cellular functions involved in stress resistance, apoptosis, and energy metabolism [127, 128]. Recently, numerous studies showed that CR could upregulate the level of Sirt1 expression and activity in several tissues, including muscle, brain, white adipose tissue, liver, and kidney [129, 130]. In addition, it has been shown that Sirt1 knockout mice failed to display a typical increase of physical activity induced by CR [131]. However, transgenic mice with overexpressed Sirt1 were found to display physiologic parameters and behaviors similar to those of calorie-restricted mice [132]. On the other hand, some studies demonstrated that Sirt1 can trigger stress tolerance of CR through deacetylation of its target proteins such as p53 and Ku70, which are essential for the process for the DNA repair system [133]. Besides, it has been proposed recently that Sirt1 plays an important role in CR-regulated glucose homeostasis by activating FOXOs and PGC-1 [134, 135]. Taken together, these studies suggest that the induction of Sirt1 by CR is an evolutionarily conserved biological stress response, which can slow down the aging process by altering the energy metabolism and increasing the stress response signaling cascade for the survival of animals.

4.2 The Regulation of Mitochondrial Function and Life Span by Sirt1

Recently, it was demonstrated that the Sirt1 activity is required for CR-mediated activation of mitochondrial biogenesis and respiration in animals [130]. In other words, Sirt1 may exert the CR-induced anti-aging effect by modulating the mitochondrial function. In fact, Sirt1 itself is one of the regulators of mitochondrial biogenesis through regulation of the acetylation status and activity of PGC-1α, a transcription coactivator involved in the regulation of some nuclear genes encoding mitochondrial proteins [136, 137]. Besides, in a mouse model, activation of Sirt1 by

its activator resveratrol was found to increase the number and activity of mitochondria in skeletal muscle and brown adipose tissues, resulting in the increase of energy expenditure and alleviation of the bioenergetic function decline in aging [138]. On the other hand, Sirt1 might regulate mitochondrial function by other mechanisms to extend the life span of animals. In a study of glucose-elicited insulin secretion, the Sirt1 transgenic mice showed repressed levels of uncoupling protein-2 (UCP-2) resulting in a more efficient coupling of mitochondrial respiration to generate ATP [139]. Besides, it has been reported that Sirt1 can protect β-cells against oxidative stress by deacetylating and increasing the activity of the forkhead protein FOXO1 [140]. Many studies have demonstrated that FOXO factors can promote longevity and reduce age-dependent diseases in invertebrates. Moreover, FOXO1 has been reported to enhance detoxification of ROS by upregulation of free radical scavenging enzymes, including Mn-SOD and catalase [141]. The increase in the resistance to oxidative stress has been established to be closely correlated with the extension of life span in laboratory animals. Therefore, the ability of Sirt1 to promote FOXO functions to cope with stress may explain why the Sirt1 proteins are involved in the extension of life span of animals fed on the restricted diet.

4.3 Other Sirtuins in Regulation of Mitochondrial Function

It has been reported that three of the mammalian sirtuins, Sirt3, Sirt4, and Sirt5, are located in the mitochondria [127]. Recent studies have shown that they can regulate the activity of several mitochondrial enzymes through deacetylation or ADP ribosylation. Evidence suggests that Sirt3-dependent deacetylation regulates the activity of acetyl coenzyme A synthetase 2 (AceCS2) [142], which catalyzes the conversion of acetate to acetyl-CoA and facilitates the use of acetate in metabolism. Besides, Sirt3 has been reported to be upregulated in white and brown adipose tissues in response to CR. It was found that overexpression of Sirt3 in brown adipocytes can regulate adaptive thermogenesis and affect the expression of genes involved in mitochondrial function, which may decrease mitochondrial membrane potential and ROS production while increasing cellular respiration [143]. Moreover, recent studies demonstrated that Sirt3 plays a role in maintaining the basal ATP levels and regulating mitochondrial electron transport through activation of Complex I function [144]. Furthermore, Sirt3 is downregulated in several genetically obese mice, and sequence variability in the human *Sirt3* gene has been linked to survivorship in elderly subjects [145].

Sirt4 does not have the deacetylase activity but has the ability to ADP-ribosylate and inhibit glutamate dehydrogenase (GDH), which can convert glutamate to α-ketoglutarate and control amino acid–stimulated insulin secretion [146]. In addition, Sirt4 expression is downregulated in response to CR-mediated effects in β-cells, which may enable the animal to use amino acids as energy sources during dietary limitation and enhances the insulin secretion in response to amino acids [146]. Recently, it was observed that Sirt5 can deacetylate mitochondrial cytochrome *c*

and carbamoyl phosphate synthetase 1 (CPS1), an enzyme that catalyzes the initial step of the urea cycle [147]. It was reported that CPS1 activity was increased during CR, and Sirt5 was able to facilitate ammonia disposal by deacetylating CPS1 [147]. Therefore, the above findings together suggest that sirtuins targeted at mitochondria can modulate mitochondrial metabolism to respond to environmental changes and energy demand. It has been suggested that during long-term diet restriction, as during fasting, the energy supply from catabolism of carbohydrates is not sufficient. Thus, Sirt3 and Sirt4 modulate the production of metabolic intermediates from acetate and amino acids in order to support energy production and other metabolic activities. Excess ammonia is produced when amino acids are used as the energy source, and at the same time Sirt5 immediately activates CPS1 to facilitate the removal of ammonia. In addition to the regulation of mitochondrial metabolism, both Sirt3 and Sirt4 have been shown to protect mammalian cells from genotoxic stress [148]. Based on the above findings, we suggest that mitochondrial sirtuins, including Sirt3, Sirt4, and Sirt5, play a critical role not only in the upregulation of mitochondrial function but also in the protection against oxidative stress and culminate in the extension of the life span of animals.

4.4 Therapeutic Agent Targeting Sirtuins in Age-Related Diseases

Recent research in the field of aging has begun to search for the key regulators of CR, which can help the development of drugs to improve health and extend life span. CR prolongs life span partly due to mitochondrial metabolism reorganization through the regulation of mitochondrial biogenesis by Sirt1 and PGC-1α. It is obvious that sirtuins may serve as an entry point for drug treatment of aging and age-related diseases. Therefore, controlling the expression and activity of sirtuins by using its activator, such as resveratrol, will have a health benefit. Resveratrol is a natural polyphenolic compound isolated from plants and has been shown to extend the life span of lower organisms by activating Sirt1 [138]. By, using a mouse model, Lagouge et al. found that resveratrol treatment protected young mice from obesity induced by high-fat diet through reducing the amount of adipose tissues. In addition to its effect on adipocytes, resveratrol was observed to increase the expression of mitochondrial genes involved in OXPHOS in skeletal muscle [149–151]. More evidence will be needed to substantiate that resveratrol can improve energy balance and enhance mitochondrial function in animals through activating Sirt1-mediated deacetylation of PGC-1α. These findings suggest that the resveratrol-induced activation of Sirt1 renders it a good therapeutic target for future treatment of various age-associated diseases such as metabolic disorder and mitochondrial disease.

5 Conclusions

In the past three decades, abundant evidence has been accumulated to support the mitochondrial theory of aging, which proposes that age-dependent decline in

mitochondrial function is a key contributor to aging [76]. The theory is based on the idea of a vicious cycle, in which somatic mutations of mtDNA cause respiratory chain dysfunction that leads to the increased production of ROS, which in turn lead to further mtDNA mutations, and finally a bioenergetic crisis culminates in overt tissue dysfunction and degeneration. This theory has recently been further examined by using transgenic mice with proofreading-deficient version of DNA polymerase γ, which displayed an accumulation of mtDNA mutations and deletions and manifested hallmarks of premature aging [45, 46]. On the other hand, many longevity genes have recently been identified, and the protein expression levels of most of them are related to the maintenance of mitochondrial structure and function [152, 153]. Therefore, it is believed that the maintenance of the activity and biogenesis capacity of mitochondria during the aging process would be one of the key factors that determine the progression of aging and age-related diseases. Interestingly, CR has been found to not only increase the life span but also reduce ROS production in mitochondria [15, 122]. This observation implies that mitochondria may increase the respiratory function and suppress the ROS production in calorie-restricted animals. In addition, CR was found to reverse the expression of age-related genes and slow down the age-associated biochemical and physiologic changes [119, 120]. On the other hand, the members of the sirtuins family have the capacity to serve as NAD^+-dependent protein deacetylases. Several of the sirtuins are involved in the regulation of the expression and posttranslational modification of mitochondrial biogenesis-related proteins including respiratory enzymes, especially in the calorie-restricted animals. Recent studies revealed that resveratrol can improve energy balance and enhance mitochondrial function through the activation of Sirt1-mediated deacetylation of PGC-1α [138]. Therefore, effective upregulation of Sirt1 and maintenance of mitochondrial integrity and respiratory function by use of some therapeutic agents or natural compounds such as resveratrol may be a potential approach to achieve healthy aging and prevent the onset of age-related diseases.

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Methylglyoxal, Oxidative Stress, and Aging

Miklós Péter Kalapos, Kaushik M. Desai, and Lingyun Wu

Abstract Methylglyoxal (MG) is a highly reactive product of mainly glucose metabolism. Reactive oxygen species (ROS) are produced during various biochemical reactions, including mitochondrial electron transport. An excess of MG production can increase ROS production and oxidative stress. MG can also form advanced glycation end products (AGEs) by reacting with proteins, DNA, and other biomolecules. The process of aging is multifactorial and involves changes at the cellular, tissue, organ, and whole-body levels that lead to decreased functioning, development of diseases, and death. MG, ROS, and AGEs are all associated with the aging process and age-related diseases such as cardiovascular complications of diabetes, neurodegenerative diseases, and connective tissue disorders. Specific and effective scavengers and cross-link breakers of MG and AGEs are being developed to slow the aging process and prevent many diseases.

Keywords Methylglyoxal · Oxidative stress · Advanced glycation end products · Aging

1 Introduction

Aging is a life process. The *Encyclopedia Britannica* [1] defines aging as a "Gradual change in an organism that leads to increased risk of weakness, disease, and death. It takes place in a cell, an organ, or the total organism over the entire adult life span of any living thing. There is a decline in biological functions and in ability to adapt to metabolic stress. Changes in organs include the replacement of functional cardiovascular cells with fibrous tissue. Overall effects of aging include reduced immunity, loss of muscle strength, decline in memory and other aspects of

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cognition, and loss of color in the hair and elasticity in the skin. Aging is part of the development sequence of the entire life span, from prenatal growth to senescence."

As defined above, the whole process of aging becomes more obvious in species with longer life spans such as humans. Many different theories have been proposed over the years to explain aging [2–5]. These theories can be broadly classified as program theories (e.g. biological clock theory, limited number of proliferation theory), error theories (e.g. disease theory, cross-linking theory, rate of living theory, free radical theory), or a combination of these two classes of theories [4]. The rate of living theory was proposed based on the observation that animals with higher metabolic rates often have shorter life spans. A connection between the metabolic rate and aging was provided by the free radical theory, which will be discussed in more detail later on.

Oxidative stress is caused when there is an excess of free radicals or reactive oxygen species (ROS) in the body [6–8]. This is due to an imbalance between production of ROS and antioxidant defenses such as antioxidant enzymes. Free radicals are highly reactive atoms or molecules with an unpaired electron in an orbit. ROS include oxygen-derived free radicals such as superoxide anions ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), as well as highly reactive nonradicals that do not have an unpaired electron in their orbit, such as hydrogen peroxide (H_2O_2).

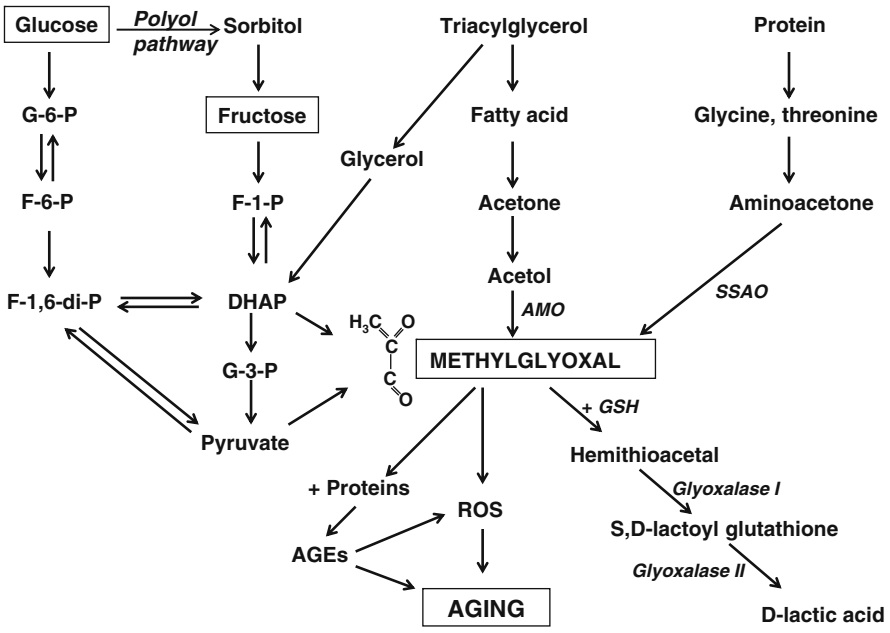

Fig. 1 Schematic of key steps of metabolic pathways of methylglyoxal. Abbreviations: AGEs, advanced glycation end products; ROS, reactive oxygen species; DHAP, dihydroxyacetone phosphate; AMO, acetol/acetone monooxygenase; SSAO, semicarbazide-sensitive amine oxidase; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1-P, fructose-1-phosphate; F-1,6-di-P, fructose-1,6-diphosphate; G-3-P, glyceraldehyde-3-phosphate

ROS react with proteins, lipids, DNA, and other biomolecules and alter their structure and function. ROS production involves many cellular biochemical pathways and enzymes such as the mitochondrial electron transport chain, nitric oxide synthase, non-mitochondrial-Fenton reaction, microsomal cytochrome P450 enzymes, peroxisomal beta-oxidation, and respiratory burst of phagocytic cells [9]. Antioxidant enzymes include superoxide dismutase (SOD), glutathione reductase, glutathione peroxidase, and catalase. Nonenzymatic antioxidant compounds include glutathione, ascorbate, urate, bilirubin, melatonin, tocopherols, carotenoids, and ubiquinol [9].

Methylglyoxal (MG) is a highly reactive electrophilic α,β-dicarbonyl aldehyde compound formed during glycolysis, mainly through spontaneous transformation of triose phosphates [10–12] (Fig. 1). MG is a major precursor of advanced glycation end products (AGEs). Plasma MG levels are elevated in diabetic patients, which underscores the clinical and pathological significance of MG [13–15]. An excess of MG and other reactive aldehydes such as glyoxal and 3-deoxyglucosone (3-DG) causes carbonyl overload and stress [6, 10, 11] in conditions such as diabetes [15, 16], hypertension [17–19], atherosclerosis [20], and neurodegenerative diseases [21, 22].

In this review, we will examine the current knowledge that implicates involvement of MG in the process of aging through increasing oxidative stress.

2 Methylglyoxal Metabolism

MG is a reactive intermediate of the metabolic network. Its formation is mainly, but not exclusively, linked to glycolysis through enzymatic or nonenzymatic conversions of dihydroxyacetone phosphate into MG [10, 11, 17]. Thus, glucose and fructose are the main precursors of MG formation [10, 23]. Other sources of MG formation include amino acid metabolism (such as glycine and threonine metabolism via aminoacetone) mediated by the enzyme semicarbazide-sensitive amine oxidase (SSAO), and lipid metabolism (such as acetone or acetoacetate) mediated by the enzymes acetol and acetone monooxygenases [10, 24, 25] (Fig. 1). Along with 3-deoxyglucosone and glyoxal, MG is believed to be a major source of intracellular and plasma AGEs [10]. MG reacts oxidatively with arginine, cysteine, or lysine residues of proteins [26–28] and forms irreversible AGEs. The reaction of MG with proteins depends on the structural configuration of protein and/or its microenvironment, and is thus selective [26, 27]. The reaction of MG with arginine produces hydroimidazolone and argpyrimidine [10, 29], whereas with lysine it forms N^{ε}-carboxymethyl-lysine (CML) and N^{ε}-carboxyethyl-lysine (CEL) [27, 30–32]. The thiol groups of cysteine residues with low pKa values are favorable nucleophiles to react with MG or glyoxal to produce AGEs such as carboxymethyl cysteine (CMC) and carboxyethyl cysteine (CEC) [33]. Subsequently, the glycated proteins form cross-links and cause malfunction [33]. AGEs can be detected immunohistochemically in tissues [34].

MG formed by all routes is mainly catabolized by the ubiquitous glyoxalase system [10, 16, 35, 36]. The glyoxalase system is composed of two enzymes, designated glyoxalase I and II, and uses a catalytic amount of reduced glutathione (GSH) [35–37] (Fig. 1). Beside the glyoxalase route, other enzymes also participate to varying degrees in the detoxification of methylglyoxal [10]. The physiologic plasma concentration of methylglyoxal in healthy humans has been reported to vary from less than 1 μM to about 3 μM and is elevated in diabetic patients [13, 14].

3 Free Radical Theory of Aging

ROS are inevitable by-products of metabolism, and all organisms are exposed to free radicals in the microenvironment within the cells of their bodies and in the macroenvironment outside their bodies. ROS include free radicals such as superoxide anion, hydrogen peroxide, and hydroxyl radical. ROS are commonly produced during the process of energy production, which in all eukaryotes involves mitochondrial respiration, which is driven by the electron transport chain [6, 38]. To handle superoxide anions produced, all aerobic organisms have evolved with SOD, an enzyme that scavenges superoxide anions [38–40] (Fig. 2). As the name suggests, ROS, and for that matter free radicals, are highly reactive and cause cellular oxidative damage by reacting with and modifying the structure and function of DNA, protein, and lipid molecules [6]. Gerschman showed that oxygen free radicals existed in vivo [41]. Based on the work of Rubner [42] and Gerschman, Denham Harman proposed in the 1950s that ROS are a cause of aging [43]. This free radical theory of aging has become one of the most popular theories of aging even though it is controversial. It hypothesizes that free radicals generation is a part of biological systems and processes and results in cumulative damage and aging. This is supported by the fact that aged organisms have increased levels of oxidatively damaged DNA [38, 39, 44].

There is substantial experimental evidence for the free radical theory. In the case of the fly *Drosophila melanogaster*, a number of studies support the role of free radicals and oxidative stress in shortening life span [45]. For example, transgenic *Drosophila* flies expressing the superoxide anion scavenging enzyme, SOD, show delayed aging and have a longer life span [46]. Similarly, flies and worms that show increased resistance to oxidative stress with elevated levels of antioxidant enzymes, such as SOD and catalase, have a longer life span [47, 48]. Catalase and/or SOD mimetic compounds extended life span of the worm *Caenorhabditis elegans* [49]. In analyzing the relationship of oxidative stress with maximum life span in different vertebrate species, it was found that the endogenous levels of enzymatic and nonenzymatic antioxidants in tissues negatively correlate with maximum life span, and the most long-living vertebrates studied in each group, pigeon or human, show the minimum levels of antioxidants [50]. Long-living animals may produce oxygen radicals at a low rate and, as such, require less antioxidants. However, administration of

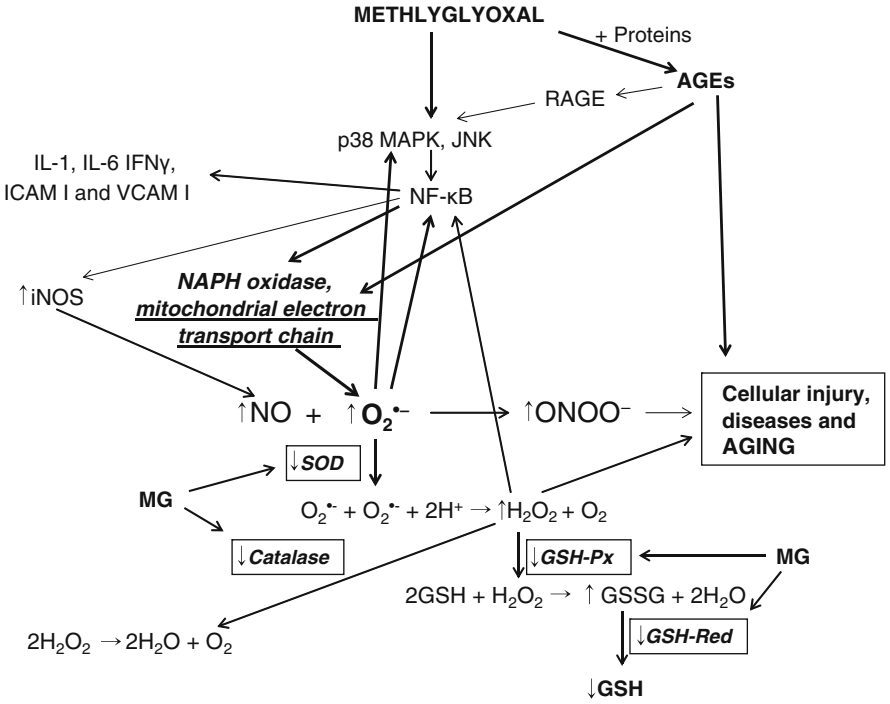

Fig. 2 Schematic diagram showing multiple effects of methylglyoxal that increase superoxide anion formation, oxidative stress, and play a role in aging. Abbreviations: AGEs, advanced glycation end products; GSH-Px, glutathione peroxidase; GSH-Red, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; ICAM 1, intracellular adhesion molecule 1; IFNγ, interferon γ; IL1α, interleukin 1α; JNK, JUN N-terminal kinase; MG, methylglyoxal; NF-κB, nuclear factor kappa B; NO, nitric oxide; $O_2^{\bullet -}$, superoxide anion; $ONOO^-$, peroxynitrite; p38 MAPK, p38 mitogen activated protein kinase; RAGE, receptor for advanced glycation end products; SOD, superoxide dismutase; VCAM 1, vascular cell adhesion molecule 1

antioxidants in the diet to mice or overexpression of SOD in rats did not extend life span [51, 52]. Muller et al. [45] believe that antioxidant administration may help alleviate certain age-related diseases, especially cancer, but may not be critical in the aging process and life span.

The mitochondria are the energy-producing centers or the "powerhouse" of the cell. The mitochondrial respiratory chain or the electron transport chain uses molecular oxygen and happens to be a major source of ROS production. Mechanistically, the mitochondrion, as both source and target of ROS, is one of the most popular ways of explaining the free radical theory of aging. The evidence to date suggests that mitochondria and mitochondrial DNA integrity may be important factors affecting aging [53]. Thus, free radicals have been proposed to damage DNA causing mutations leading to altered enzymes in the electron transport chain. Dysfunction of

the electron transport chain produces more free radicals and further mitochondrial DNA mutations. This "vicious cycle" ultimately accelerates age-related changes [54]. Barja et al. [55] measured the steady-state levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), as a marker of DNA damage, in the mitochondrial and nuclear DNA from the hearts of eight and the brains of six mammalian species ranging in maximum life span from 3.5 to 46 years. Significantly higher (3- to 9-fold) 8-oxodG/deoxyguanosine (dG) values were found in mitochondrial DNA than in nuclear DNA in all the species studied in both tissues. 8-OxodG/dG in mitochondrial DNA was inversely correlated with maximum life span both in heart and brain. The authors conclude that mitochondrial free radical generation is lower in long-lived than in short-lived species and that oxygen radicals of mitochondrial origin oxidatively damage mitochondrial DNA in a way related to the aging rate of each animal species [55].

Ku et al. [56] compared the rates of mitochondrial superoxide anion and hydrogen peroxide production and oxygen consumption in the kidney and the heart of seven different mammalian species, namely, mouse, hamster, rat, guinea pig, rabbit, pig, and cow, whose maximum life span varies from 3.5 to 30 years. The results indicated that under identical conditions, mitochondria from shorter-lived species produce relatively higher amounts of ROS than those from the longer-lived species, thus supporting the free radical theory of aging [56]. Basal and maximum rates of hydrogen peroxide production, oxygen consumption, and free radical leak in the respiratory chain were higher in heart mitochondria of the short-lived rat (4 years) than in the long-lived pigeon (35 years) [57].

Schriner et al. [58] generated transgenic mice that overexpress human catalase localized to the peroxisome, the nucleus, or mitochondria. The results showed an increase in median (average of 5 months) and maximum life spans (average of 5.5 months) in the transgenic mice. Hydrogen peroxide production and hydrogen peroxide–induced aconitase inactivation were attenuated along with reduced oxidative damage. Thus, mitochondrial deletions and cardiac pathology were reduced, and cataract development was delayed. These results also support the free radical theory of aging and point to the mitochondria as an important source of these radicals [58].

Sejersen and Rattan have proposed an in vitro cellular model of aging using human skin fibroblasts. Treatment of these cells with MG (400 μM) or glyoxal (1 mM) caused the appearance of senescent phenotype within 3 days. The parameters used included morphologic phenotype, irreversible growth arrest and G(2) arrest, increased senescence-associated β-galactosidase activity, increased hydrogen peroxide level, increased MG-induced AGE, CML protein level, and altered activities of SOD and catalase antioxidant enzymes [59].

Higher glucose and total calories intake increases free radical production and oxidative stress, which hastens aging and reduces life span. Many studies support this statement. On the other hand, stress conditioning can induce a positive compensatory response (hormesis) that protects against oxidative damage and extends life span [38, 39, 60].

4 Methylglyoxal and Oxidative Stress

The capacity of MG to initiate free radical formation was shown in vitro as early as the 1970s by Szent-Györgyi and associates [61]. However, during the ensuing two decades, there was no follow-up until a paper was published describing MG-provoked oxidative stress in cell culture [62]. In recent years, the correlation of MG and ROS has drawn more attention, involving the production of superoxide anion, hydrogen peroxide, peroxynitrite ($ONOO^-$), and nuclear factor kappa B (NF-κB). Herewith these findings are summarized in brief, and a schematic overview of MG-induced increase in oxidative stress is presented (Fig. 2).

4.1 Methylglyoxal and Superoxide Anion Formation

There is substantial evidence to support a direct stimulatory effect of MG on superoxide anion production. For example, incubation of cultured rat aortic vascular smooth muscle cells (VSMCs) with MG (10–300 μM) for 4 h significantly increased superoxide production in a concentration-dependent manner [63]. Superoxide scavenger SOD or NAD(P)H oxidase inhibitor diphenylene iodonium (DPI) prevented MG-induced superoxide production [63]. Similarly, incubation of neutrophils with MG (1 μM to 1 mM) for 60 min at 37°C resulted in a concentration-dependent increase of basal as well as formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated production of superoxide. MG was shown to stimulate NADPH oxidase through p38 MAPK [64]. MG 100 μM for 2 h also increased NADPH oxidase–mediated superoxide production in rat kidney mesangial cells, which was prevented by SOD [65]. Elevated plasma and aortic MG levels are associated with increased levels of superoxide in the aortas of 13-week-old spontaneously hypertensive rats (SHR) with elevated blood pressure compared with age-matched Wistar Kyoto (WKY) rats [18].

We investigated the effects of MG on mitochondrial function [66]. Incubation of vascular smooth muscle A-10 cells with MG (30 μM) increased mitochondrial superoxide anion production by 69.9% compared with untreated cells. The SOD mimetic 4-hydroxy-tempo (Tempol; 500 μM) decreased mitochondrial superoxide production induced by MG by 85.8%. MG (30 μM) decreased the activity of Mn-SOD, the first line of defense against superoxide in mitochondria, by 24.5%. Furthermore, MG decreased respiratory complex III activity and ATP synthesis in mitochondria, indicating an impaired mitochondrial respiratory chain. By inhibiting complex III activity, MG induces mitochondrial oxidative stress and reduces ATP production [66].

In mitochondria from streptozotocin-induced diabetic rat kidney cortex, MG significantly increased superoxide anion production and damaged mitochondrial proteins through formation of AGEs. Aminoguanidine improved mitochondrial respiration and decreased MG-induced oxidative damage to mitochondrial proteins [67].

All of the above data, including the effect of MG on mitochondrial respiratory chain and increased superoxide anion production, implicate excess MG-induced oxidative stress in accelerating the aging process (Fig. 2).

4.2 Methylglyoxal and Hydrogen Peroxide Production

A dose-dependent effect of MG on ROS production (measured by luminol/peroxidase method mainly detecting hydrogen peroxide) in cultured rat hepatocytes was seen after a lag period [62]. The release of hydrogen peroxide was not detectable under anaerobic conditions, whereas MG proved toxic to the cells [62]. The release of hydrogen peroxide became only significant when a large portion of hepatocytes had already lost their viability, thus it was concluded that hydrogen peroxide was not essentially involved in MG related toxicity [62]. Treatment of human platelets with MG stimulated hydrogen peroxide formation [62, 68]. Cotreatment of platelets with MG and thrombin resulted in an even greater hydrogen peroxide production [68]. Depletion of GSH was implicated in the action of MG to produce hydrogen peroxide in platelets [69, 70]. Incubation of rat aortic VSMCs with MG increased hydrogen peroxide generation as measured by the 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) probe along with nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) [63]. Incubation of VSMCs with MG (50–500 μM) for 24 h caused a significantly greater increase in oxidative stress in VSMCs from SHR compared with those from WKY rats [19]. Incubation of neutrophils with MG (1 μM to 1 mM) for 60 min at 37°C resulted in a concentration-dependent increase of hydrogen peroxide [64].

4.3 Methylglyoxal and Nitric Oxide/Peroxynitrite Production

MG increased nitric oxide (NO) generation in rat aortic VSMCs. As described earlier, MG also increased production of superoxide, which interacts with NO and leads to formation of peroxynitrite [63]. Inducible nitric oxide synthase (iNOS) seems to be the source of increased NO formation in response to MG because immunocytochemical staining showed increased expression of iNOS in cells treated with MG (100 μM for 18 h) compared with the untreated group.

4.4 Methylglyoxal and p38 MAPK

MG primes and activates neutrophils [64, 71, 72], through activation of p38 MAPK, to produce ROS and proteolytic enzymes to kill bacteria in neutrophils. MG also activates p38 MAPK in human endothelial cells [73] and rat kidney mesangial cells [74]. MG seems to be activating p38 MAPK directly [64].

4.5 Methylglyoxal and Activation of NF-κB

NF-κB is involved in inflammation. Within 3 h of treatment of VSMCs from SHR with 300 μM MG, there was a significant increase in the nuclear level of NF-κB p65 and a decrease in cytoplasmic level of IκBα protein, an inhibitory protein for NF-κB. The level of cytoplasmic IκBβ was not changed [75]. MG (300 μM) activated NF-κB in VSMCs from WKY rats. The activation of NF-κB in SHR VSMCs was associated with a large increase of intracellular adhesion molecule-1 (ICAM-1) 24 h after exposure to 100–300 μM MG. A smaller but significant increase in ICAM-1 was also seen in WKY VSMCs 24 h after exposure to 300 μM, but not 100 μM, MG.

MG-induced activation of NF-κB is likely mediated through production of strong oxidants such as superoxide anion and hydrogen peroxide [76, 77]. MG-induced increased oxidative stress and activation of NF-κB have also been observed in human endothelial cells [78, 79]. Activation of NF-κB affects its downstream signals, such as altered expression of proinflammatory genes [80]. An increased activation of NF-κB by MG was also reported in macrophages from deoxycorticosterone acetate hypertensive rats [81].

JNK, NF-κB, and peroxisome proliferator-activated receptor α (PPARα) are stress-response factors, and their expressions are regulated by redox cellular changes [82–86]. MG was shown to activate JNK and apoptosis signal-regulating kinase 1 (ASK1), associated with an increase in superoxide production in Jurkat leukemia T cells [87, 88]. MG increased expression of JNK and PPARα in SH-SY 5Y neuroblastoma cells. These cells have defective antioxidant ability, making them more susceptible to MG-induced toxicity [89]. Human glioblastoma ADF cells are able to scavenge MG and MG-induced ROS effectively through antioxidants and hence are less susceptible to its toxic effects [89].

MG has also been shown to increase oxidative stress through an induction of proinflammatory cytokines, IL-1β and IL-6, in cultured neural cells from rat hippocampus and neutrophils from nondiabetic human subjects [13, 15, 90]. This induction was prevented by pretreatment of neural cells with the antioxidant *N*-acetylcysteine (NAC) or of neutrophils with GSH or metformin [13, 15, 90].

4.6 Methylglyoxal, Antioxidant Enzymes, and Reduced Glutathione

MG has been shown to impair both enzymatic and nonenzymatic antioxidant defense systems. MG can increase oxidative stress by reacting with and inactivating antioxidant enzymes such as glutathione reductase and glutathione peroxidase [91, 92].

MG is detoxified by the glyoxalase system, which depends on the availability of GSH. In vitro incubation of the cytosolic fraction of mice liver cells with MG (1 mM) for 15 min inhibited glyoxalase I activity by about 20% [93]. MG has been shown to deplete GSH, making cells more sensitive to oxidative stress [94–96].

This establishes a vicious cycle that leads to continuous elevation of MG levels. Incubation of VSMCs from SHR and WKY rats with MG (500 μM) reduced the GSH content of these cells [19]. MG (300 μM for 24 h) also increased the levels of oxidized glutathione (GSSG), which was inhibited by pretreatment of the cells with NAC (600 μM for 30 min), indicating involvement of oxidative stress [19]. MG (100 μM) decreased glutathione peroxidase activity in both cell types but more so in SHR VSMCs. Glutathione peroxidase removes hydrogen peroxide with the help of GSH, which is in turn oxidized to GSSG [6, 78, 79]. In VSMCs from SHR as well as WKY rats, MG (300–500 μM) also reduced the activity of glutathione reductase, which acts as an antioxidant by converting GSSG to GSH [19, 97].

5 Methylglyoxal, Oxidative Stress, and Aging

The role of MG in aging has been investigated with regard to the generation of oxidative stress or formation of AGEs [10, 16, 23, 26, 27]. The aging process affects collagen and other long-lived proteins, which accumulate AGEs. The effects of these changes in basement membrane and matrix proteins are seen in blood vessels, which gradually lose their elasticity and become stiff; and in the eye lens, which becomes cataractous. Arterial aging has been suggested as the key factor for increased susceptibility of older subjects to cardiovascular disease [98, 99]. In vitro incubation with MG inactivated creatine kinase, an enzyme that controls the creatine–creatine phosphate shuttle [98].

Using a specific monoclonal antibody against glyoxalase I, Mailankot et al. [100] showed strong glyoxalase I immunoreactivity in the anterior epithelial cells of the human lens. The glyoxalase I activity and immunoreactivity both decreased with age, which may result in elevated MG levels and increased modification of the aging lens proteins [100].

MG-induced AGEs, such as CEL and CML, accumulate in long-lived tissue proteins with age and are implicated in the aging of tissue proteins. CEL is formed during the reaction of MG with lysine residues in the proteins RNase and collagen. CEL was also detected in human lens proteins at a concentration similar to that of CML and increased with age in parallel with the concentration of CML [31]. In another study on 172 young (<45 years old) and elderly (>60 years old) people, serum levels of MG-induced AGEs and the oxidative stress level were measured. CML and other MG derivatives were higher in elderly people and correlated with dietary AGEs intake and with markers of oxidative stress, 8-isoprostanes, and C-reactive protein [101].

The roles of MG, glyoxalase I that catalyzes MG, and MG-induced ROS formation in aging and life span were studied in the mitochondria of the worm *Caenorhabditis elegans*. In this study, Morcos et al. [102] showed that the activity of glyoxalase I was markedly reduced with age. This led to accumulation of MG-derived adducts and oxidative stress markers, causing further inhibition of glyoxalase I expression. Overexpression of the *C. elegans* glyoxalase I ortholog (CeGly) decreased MG-induced modifications of mitochondrial proteins,

mitochondrial ROS production, and prolonged the life span of *C. elegans,* whereas knock-out of CeGly had the opposite effects [102].

In SH-SY5Y neuroblastoma cells, MG increased the formation of intracellular ROS and lactate and decreased mitochondrial membrane potential and intracellular ATP levels. These harmful effects of MG can contribute to accelerated aging and neurodegeneration. MG scavengers aminoguanidine and tenilsetam attenuated these harmful effects of MG [103].

Glycated proteins and AGEs also induce oxidative stress, partly through induction of cytokines and growth factors [6, 11, 104–108]. Furthermore, AGEs can interact with the receptor for AGEs (RAGE) and scavenger receptors, which are present in endothelial cells, VSMCs, and mononuclear phagocytes [78, 106, 107, 109]. Activation of these receptors results in a strong oxidant production [106, 109, 110]. The interaction of AGEs with RAGEs promotes expression of cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1, in endothelial cells, and causes general vasculopathy, which is likely mediated by NF-κB activation caused by the oxidative stress associated with RAGE activation [78, 79].

To reiterate, MG *via* increases in oxidative stress [6, 23, 66] and formation of AGEs [10, 12, 15, 16, 111–113] is implicated in accelerating the aging process in many tissues and organs of the cardiovascular system, nervous system, and other systems of the body.

6 Prevention of Methylglyoxal-Induced Aging

Compounds that scavenge MG, such as aminoguanidine and metformin, or compounds that break AGEs cross-links, such as alagebrium (3-phenacyl-4,5-dimethylthiazolium chloride, ALT-711) [10, 112], may potentially slow the aging process [114, 115]. The effects of cross-link breakers may be mediated in part *via* reduction in oxidative stress and profibrotic cytokines [116]. Alagebrium has been shown to reduce vascular and ventricular stiffness. For example, 1-month treatment of aged dogs with alagebrium caused approximately 40% reduction of age-related left ventricular stiffness and improved cardiac function [116]. In clinical studies, alagebrium has been shown to improve arterial compliance in elderly patients with vascular stiffening and improved cardiac function in patients with heart failure [117]. After older subjects with systolic hypertension were treated for 8 weeks with alagebrium, there was improvement in endothelial function (measured as brachial artery flow–mediated dilation) [118]. This improvement correlated with reduced vascular fibrosis and inflammation [118]. Alagebrium treatment also influences vascular remodeling independently of blood pressure changes [99, 118–120].

Aging causes a progressive deterioration of cardiac function. Thus, in the aging heart there is an increased mitochondrial DNA deletion and a twofold increase in AGEs [121]. At the same time, the antioxidant SOD and glutathione peroxidase activities were reduced by 50%. Treatment with alagebrium preserved cardiac diastolic function, reduced mitochondrial DNA deletion, and decreased

AGEs by about 30%. Alagebrium also increased SOD and glutathione peroxidase activities in aging hearts as well as in cultured cardiomyocytes [121].

Coughlan et al. [108] show that, under hyperglycemic conditions in vitro or in diabetic rats, increased availability of NADH leads to increased formation of superoxide from mitochondrial complex I, which uses NADH as substrate. AGEs acting on the receptor RAGE increase generation of cytosolic ROS, which leads to mitochondrial permeability transition. Inhibition of cytosolic ROS production with apocynin or reducing AGEs with alagebrium attenuated mitochondrial superoxide generation. Thus, AGEs cross-link breakers may prove useful in treatment of diabetic nephropathy.

Caloric restriction has been shown to increase longevity in organisms ranging from yeast to mammals. Caloric restriction reduces metabolism and ROS production, which in turn can prevent oxidative damage to biomolecules, thus slowing the aging process and prolonging life span. Reduction of glucose concentration increased life span and resistance to oxidative stress of fission yeast *Schizosaccharomyces pombe* [60]. Caloric restriction in rats reduced generation of ROS from isolated mitochondrial preparations and attenuated the accumulation of oxidative damage [122]. Hipkiss [123] proposed a mechanism linking diet, exercise, and mitochondria-dependent changes in NAD/NADH ratio to intracellular protein modifications induced by MG. According to Hipkiss, ad libitum feeding decreases NAD availability, which in turn increases MG formation through decreased metabolism of glycolytic intermediates such as glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Glycolytic intermediates can spontaneously decompose into MG. MG and MG-induced AGEs would increase ROS formation and cause mitochondrial dysfunction and affect gene expression and intracellular signaling. On the other hand, after dietary restriction, NAD would be regenerated due to oxidation of NADH in the mitochondria. This would also suppress MG formation. This proposal can explain the apparent paradox whereby increased aerobic activity suppresses formation of glycoxidized proteins and extends life span [123]. Hipkiss [124, 125] also proposes that intermittent feeding can produce metabolic effects similar to those produced by dietary calorie restriction such as reduced formation of MG and help delay aging and prolong life span. It is believed that ad libitum feeding results in more or less continuous glycolysis and continuous generation of excess MG, which overwhelms the anti-MG defense systems. An excess of MG damages mitochondria and induces a prooxidant state to create a senescent-like condition. Caloric restriction also induces synthesis of glyoxalase-1 and antiglycating agents (carnosine and polyamines) and reduces ROS production. This is an adaptive response, known as hormesis, that increases life span.

7 Conclusions

MG can generate oxidative stress and cause AGEs formation. Under normal conditions, MG is efficiently metabolized to D-lactate by GSH and the glyoxalase enzymes so that its harmful effects are limited. Glucose and fructose are the main

precursors of MG formation in the body. The balance between MG production and anti-MG defense mechanisms can swing toward excess accumulation of MG, ROS, and AGEs with daily excess carbohydrate intake, which is seen especially with the Western diet or with uncontrolled hyperglycemia of diabetes. Due to its high reactivity, MG reacts with antioxidant enzymes such as glutathione peroxidase and SOD, thus impairing degradation of MG and setting up a vicious cycle. MG-induced ROS and AGEs can impair mitochondrial function and lead to more production of ROS and further damage. The end result is modification of DNA, vital enzymes, and long-lived proteins such as collagen and elastin, all of which accelerate the aging process and trigger the onset of cardiovascular, neurologic, and collagen/connective tissue–associated diseases. Scavengers of MG and AGEs have the potential to reverse or slow the aging process and the age-associated diseases. New AGEs cross-link breaking drugs such as alagebrium show promise to slow the age-related changes. Dietary calories restriction is also showing a beneficial influence on aging *via* reduced MG production.

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Part II
The Cardiovascular System

Novel Strategies for Neurovascular Longevity During Aging

Kenneth Maiese, Zhao Zhong Chong, Jinling Hou, and Yan Chen Shang

Abstract Globally, approximately 400 million individuals suffer from central nervous system disorders that can involve the neurovascular system and are related to aging, such as presenile dementia, Alzheimer's disease (AD), Parkinson's disease, ischemic brain disease, and cancer-related disorders. However, what may be of even greater significance to the brain and body is the association of these disorders with oxidative stress and the release of reactive oxygen species. Oxidative stress leads to the destruction of a number of cell types and tissues through apoptotic pathways that involve both early membrane changes and immune cell activation as well as end-stage nuclear degradation. For the development of new therapeutic strategies that are directed against disorders of the neurovascular system, novel investigations that blend basic research with clinical trials are necessary to expand our understanding of the multiple cellular pathways involved. In this regard, pathways such as those that pertain to the mammalian forkhead family of the "O" class (FoxOs), the growth factor and cytokine erythropoietin (EPO), protein kinase B, and *Wingless* may provide hope for individuals that suffer from neurovascular disorders related to aging and oxidative stress. In addition, these pathways not only serve as biomarkers that sometimes predict clinical outcome but also govern the development of stem cells, cardiovascular function, immune system recognition, cellular survival and longevity, metabolic disease, and cancer progression. As our knowledge increases of the intimate relationships that link these unique cellular pathways, initiation of new treatments for aging and oxidative stress should allow for highly successful clinical outcomes with limited or absent detrimental results.

Keywords Aging · Alzheimer's disease · Angiogenesis · Apoptosis · Cancer · Cardiac · Diabetes · Erythropoietin · Forkhead transcription factors · Immune system · Ischemia · Neurodegeneration · Oxidative stress · Stem cells · Wnt · Wingless

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1 Introduction

1.1 Aging and Oxidative Stress

It is estimated that greater than 25 million people in the United States suffer from central nervous system (CNS) disorders. Throughout the world, this number reaches a level approaching 400 million people. These disorders can involve the neurovascular system and are related to aging and consist of presenile dementia, Alzheimer's disease (AD), Parkinson's disease, ischemic brain disease, and cancer-related disorders. For the clinical care of these disorders, the cost of physician services, hospital and nursing home care, and medications continues to rise dramatically. In addition, the medical costs for neurodegenerative disease and cancer treatment parallel a progressive loss of economic productivity with rising morbidity and mortality, ultimately resulting in an annual deficit to the economy that is estimated to be greater than $450 billion. Yet, the most significant portion of this economic loss can be confined to only a few neurodegenerative disease entities, such as AD. For example, the annual cost per patient with AD may easily reach $250,000 with an annual population aggregate cost of $100 billion [1–3].

If one considers the cellular pathology of AD, this progressive neurodegenerative disorder leads to a deterioration of cognitive function with memory loss and injury to hippocampal neurons. The generation of extracellular plaques of amyloid-β peptide aggregates composed of a 39- to 42-amino-acid peptide (Aβ) are considered to be one of the pathologic mechanisms that may promote the development of AD [2]. Accumulation of Aβ can lead to apoptotic injury with chromatin condensation, DNA fragmentation, and cellular membrane phosphatidylserine (PS) exposure [2, 3]. In addition, Aβ can precipitate a significant inflammatory response with microglial activation [4, 5] and the secretion of tumor necrosis factor-α (TNF-α) [6]. Yet, what may be of greater systemic importance to the brain and body is the association of neurodegenerative and cancer-related disorders to oxidative stress and the release of reactive oxygen species (ROS). With regard to AD, Aβ can release ROS and lead to apoptotic cell injury [1, 2].

Release of ROS that consist of oxygen free radicals and other chemical entities occurs during oxidative stress. Oxygen free radicals can be generated in elevated quantities during the reduction of oxygen and lead to cell injury. ROS can involve superoxide free radicals, hydrogen peroxide, singlet oxygen, nitric oxide (NO), and peroxynitrite [1, 7, 8]. Most species are produced at low levels during normal physiologic conditions and are scavenged by endogenous antioxidant systems that include superoxide dismutase (SOD), glutathione peroxidase, catalase, and small-molecule substances such as vitamins C and E. Other closely linked pathways to oxidative stress may be tempered by different vitamins, such as vitamin D_3 [9] and the amide form of niacin or vitamin B_3, nicotinamide [10–16].

Initial investigations by Pearl proposed that increased exposure to oxygen through a high metabolic rate could lead to a shortened life span [17]. Additional studies by other investigators have demonstrated that increased metabolic rates could be detrimental to animals in an elevated oxygen environment [18]. Current

studies show that oxygen free radicals and mitochondrial DNA mutations have become associated with cellular injury, aging mechanisms, and accumulated toxicity for an organism [19].

Oxidative stress leads to the destruction of multiple cell types through apoptotic pathways [20–22]. Apoptosis is a dynamic process that consists of both the early exposure of membrane PS residues and the late destruction of genomic DNA [3, 23]. Externalization of membrane PS residues is an early event during cell apoptosis [24, 25] and can become a signal for the phagocytosis of cells [22, 26, 27]. The loss of membrane phospholipid asymmetry leads to the exposure of membrane PS residues on the cell surface and assists microglia to target cells for phagocytosis [16, 28–31]. This process occurs with the expression of the phosphatidylserine receptor (PSR) on microglia during oxidative stress [32, 33]. It has been shown that blockade of PSR function in microglia prevents the activation of microglia [30, 34]. Externalization of membrane PS residues occurs in neurons, vascular cells, and inflammatory microglia during reduced oxygen exposure [22, 35–38], Aβ exposure [4, 5], NO exposure [39–43], and during the administration of agents that induce the production of reactive oxygen species, such as 6-hydroxydopamine [44]. Membrane PS externalization on platelets also has been associated with clot formation in the vascular system [45].

The cleavage of genomic DNA into fragments [37, 46, 47] usually occurs after membrane PS exposure [48] and is considered to be a later event during apoptotic injury [29, 47, 49, 50]. Several enzymes responsible for DNA degradation include the acidic, cation-independent endonuclease (DNase II), cyclophilins, and the 97-kDa magnesium-dependent endonuclease [1, 51]. Three separate endonuclease activities also have been found in neurons that include a constitutive acidic cation-independent endonuclease, a constitutive calcium/magnesium-dependent endonuclease, and an inducible magnesium-dependent endonuclease [52, 53].

During oxidative stress, mitochondrial membrane transition pore permeability also is increased [15, 29, 54, 55], a significant loss of mitochondrial NAD^+ stores occurs, and further generation of superoxide radicals leads to cell injury [16, 56]. Mitochondria are a significant source of superoxide radicals that are associated with oxidative stress [1, 57]. Blockade of the electron transfer chain at the flavin mononucleotide group of complex I or at the ubiquinone site of complex III results in the active generation of free radicals, which can impair mitochondrial electron transport and enhance free radical production [32, 51]. Furthermore, mutations in the mitochondrial genome have been associated with the potential development of a host of disorders, such as hypertension, hypercholesterolemia, and hypomagnesemia [58, 59]. ROS also may lead to cellular acidosis and subsequent mitochondrial failure [2]. Apoptotic-induced oxidative stress in conjunction with processes of mitochondrial dysfunction [60–62] can contribute to a variety of disease states such as diabetes, ischemia, cognitive loss, AD, cancer, and trauma [1, 2, 63–65]. Oxidative stress can lead to apoptosis in neurons, endothelial cells (ECs), cardiomyocytes, and smooth muscle cells that involve separate as well as overlapping pathways [29, 63, 66–69].

Interestingly, novel cellular pathways, such those that involve the mammalian forkhead family of the "O" class (FoxOs), may illustrate the existence of innate defense mechanisms of the body against oxidative stress. During oxidative stress,

FoxOs can become activated and control cell cycle regulation to limit tumor cell invasion. As a result, these transcription factors may represent host body defenses that are attempting to restrict neoplastic growth [70–72]. Other unique pathways of the body that can become activated during oxidative stress, such as the growth factor and cytokine erythropoietin (EPO), also may provide hope for individuals that suffer from cognitive disability and neurodegenerative disorders related to aging. For example, the increased expression of EPO during periods of oxidative stress may suggest that protective mechanisms against ROS are being set into motion by the brain [73–75], as EPO in animal studies has been shown to reduce cognitive loss during mechanical injury to the hippocampus [76]. Both FoxOs and EPO also may serve as biomarkers that can offer the additional benefit to function as a surrogate marker to be able to be used to predict clinical outcome in some cases. For biological systems, a "biomarker" can consist of any entity that occurs in the body and that can be measured to predict the diagnosis, onset, or progression of a disease process. Application of biomarkers can be used for the determination of specific genes, proteins, products of cellular and biological processes, as well as the response of cells or tissues to therapeutic strategies [77]. Yet, it should be noted that additional pathways that occur in combination with a particular biomarker during oxidative stress also may influence outcome. Therefore, it becomes imperative to elucidate the components and function of novel pathways for agents such as FoxOs and EPO during oxidative stress to understand their role not only as biomarkers, but also as therapeutic strategies to offer new insight for clinical care for neurovascular disease entities.

2 FoxOs

2.1 Background, Expression, and Regulation of FoxOs

Mammalian forkhead transcription factors of the O class (FoxOs) can either block or activate target gene expression by binding to DNA through the forkhead domain that uses 14 protein–DNA contacts [72]. The forkhead domain in Fox proteins consists of three α-helices, three β-sheets, and two loops that are referred to as the wings [78], but not all winged helix domains are considered to be Fox proteins [79]. The forkhead domain is described as a "winged helix" as a result of a butterfly-like appearance on X-ray crystallography [78] or nuclear magnetic resonance imaging [80]. High sequence homology is present in the α-helices and β-sheets with variations described in either absent β-sheets and loops or additional α-helices. Although both the first and second loops make contact with DNA, it is the second loop that can influence the stability of DNA binding. In addition, posttranslational modification of FoxO proteins, such as phosphorylation or acetylation that block FoxO activity, alter the binding of the C-terminal basic region to DNA to prevent transcriptional activity [81]. Yet, other mechanisms may influence DNA binding of forkhead proteins, such as variations in the N-terminal region of the DNA recognition helix, changes in electrostatic distribution, and the ability of forkhead proteins to be shuttled to the cell nucleus [70, 82].

At least 100 forkhead genes and 19 human subgroups that range from *FOXA* to *FOXS* are now known to exist since the initial discovery of the fly *Drosophila melanogaster* gene *forkhead* [83]. The original nomenclature for these proteins, such as forkhead in rhabdomyosarcoma (*FKHR*), the *Drosophila* gene *forkhead* (*fkh*), and forkhead related activator (FREAC)-1 and -2, has been replaced. The current nomenclature for human Fox proteins places all letters in uppercase, otherwise only the initial letter is listed as uppercase for the mouse, and for all other chordates the initial and subclass letters are in uppercase [84]. FoxOs were first reported in fusion genes in human soft tissue tumors and leukemias. FOXO1, termed forkhead in rhabdomyosarcoma (FKHR), and FOXO3a, also known as forkhead in rhabdomyosarcoma like protein 1 (FKHRL1), and their genes were identified through chromosomal translocations in alveolar rhabdomyosarcoma tumors [85]. The acute leukemia fusion gene located in chromosome X (*AFX*), also known as the *FOXO4* gene, was demonstrated as a gene that fused to mixed lineage leukemia (MLL) transcription factor as a result of the *t(X; 11)* chromosomal translocation in acute lymphoblastic leukemia [86]. A fusion between FOXO2 and MLL also occurs in some cases of acute myeloid leukemia that may be identical to FOXO3a [87].

FoxO proteins (FoxO1, FoxO3, FoxO4, and FoxO6) occur throughout the body and are expressed in tissues of the reproductive system of males and females, skeletal muscle, the cardiovascular system, lung, liver, pancreas, spleen, thymus, and the nervous system [70, 88–94]. However, FoxO proteins are not equally expressed in all tissues, suggesting that individual FoxO proteins may have specificity with regard to cellular function [93]. For example, FoxO6 expression is found in several regions of the brain that play a significant role in cognitive function and emotion, such as the hippocampus, the amygdala, and the nucleus accumbens [91]. In contrast, FoxO1 may be more suited for the control of motor function and memory formation, as the expression of this protein is primarily in the striatum and subregions of the hippocampus [91]. In addition, FoxO3 is more diffusely represented in the hippocampus, cortex, and cerebellum, suggesting a complementary role for this FoxO protein to control cognitive and motor function. FoxO expression can be variable in other tissues [95]. Although studies in mice have shown that the mRNA distribution of Foxo1, Foxo3a, and Foxo4 is similar in the embryo and adult [89], Foxo1 expression was highest in adipose tissue, Foxo3a expression was greatest in the liver, and Foxo4 expression was strongest in muscle [89]. Subsequent work in mice has described Foxo1 expression in all tissues with high levels in the ovaries [96]. Foxo3a also is expressed in all tissues, and Foxo4 expression was considered to be more tissue specific in skeletal muscle [96].

Posttranslational control of FoxO proteins employs pathways associated with ubiquitylation and acetylation [97, 98]. IκB kinase (IKK) can phosphorylate and block the activity of FoxO proteins, such as FoxO3a [70, 85]. This leads to the proteolysis of FoxO3a via the Ub-dependent proteasome pathway [70, 85, 99–101]. FoxO proteins also are acetylated by histone acetyltransferases that include p300, the CREB-binding protein (CBP), and the CBP-associated factor. In addition, FoxO proteins are deacetylated by histone deacetylases. These include Sirt1, a NAD^+-dependent deacetylase and the mammalian ortholog of the silent information regulator 2 (Sir2) protein [70], which can control multiple processes

such as cell injury, life span, and metabolism [102, 103]. Acetylation of FoxO proteins provides another avenue for the control of these proteins. Once acetylated such as by CBP, FoxO proteins may translocate to the cell nucleus but have diminished activity, as acetylation of lysine residues on FoxO proteins has been shown to limit the ability of FoxO proteins to bind to DNA [104]. Acetylation also can increase phosphorylation of FoxO proteins by the serine–threonine kinase protein kinase B (Akt) [104].

In addition to acetylation and ubiquitylation, posttranslational modulation of FoxO proteins also involves pathways associated with phosphorylation [70, 85, 99–101]. Protein phosphorylation is a critical pathway in the scheme for protein regulation [105]. Akt is a primary mediator of phosphorylation of FoxO1, FoxO3a, and FoxO4 that can block activity of these proteins [85, 106]. Akt phosphorylation of FoxO proteins not only retains these transcription factors in the cytoplasm but also leads to ubiquitination and degradation through the 26S proteasome [98, 99]. The serum- and glucocorticoid-inducible protein kinase (Sgk), a member of a family of kinases termed AGC (protein kinase A/protein kinase G/protein kinase C) kinases that includes Akt, also can phosphorylate and retain FoxO3a in the cytoplasm [107]. Knowledge that Sgk and Akt can phosphorylate FoxO3a at different sites suggests other avenues to more effectively prevent apoptotic cell injury that may be mediated by FoxO3a activity. Yet, phosphorylation of FoxO proteins does not always lead to negative regulation. The protein kinase mammalian sterile 20-like kinase-1 also can phosphorylate FoxO proteins directly and lead to their activation [108]. The ability of sterile 20-like kinase-1 to activate FoxO proteins may be linked to c-Jun N-terminal kinase (JNK), as sterile 20-like kinase-1 can increase JNK activation [109].

Activation of Akt in pathways that involve EPO or FoxOs is usually cytoprotective but may mediate other processes. For example, Akt either alone or through EPO can lead to cell proliferation [110], blood–brain barrier permeability [111], or cell protection during inflammation [112, 113], neurodegeneration [114], hyperglycemia [115], hypoxia [116], Aβ toxicity [4, 117–120], excitotoxicity [121], cardiomyopathy [122], cellular aging [123], and oxidative stress [29, 30, 66]. In addition, Akt can prevent cellular apoptosis through the phosphorylation of FoxO proteins [8]. Posttranslational phosphorylation of FoxO proteins, such as during EPO administration, will maintain FoxO transcription factors in the cytoplasm by association with 14-3-3 proteins and prevent the transcription of proapoptotic target genes [75, 124]. An exception to these observations involving the subcellular trafficking of FoxO proteins involves FoxO6. This FoxO protein usually resides in the nucleus of cells and is phosphorylated by Akt in the nucleus. FoxO6 does not contain a conserved C-terminal Akt motif, which limits nuclear shuttling of this protein, but FoxO6 transcriptional activity can be blocked by growth factors independent of shuttling to the cytosol through a FoxO6 N-terminal Akt site [125].

Modulation of Akt activity also controls apoptotic pathways of caspases that may offer an alternative mechanism to regulate FoxO proteins [71]. Caspases are a family of cysteine proteases that are synthesized as inactive zymogens that are proteolytically cleaved into subunits at the onset of apoptosis [32, 126, 127]. The caspases 1 and 3 have been linked to the apoptotic pathways of genomic DNA cleavage, cellular

membrane PS exposure, and activation of inflammatory cells [34, 48, 55]. Caspase pathways may be tied to the forkhead transcription factor FoxO3a, as increased activity of FoxO3a can result in cytochrome c release and caspase-induced apoptotic death [124, 128–130]. Pathways that can inhibit caspase-3 appear to offer a unique regulatory mechanism. For example, studies suggests that cell death pathways that rely upon FoxO3a also appear to involve caspase-3 activation [5]. FoxO3a activity promotes caspase-induced apoptotic death [124, 128–130], but inhibition of caspase-3 also can maintain the phosphorylated "inactive" state of FoxO3a to prevent cell injury [124, 128, 129]. Other work has shown that caspase-3 activity and cleavage is promoted during transfection of a triple mutant FoxO3a expression in which three phosphorylation sites have been altered to prevent inactivation of FoxO3a [131]. Furthermore, FoxO3a may control early activation and subsequent apoptotic injury in microglia during Aβ exposure through caspase-3 [5] (Fig. 1). As Aβ exposure can facilitate the cellular trafficking of FoxO3a from the cytoplasm to

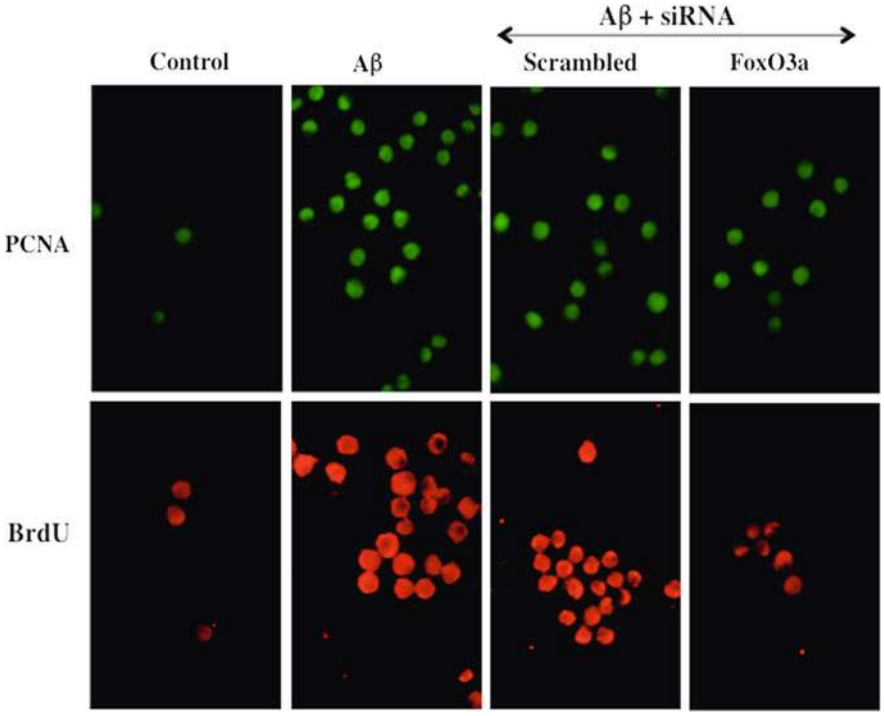

Fig. 1 Gene silencing of FoxO3a blocks early microglial activation, and proliferation occurs after amyloid (Aβ) exposure. Representative images illustrate that gene silencing with FoxO3a small interfering RNA (siRNA) decreases the activation of microglia as evidenced by the expression of proliferating cell nuclear antigen (PCNA) and the proliferation of microglia as evidenced by the uptake of bromodeoxyuridine (BrdU) at 6 h after $A\beta_{1\text{-}42}$ (10 μM) exposure ($^{*}p < 0.01$ vs. control or untreated cells). In contrast, during $A\beta_{1\text{-}42}$ (10 μM) exposure alone or with scrambled siRNA, significant microglial activation with PCNA and microglial proliferation with BrdU occurs. In all cases, control = untreated cells and scrambled = scrambled siRNA that is not specific for FoxO3a

the cell nucleus to potentially lead to "proapoptotic" programs by this transcription factor [5], one program in particular that may be vital for apoptotic injury appears to involve the activation of caspase-3. Aβ exposure leads to a rapid and significant increase in caspase 3 activity within 6 h after Aβ administration, but this induction of caspase-3 activity by Aβ requires FoxO3a, as loss of FoxO3a through gene silencing prevents the induction of caspase-3 activity by Aβ.

3 Erythropoietin

3.1 Background, Structure, and Expression for EPO

EPO was first described as "hemopoietine," an agent that could stimulate new red blood cell development. Carnot and Deflandre in 1906 demonstrated that plasma removed from rabbits after a bleeding stimulus that was later injected into control untreated rabbits would lead to the development of immature red blood cells [73, 74, 132, 133]. A number of other investigators followed these studies and found similar results demonstrating that plasma from bled animals would yield a significant reticulocytosis [134–136]. More elegant experiments eventually demonstrated that a rise in hemoglobin levels with reticulocytosis occurred in parabiotic rats when only one partner was exposed to hypoxia, illustrating that depressed oxygen tensions could stimulate EPO production [137]. Later, human EPO protein was purified that led the way for the cloning of the EPO gene and the development of recombinant EPO for clinical use [138, 139].

The EPO gene is located on chromosome 7, exists as a single copy in a 5.4-kb region of the genomic DNA, and encodes a polypeptide chain containing 193 amino acids. During the production and secretion of EPO, a 166-amino-acid peptide is initially generated after the cleavage of a 27-amino-acid hydrophobic secretory leader at the amino-terminal. In addition, a carboxy-terminal arginine in position 166 is removed both in the mature human and recombinant human EPO (rhEPO) resulting in a circulatory mature protein of 165 amino acids [75, 140]. Once a mature protein, EPO becomes a 30.4-kDa glycoprotein with approximately half of its molecular weight derived from carbohydrates that can vary among species [75]. EPO contains four glycosylated chains including three N-linked and one O-linked acidic oligosaccharide side chains. The glycosylated chains are important for the biological activity of EPO and can protect EPO from oxygen radical degradation. EPO is stabilized by the carbohydrate chains [141], and the oligosaccharides in EPO also may protect the protein from oxygen radical activity [142]. The N-glycosylated chains are believed to contribute to the thermal stability of EPO [143]. In addition, the N- and O-linked chains may be necessary for the production and secretion of the mature EPO [144]. The presence of the carbohydrates also are important in the control of the metabolism of EPO, as EPO molecules with high sialic acid content can be easily cleared by the body through specific binding in the liver [145]. In addition, the biological activity of EPO also relies upon two disulfide bonds formed between cysteines at positions 7 and 160 and at positions 29 and 33 [140].

The principal organs of EPO production and secretion are the kidney, liver, brain, and uterus. EPO production and secretion occurs foremost in the kidney [146]. The kidney peritubular interstitial cells are responsible for the production and secretion of EPO [133]. With the use of cDNA probes derived from the EPO gene, peritubular ECs, tubular epithelial cells, and nephron segments in the kidney also have been demonstrated to be vital cells for the production and secretion of EPO [147, 148]. During periods of acute renal failure, EPO may provide assistance for the protection of the kidneys and nephrons [149–151]. Other sites of EPO production and secretion occur in the liver and the uterus [152]. Hepatocytes, hepatoma cells, and Kupffer cells of the liver can produce EPO, and, in turn, EPO may protect these cells from injury and assist with regeneration [153, 154]. With regard to the uterine production of EPO, it is believed that the occurrence of neonatal anemia that can take place in the early weeks after birth may partly result from the loss of EPO production and secretion by placenta [155]. In addition, increased levels of EPO in the fetal plasma and amniotic fluid during gestation may function as a biomarker of intrauterine hypoxia [156].

Although EPO is approved by the Food and Drug Administration for the treatment of anemia, recent studies have demonstrated that EPO is not only required for erythropoiesis but also functions in other organs and tissues, such as the brain, heart, and vascular system [34, 116, 124, 157–159]. EPO production is believed to occur throughout the body [8, 75, 160] and can be detected in the breath of healthy individuals [161]. In addition, it has been suggested that EPO may provide developmental cognitive support. In experimental animal models, EPO may reduce apoptotic pathways during periods of hyperoxia in the developing brain [162, 163]. Furthermore, clinical disorders may have periods of hyperoxia followed by cerebral hypoperfusion and hypoxia that can lead to cerebral injury with associated oxidative stress [164]. In these circumstances, EPO also may be protective, as it can promote neurite outgrowth [165] and also may regulate hemoglobin levels that have recently been associated with cognitive decline [166]. In other work, elevated EPO concentrations during infant maturation have been correlated with increased Mental Development Index scores [167], and EPO may prevent toxic effects of agents used to control cognitive function such as haloperidol [168].

New knowledge that EPO and its receptor are present in the nervous and vascular systems has generated great enthusiasm for the potential clinical applications of EPO, such as in AD, cardiac insufficiency [169, 170], and cardiac transplantation [171, 172]. In the nervous system, primary sites of EPO production and secretion are in the hippocampus, internal capsule, cortex, midbrain, cerebral ECs, and astrocytes [75, 140, 173, 174]. Further work has revealed several other organs as secretory tissues for EPO that include peripheral ECs [175], myoblasts [176], insulin-producing cells [177], and cardiac tissue [75, 146].

3.2 Cellular Signaling for EPO and the EPO Receptor

EPO controls erythroid cell proliferation, differentiation, and survival through its binding to a target cell surface receptor, the EPO receptor (EPOR) [178]. The

EPOR also is expressed in numerous nonerythroid blood lines that include neurons, microglia, astrocytes, and in cerebral ECs [75, 140, 146, 174, 175], as well as on myelin sheaths of radicular nerves in human peripheral nerves [179], suggesting both a developmental and potential protective role for EPO in the central and peripheral nervous systems. During gestation, EPO production is increased but later becomes suppressed after birth to be regulated by the tissue oxygen supply [180]. The EPOR also is expressed in primary cerebral ECs [55, 181], as well as in human umbilical veins, bovine adrenal capillaries, and rat brain capillaries [175, 182].

Despite the fact that EPO is a critical modulator of erythropoiesis, the presence of a diminished oxygen tension is required rather than a low concentration of red blood cells [8, 73, 74, 183]. In most tissues including the brain, hypoxia-dependent expression of EPO and EPOR are controlled by hypoxia-inducible factor 1 (HIF-1). HIF-1 is essential for the production and secretion of EPO in response to hypoxia. At the transcriptional level, the hypoxia-dependent gene transcription of EPO and EPOR directly results from the activation of the HIF-1 pathway under hypoxic conditions. Gene transcription of EPO is mediated by the transcription enhancer located in the 3′-flanking region of the EPO gene that specifically binds to HIF-1 [75, 140]. Yet, hypoxia is not the only condition that can alter the expression of EPO and the EPOR. The production and secretion of EPO in female reproductive organs is estrogen-dependent. During the cyclic development of the uterine endometrium, 17β-estradiol can lead to a rapid and transient increase in EPO mRNA in the uterus [184], oviducts, and ovaries [185]. Hypoxia-induced EPO mRNA expression in uterine tissue occurs only in the presence of 17β-estradiol. EPO mRNA expression by hypoxia in the uterus is less pronounced than the EPO expression that occurs in the kidney and the brain [186]. In addition, a variety of cellular disturbances may lead to either increased or decreased EPO expression through the control of HIF, such as hypoglycemia, cadmium exposure, raised intracellular calcium, or intense neuronal depolarizations generated by mitochondrial reactive oxygen species [174, 180, 187]. Anemic stress, insulin release, and several cytokines, including insulin-like growth factor, tumor necrosis factor-α (TNF-α) [188], interleukin-1β (IL-1β), and interleukin-6 (IL-6) [189], also can lead to increased expression of EPO and the EPOR [75, 140] and may provide a feedback loop that is regulated by EPO such as TNF-α [190].

4 FoxOs, EPO, and the Control of Cell Injury

During a number of scenarios, FoxO proteins and EPO directly govern cell survival. For example, FoxO transcription factors can lead to apoptosis during oxidative stress [8]. Forkhead transcription factors such as FoxO1 and FoxO3a must be present for oxidative stress to result in apoptotic cell injury [191]. FoxO3a in conjunction with JNK also has been shown to modulate an apoptotic ligand activating a Fas-mediated death pathway in cultured motoneurons [192], to lead to apoptosis through tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and BH3-only proteins

Noxa and Bim in neuroblastoma cells [130], and to promote proapoptotic activity of p53 [193]. In addition, loss of FoxO expression during oxidative stress is protective to cells. Protein inhibition or gene knockdown of FoxO1 or FoxO3a can lead to reduction in ischemic infarct size in the brain [194], mediate protection of metabotropic glutamate receptors during vascular injury [128], enhance pancreatic β-cell or neuronal survival through NAD^+ precursors during oxidative stress [129], and provide trophic factor protection with EPO [124] and neurotrophins [195]. Furthermore, the canonical wingless-int (Wnt) pathway [196, 197] derived from the *Drosophila Wingless* (*Wg*) and the mouse *Int-1* genes that involves β-catenin [33, 198] also appears to link FoxO proteins and Wnt signaling together [23]. The Wnt proteins are secreted, cysteine-rich, glycosylated proteins that can control cell proliferation [199], differentiation, survival, and tumorigenesis [33, 198]. These genes are present in several cellular populations [200], such as neurons, cardiomyocytes, endothelial cells, cancer cells, and preadipocytes [7]. In relation to AD, Aβ is toxic to cells [4, 120, 201] and is associated with the phosphorylation of FoxO1 and FoxO3a that can be blocked with ROS scavengers [202]. A common denominator in the pathways linked to Aβ toxicity involves Wnt signaling [4, 203] and β-catenin. β-catenin may increase *FoxO* transcriptional activity and competitively limit β-catenin interaction with members of the lymphoid enhancer factor/T-cell factor family [204]. This may lead to cell injury, as β-catenin has been demonstrated to be necessary for protection against Aβ toxicity in neuronal cells [4]. However, not all conditions with FoxOs may lead to cell demise. Some studies suggest that the loss of FoxO1, FoxO3a, and FoxO4 protein expression may actually lead to an increase in free radical release that can be responsible for oxidative stress [205]. Furthermore, FoxO proteins may be protective during aging and exercise, as FoxO3a activity may enhance vascular smooth muscle antioxidant properties in aged animals and be beneficial to the cardiovascular system during physical exertion [206].

Similar to circumstances where FoxOs may be protective, EPO can prevent cell injury during hypoxia [34, 116, 207–210], excitotoxicity [211–213], parasitic disease [214–216], endotoxin shock [217, 218], free radical exposure [39, 55, 212], cardiac disease [219, 220], amyloid toxicity [120, 221], and pulmonary disease [222, 223]. EPO also represents a potential option for the prevention of retinal degeneration or neovascularization [224–227] as well as glaucoma [228]. Systemic application of EPO also can improve functional outcome and reduce cell loss during spinal cord injury [229, 230], traumatic cerebral edema [231], cortical trauma [232], and epileptic activity [157, 233, 234].

5 FoxOs, EPO, and the Immune System

As FoxOs and EPO are intimately linked to cell survival, it may come as no surprise that these proteins are also closely associated with modulation of the immune system [72, 235]. For example, in the brain, microglia lead to the phagocytic removal of both neurons and vascular cells [26, 29, 66]. During inflammation, microglial

cells require the activation of intracellular cytoprotective pathways [27, 67] to proliferate and remove injured cells [31, 236]. Microglia also can form a barrier for the removal of foreign microorganisms from the central nervous system and promote tissue repair during neuronal and vascular cell injury [67, 237]. Yet, microglia may lead to cell injury through the generation of reactive oxygen species [57, 238] and through the production of cytokines [239, 240].

Forkhead transcription factors have an important role in maintaining immune system function. The forkhead family member FoxP3 can control the development and function of thymic-derived CD4(+)CD25(+) regulatory T cells (Treg) that impart autoimmunity. Loss of FoxP3 can result in autoimmune disorders [241]. Additional studies demonstrate the expression of FoxP3 in tumor cells, such as melanoma [242], as well as in Tregs, which may significantly affect patient mortality as the increased presence of Tregs in cancer patients combined with FoxP3 expression in tumors may impair antitumor autoimmune responses and lead to high mortality [243].

In consideration of the specific role for FoxO proteins, these transcription factors also may influence early apoptotic membrane PS externalization. The ability to regulate early apoptotic membrane PS exposure [34] and inflammatory cell activity [29] can ultimately affect cell survival because activated immune cells can lead to the phagocytic removal of injured cells or tumor cells [26, 51]. Recent work suggests a relationship between the regulation of immune system activity and the induction of apoptotic pathways that are dependent upon FoxO proteins. In microglial cells, FoxO3a is responsible for the activation and proliferation of these inflammatory cells during oxidative stress models, as gene silencing of FoxO3a can prevent the subsequent activation and proliferation of microglia [5] (Fig. 1). Prevention of inflammatory activation and apoptosis in the nervous system such as in systemic lupus erythematosus in animal models also may require the upregulation of different Fox proteins, such as FoxJ1 and FoxO3a, that can block NF-κB activation and interferon-γ secretion [244]. FoxO proteins also may work in concert with Fas signaling to clear activated T cells after a decrease in cytokine stimulation in patients with autoimmune lymphoproliferative syndromes [245], suggesting that activation of specific FoxO proteins may be beneficial for autoimmune disorders but may impair treatments designed to target tumor cells through immune-mediated pathways. Furthermore, in mice deficient for *Foxo3a*, lymphoproliferation, organ inflammation of the salivary glands, lung, and kidney, and increased activity of helper T cells results, supporting an important role for FoxO3a in preventing T-cell hyperactivity [246]. FoxO3a also appears to be necessary for neutrophil activity, as *Foxo3a* null mice are resistant to models of neutrophilic inflammation that involve immune complex–mediated inflammatory arthritis [247]. Patients with rheumatoid arthritis and osteoarthritis show phosphorylation of FOXO3a in T lymphocytes as well as FOXO1 and FOXO4 in synovial macrophages, suggesting that loss of functional FOXO family members may lead to inflammatory cell activation in these disorders [248]. *FOXO1* gene transcript levels also are downregulated in peripheral blood mononuclear cells of patients with systemic lupus erythematosus and rheumatoid arthritis [249], illustrating a potential etiology through the loss of functional

FOXO proteins for these disorders and possibly providing a biomarker of disease activity. Other studies show that FOXO1 protein controls L-selectin expression that can regulate human T-lymphocyte trafficking [250].

EPO also can modulate immune system function and can reduce cytokine gene expression in endothelial cells exposed to tumor necrosis factor [251], prevent ulcer progression in cases of scleroderma [252], reduce inflammation in murine arthritis models [253], and block primary microglial activation and proliferation during oxidative stress [34, 120] to prevent phagocytosis of injured cells through pathways that involve cellular membrane PS exposure, protein kinase B [66], and the regulation of caspases [34, 55, 254]. EPO can directly inhibit several proinflammatory cytokines, such as IL-6, TNF-α, and monocyte chemoattractant protein 1 [75, 255], and reduce leukocyte inflammation [256]. EPO also may foster the preservation of microglial cells for neuronal and vascular restructuring by preventing apoptotic injury in microglia [27, 257].

6 FoxOs, EPO, Stems Cells, and Tissue Development

The existence of FoxO proteins in soft tissue tumors and leukemias, neoplasms now believed to contain cancer stem cells for tumor self-renewal [258], as well as the knowledge that EPO may promote tumor proliferation [259, 260], suggests that EPO and FoxO proteins may be closely tied to stem cell proliferation and differentiation. When one considers progenitor cell proliferation for FoxO proteins, either simultaneous deletion of *Foxo1*, *Foxo3a*, and *Foxo4* or single deletion of *Foxo3a* in mice prevents the repopulation of hematopoietic stem cells and leads to apoptosis in these stem cell populations [205, 261]. With regard to the reproductive potential of an organism, deletion of the *FoxO3a* gene results in the depletion of oocytes and subsequent infertility [262]. Other work using a mouse model of FoxO3a overexpression in oocytes suggests that FoxO3a also may retard oocyte growth and follicular development and leads to anovulation and luteinization of unruptured follicles [263]. In clinical studies, a small percentage of women who suffer from premature ovarian failure have mutations in *FOXO3a* and *FOXO1a* [264]. In neuronal populations, FoxOs also may prevent stem cell proliferation, as the proliferation of human neural progenitor cells appears to require the inhibitory phosphorylation of FOXO3a [265].

FoxO proteins also play a significant role to modulate new vessel growth that can impact upon cardiovascular development. FoxO proteins are intimately involved in endothelial cell development and angiogenesis. For example, *Foxo3a*$^{-/-}$ and *Foxo4*$^{-/-}$ mice develop without incidence and are indistinguishable from control littermates. However, mice that are singly deficient in *Foxo1* die by embryonic day 11 and lack development of the vascular system [266]. Additional studies illustrate that endothelial cell colonies in *Foxo1*-deficient mice fail to respond to vascular endothelial growth factor in a manner similar to wild-type endothelial cells [267], suggesting that FoxOs are necessary for the development of vascular cells as well as for the biological response to cellular mediators.

During cardiac development, FoxO proteins also appear to be necessary to modulate cardiomyocyte proliferation. Both FoxO1 and FoxO3 are expressed during embryonic through prenatal stages in the developing myocardium. The expression of these FoxO proteins is believed to negatively regulate cardiomyocyte growth, as overexpression of FoxO1 blocks cardiomyocyte proliferation but expression of dominant negative FoxO1 leads to enhanced cardiomyocyte growth [268]. These observations may provide clues into the roles of FoxO proteins during cardiac hypertrophy. Atrogin-1, a protein that can block cardiac hypertrophy, may rely upon the upregulation of Foxo1 and Foxo3a to disrupt cardiac hypertrophy, as mice lacking atrogin-1 are susceptible to cardiac hypertrophy and do not yield increased expression of Foxo1 and Foxo3a [269]. With regard to smooth muscle cell growth, gene transfer of FoxO3a can inhibit neointimal hyperplasia through the prevention of vascular smooth muscle growth [270]. However, not all FoxO proteins may exert an inhibitory effect upon vascular smooth muscle cells. FoxO4 may inhibit smooth muscle cell differentiation through the repression of the transcriptional coactivator of smooth muscle genes myocardin [271], but other work suggests that FoxO4 also can increase matrix metalloproteinase-9 expression to promote vascular smooth muscle migration and foster neointimal hyperplasia [272].

In consideration of the ability of FoxO proteins to regulate vascular smooth muscle cell proliferation, these transcription factors may have a significant clinical role with regard to disorders that involve hypertension and cardiac failure. Vascular smooth muscle cells are vital for the regulation of vascular tone and systemic arterial blood pressure. High flow states in vessels can reduce FoxO1 activity, resulting in the potential proliferation of vascular smooth muscle cells, vascular neointimal hyperplasia, and subsequent pathologic states such as hypertension [273]. Furthermore, α1-adrenergic agonists that increase systemic blood pressure can have the reverse effect and stimulate the expression of FoxO1 and its nuclear translocation that ultimately may lead to apoptotic endothelial cell injury [274]. More than moderate levels of vessel cyclic stretch that can occur during hypertension may lead to the phosphorylation and inhibition of Foxo1 and Foxo3a in smooth muscle cells to further contribute to pathologic smooth muscle cell proliferation [275]. In human as well as murine models of cardiac failure, increased expression of Fox transcription factors, such as FoxO1a, also have been observed to suggest a potential association of FoxO proteins with imminent cardiac failure [276].

With regard to cell development for EPO, EPO has been shown to promote angiogenesis [55, 116, 152]. EPO has both a mitogenic and chemotactic effect that can lead to matrix metalloproteinase-2 production, cell proliferation, and vessel formation in EC lines [75, 140]. In cultured human and bovine ECs, EPO stimulates EC proliferation and fosters the migration of ECs [277]. In neonatal mesenteric microvascular ECs, EPO also leads to vasculogenesis [278]. Angiogenesis by EPO offers an additional level of cytoprotection in various cell systems. For example, in models of cerebral ischemia, EPO promotes factors for angiogenesis such as Tie-2 and angiopoietin-2 that may assist with the restoration of cerebral blood flow to preischemic levels [279]. EPO-controlled angiogenesis also may play a significant role during renal inflammation and prevention of allograft rejection [280]. In

addition, EPO may promote the viability of transplanted marrow stromal cells and enhance capillary density during experimental cardiac ischemia [281]. Although EPO induced angiogenesis may impart beneficial effects to ischemic cells of the nervous and cardiovascular systems for nutrient and oxygen supply, other scenarios that involve ocular neovascularization may also seek to block or limit angiogenesis by EPO to prevent disease progression [282]. In clinical studies, EPO serum levels also are significantly associated with the number and function of circulating endothelial progenitor cells, and EPO can stimulate postnatal neovascularization by increasing endothelial progenitor cell mobilization from the bone marrow [283]. Recently, EPO has been shown to increase the motility of human bone marrow multipotent stromal cells [284], suggesting that EPO may lead to increased cell viability during oxidative stress via progenitor cell recruitment [285–287]. Interestingly, the ability of EPO to foster erythroid progenitor cell development is dependent upon the inhibition of FoxO3a activity [74, 75] but also may require regulation of specific gene expression through an EPO–FoxO3a association to promote erythropoiesis in cultured cells [288]. In addition, a close association with EPO [75, 160, 289] also may be required to modulate FoxO protein activity such as during erythroid progenitor cell development [73, 74], further indicating that use of EPO in patients with combined anemia and cancer may have unexpected detrimental effects [74, 75].

7 FoxOs, EPO, Diabetes, and Metabolic Pathways

Both FoxOs and EPO play a significant role during metabolic pathways and especially those that involve diabetes mellitus (DM). DM is a significant health concern for both young and older populations [290, 291]. Almost 18 million to 20 million individuals in the United States and more than 165 million individuals worldwide suffer from DM. By the year 2030, it is predicted that more than 360 million individuals will be afflicted with DM and its debilitating conditions. Type 2 DM occurs in at least 80% of all diabetics and is dramatically increasing in incidence as a result of changes in human behavior and increased body mass index [290, 292]. Type 1 insulin-dependent DM is present in 5–10% of all diabetics but is increasing in adolescent minority groups [290, 292]. Furthermore, the incidence of undiagnosed diabetes and impaired glucose tolerance in the population raises additional concerns.

Patients with DM can develop immune dysfunction [293], cognitive disorders [293, 294], hepatic dysfunction [295], renal disease [296], hematologic disease [297], neurodegenerative disorders [7, 183, 292], and cardiovascular disease [292, 298]. Interestingly, the development of insulin resistance and the complications of DM can be the result of cellular oxidative stress [290, 292]. Hyperglycemia can lead to increased production of ROS in endothelial cells, liver cells, and pancreatic β-cells [290–292]. Recent clinical correlates support these experimental studies to show that elevated levels of ceruloplasmin are suggestive of increased ROS [290–292]. Furthermore, acute glucose swings in addition to chronic hyperglycemia

can trigger oxidative stress mechanisms, illustrating the importance for therapeutic interventions during acute and sustained hyperglycemic episodes [290, 292].

In relation to FoxO proteins, metabolic signaling with FoxOs is conserved among multiple species including *C. elegans, D. melanogaster,* and mammals. FoxO proteins are homologous to the transcription factor Dauer formation-16 (DAF-16) in the worm *C. elegans* that can determine metabolic insulin signaling and lead to lifespan extension [299, 300], suggesting a significant role for FoxO proteins in relation to mammalian cell function [70, 85]. FoxO proteins can stimulate the insulin-like growth factor binding protein-1 (IGFBP1) promoter by binding to the insulin-responsive sequence (IRS) [301]. Both insulin and insulin-like growth factor-1 (IGF-1) can suppress this activity through activation of Akt [301, 302].

Analysis of the genetic variance in *FOXO1a* and *FOXO3a* on metabolic profiles, age-related diseases, fertility, fecundity, and mortality in patients have observed higher HbA_{1c} levels and increased mortality risk associated with specific haplotypes of *FOXO1a* [303]. These clinical observations may coincide with the demonstration in human endothelial progenitor cells that elevated glucose levels can reduce post-translational phosphorylation of FOXO1, FOXO3a, and FOXO4 and allow for the nuclear translocation of these proteins to initiate an apoptotic program in endothelial progenitor cells [304]. In experimental models, FoxO proteins may prevent the toxic effects of high serum glucose levels. Interferon-γ–driven expression of tryptophan catabolism by cytotoxic T-lymphocyte antigen-4 may activate Foxo3a to protect dendritic cells from injury in nonobese diabetic mice [305]. Additional studies have demonstrated that adipose tissue–specific expression of Foxo1 in mice improved glucose tolerance and sensitivity to insulin during an elevated fat diet [306]. FoxO proteins also may protect against diminished mitochondrial energy levels known to occur during insulin resistance such as in the elderly populations [290–292]. In calorie-restricted mice that have decreased energy reserves, Foxo1, Foxo3a, and Foxo4 mRNA levels were noted to progressively increase over a 2-year course [90]. These observations complement studies in *Drosophila* and mammalian cells that demonstrate an increase in insulin signaling to regulate cellular metabolism during the upregulation of FoxO1 expression [307].

However, the ability for FoxO proteins to maintain proper physiologic controls over cellular metabolism may be limited and occur only during specific circumstances. For example, mice with a constitutively active Foxo1 transgene have increased microsomal triglyceride transfer protein and elevated plasma triglyceride levels [308]. Studies in cardiomyocytes also suggest detrimental results with enhanced FoxO activity. Increased transcriptional activity of FoxO1, such as by the Sirt1 activator resveratrol, can diminish insulin-mediated glucose uptake and result in insulin resistance [309]. Overexpression of Foxo1 in skeletal muscles of mice also can lead to reduced skeletal muscle mass and poor glycemic control [310], illustrating that activation of FoxO proteins also may impair cellular energy reserves. Other studies that block the expression of Foxo1 in normal and cachectic mice [311] or reduce FoxO3 expression [312] show the reverse with an increase in skeletal muscle mass or resistance to muscle atrophy. These results become especially relevant in patients with cancer and cachexia, as FoxO protein expression may further

muscle wasting for these individuals. With this in mind, one potential agent to consider for the maintenance of cellular metabolism in patients is nicotinamide [16, 32], an agent that also can inhibit FoxO protein activity [129]. In patients with DM, oral nicotinamide protects β-cell function, prevents clinical disease in islet-cell antibody-positive first-degree relatives of type-1 DM, and can reduce HbA_{1c} levels [16, 32, 290]. Nicotinamide, which is closely linked to cell longevity pathways [313, 314], may derive its protective capacity through two separate mechanisms of posttranslational modification of FoxO3a. Nicotinamide not only can maintain phosphorylation of FoxO3a and inhibit its activity but also can preserve the integrity of the FoxO3a protein to block FoxO3a proteolysis that can yield proapoptotic amino-terminal fragments [129].

With regard to EPO during metabolic disorders, plasma EPO is often low in diabetic patients with anemia [315] or without anemia [316]. The inability of these individuals to produce EPO in response to a declining hemoglobin level suggests an impaired EPO response in diabetic patients [317]. However, increased EPO secretion during diabetic pregnancies may represent the body's attempt at endogenous protection against the complications of DM [318]. Similar to the potential protective role of insulin [319], EPO administration has been shown both in diabetic individuals as well as in nondiabetic individuals with severe, resistant congestive heart failure to decrease fatigue, increase left ventricular ejection fraction, and significantly decrease the number of hospitalization days [320]. In vitro studies with vascular cells exposed to elevated glucose also have demonstrated that EPO can significantly improve EC survival in a 1.0 ng/ml range [321]. EPO administration in patients also can significantly increase plasma levels of EPO well above this range of 1.0 ng/ml that has been associated with potential EPO cellular protection in patients with cardiac or renal disease [322, 323], suggesting that the effects of EPO observed during in vitro studies may parallel the cellular processes altered by EPO in patients with DM [167]. Furthermore, EPO during elevated glucose and similar to other models of oxidative stress can block neuronal degeneration [324] and apoptotic DNA degradation in ECs in cardiac and vascular cell models [55, 116, 124, 158, 251]. Protection by EPO also is related to the maintenance of mitochondrial membrane potential ($\Delta\Psi_m$). Loss of $\Delta\Psi_m$ through the opening of the mitochondrial permeability transition pore represents a significant determinant for cell injury and the subsequent induction of apoptosis [57, 64]. EPO has the capacity to prevent the depolarization of the mitochondrial membrane that also affects the release of cytochrome c [39, 116, 325].

Additional work suggests that Wnt proteins may be associated with the complications of DM [23]. Abnormalities in the Wnt pathway, such as with transcription factor 7–like 2 gene, may impart increased risk for type 2 diabetes in some populations [326–328] as well as have increased association with obesity [329]. Yet, intact Wnt family members may offer glucose tolerance and increased insulin sensitivity [330] as well as protect glomerular mesangial cells from elevated glucose-induced apoptosis [331]. These observations suggest a potential protective cellular mechanism for EPO through Wnt signaling. Cell culture studies demonstrate that the Wnt1 protein is necessary and sufficient to impart cellular protection during elevated

glucose exposure [321]. EPO maintains the expression of Wnt1 during elevated glucose exposure and prevents loss of Wnt1 expression that would occur in the absence of EPO during elevated glucose. In addition, blockade of Wnt1 with a Wnt1 antibody can neutralize the protective capacity of EPO, illustrating that Wnt1 is a critical component in the cytoprotection of EPO during elevated glucose exposure [321].

8 Clinical Strategies and Future Perspectives

As agents for the treatment of a variety of potential disorders, FoxOs and EPO have elicited great enthusiasm to yield new strategies for the treatment of neurovascular and related disorders. For example, FoxO proteins offer the potential to target and prevent neoplastic progression. The ability of FoxO proteins to control cell cycle progression and promote apoptosis supports the premise that FoxOs may be an important component for new strategies directed against tumorigenesis. FoxO3a and FoxO4 can promote cell cycle arrest in mouse myoblastic cell lines through modulation of growth-arrest and DNA-damage-response protein 45 [70, 73]. Treatment of chronic myelogenous leukemia cell lines with the Bcr–Abl tyrosine kinase inhibitor imatinib requires FoxO3a activation to antagonize cell proliferation and promote apoptotic cell death through increased TRAIL production [332]. In addition, the transcription factor E2F-1 that controls the induction of the cell cycle has been reported in cell lines to increase the endogenous expression of FoxO1 and FoxO3a to lead to cell cycle arrest [333]. In contrast, the loss of FoxO3a activity in association with c-myc, p27, and nuclear factor kappa B (NF-κB) can result in cell cycle induction and malignant transformation of mouse cells in the presence of oncogene activation [70, 85]. Other work suggests that FoxO proteins utilize the p53 upstream regulator p19(Arf) through myc to block cell cycle induction and lymphoma progression [334].

Additional work with prostate cancer has shown that the tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) is mutated in approximately 80% of tumors with the loss of FOXO1 and FOXO3a activity. In cell cultures, overexpression of FoxO1 and FoxO3a in prostate tumor cell lines also leads to apoptosis, suggesting that FoxO1 and FoxO3a are necessary for limiting prostate cell tumor growth [94]. Inhibition of FoxO3a activity can result in enhanced prostate tumor cell growth [335], whereas agents that increase FoxO3a activity in both androgen-sensitive and androgen-insensitive prostate cell lines prevent prostate cancer cell progression [336]. Therapeutic strategies that rely upon the overexpression of a non-phosphorylatable form of FoxO3a that cannot be inactivated can also sensitize prostate cancer cells to androgen-withdrawal-induced apoptosis [337]. However, in prostate cell lines, FoxO3a can be a positive regulator of androgen receptor expression and therefore may play a complex role in prostate cancer cell proliferation and growth inhibition [338]. Other factors that control FoxO protein function also may play a role during prostate tumor progression. In prostate cancer cells, cyclin-dependent kinase 1 (CDK1) can become overexpressed and

subsequently phosphorylate FOXO1 to block its transcriptional activity and contribute to prostate tumorigenesis [339]. In a similar manner, it has been shown that astrocyte-elevated gene-1 (AEG-1) can be upregulated in clinical prostate cancer [340], possibly leading to activation of Akt that suppresses FOXO3a [341] and apoptosis in prostate tumor cells.

Initial investigations of FOXO3a in clinical breast cancer suggested that activation of FOXO3a was associated with lymph nodal metastasis and a poor prognosis [342]. In contrast with these observations, other work has shown that FOXO3a was inactivated by IKK and that inactivation of FOXO3a was associated with a poor prognosis in breast cancer [343], suggesting that FOXO3a subcellular localization and pathways that enhance its activity could be used not only as a biomarker assay but also as therapeutic targets. Additional studies in breast cancer cells demonstrate the tumor repressive ability of FoxOs by illustrating that increased activity of FoxO3a in association with JNK in breast cancer cell lines [344] or in association with cyclin-dependent kinase inhibitor p27 in isolated human breast cancer cells can prevent breast cancer growth [345]. Furthermore, FoxO proteins may be able to modulate estrogen function and indirectly block breast cancer growth. Overexpression of FoxO3a in breast cancer cell lines can decrease the expression of estrogen receptor–regulated genes and inhibits 17β-estradiol (E2)-dependent breast cancer growth [346].

FoxO proteins also may represent a viable option to control tumor progression in other tissues. FoxO proteins can function as redundant repressors of tumor growth. For example, somatic deletion in mice of *Foxo1*, *Foxo3a*, and *Foxo4* results in the growth of thymic lymphomas and hemangiomas [347]. Other work illustrates that FoxO3a activation in colon carcinoma cell lines prevents tumor proliferation through Myc target genes that involve the Mad/Mxd family of transcriptional repressors [348]. In addition, the loss of FoxO3a activity may participate in oncogenic transformation in B-chronic lymphocytic leukemia [349] and in the progression of chronic myelogenous leukemia cell lines [332]. Furthermore, studies suggest that some proteins, such as the Kaposi's sarcoma–associated herpes virus latent protein LANA2, may specifically block the transcriptional activity of FoxO3a to lead to tumor growth [350]. In cell models of endometrial cancer, presensitization of cells to block Akt activation and foster transcription activity of FoxO1 enhances the effect of chemotherapy to limit tumor growth [351]. Use of triple mutant FoxO1 or FoxO3a expression in which three phosphorylation sites have been altered to prevent inactivation of this protein has been proposed to block melanoma tumors [131] and endometrial cancer [352].

FoxO proteins also may function as biomarkers of cancer growth. Downregulation of the phosphatidylinositol 3 kinase and Akt pathways has been associated with increased transcript levels for FOXO1a and FOXO3a in clinical prostate cancer samples and may indicate the onset of precancerous changes or the progression of ongoing tumor growth [353]. Although loss of Akt activity in prostate cancer cells can result in enhanced FoxO3a activity and subsequent apoptosis of tumor cells [340], it is conceivable that early stages of cancer may lead to reduced Akt activity with insufficient levels of active forkhead transcription factors

to limit tumor progression. In addition, the early and persistent expression of phosphorylated FOXO1a in gastric tumors may not only indicate the onset of cancer but also suggest an improved prognosis for patients [354].

The known mutations in FoxO proteins that exist in several disease entities also may provide novel insights for the treatment of other disorders. Future analysis in larger populations of patients with premature ovarian failure and diabetes could strengthen our understanding of the role of FoxO proteins in these disorders. In addition, targeting the activity of FoxO1, FoxO3a, or FoxO4 in cardiac and endothelial cells may prevent the onset of pathologic cardiac hypertrophy and neointimal hyperplasia that may result in atherosclerosis. Recent studies also suggest that the utilization and combination of multiple biomarkers may improve risk assessment for patients suffering from cardiovascular disorders [355]. These studies illustrate that FoxO proteins may serve as biomarkers of disease activity such as in individuals with imminent cardiac failure [276].

However, FoxO transcription factors may have complicated and sometimes detrimental clinical outcomes. For example, FoxO protein inhibition of cell cycle progression may not consistently lead to apoptotic cell death. Some investigations suggest that during oxidative stress, FoxO3a activation in association with Sirt1 can lead to cell cycle arrest but not result in apoptotic cell injury [356]. Furthermore, during hypoxic stress, forkhead transcription factors, such as FOXO3a, may potentiate antiapoptotic pathways in breast cancer cells to further tumor growth [357]. FoxO proteins also have been linked to potential chemotherapy drug resistance with increased expression of MDR1 (P-glycoprotein) that has been associated with chemotherapy drug resistance in breast cancer cells. FoxO1 can stimulate the transcriptional activity of MDR1 that may promote increased tolerance of tumor cells [358]. In addition, the common pathways shared between Wnt and forkhead proteins may lead to other outcomes that alter the ability to control tumor growth [33, 359]. FoxO proteins may assist with β-catenin activation in the Wnt pathway and lead to tumor cell proliferation [198]. In the presence of Wnt deregulation and increased β-catenin activity, tumorigenesis may ensue, such as with the proliferation of medulloblastoma tumors [258]. Therefore, the role of FoxO protein involvement in several disorders may not be consistently known and may be influenced by multiple parameters such as tissue characteristics, cellular metabolic state, and the age of an individual.

In reference to EPO, U.S. annual sale revenues for EPO have recently been reported to approach $9 billion [360], and more than 100 trials registered at the National Institutes of Health website (clinicaltrials.gov) currently exist that are either recruiting or in preparation to examine the role of EPO in patients with a variety of disorders that include anemia, cancer, cardiac ischemia, or spinal cord trauma. Although some cardiac injury studies do not always demonstrate a benefit with EPO [361, 362], early studies in patients with anemia or on chronic hemodialysis have suggested a direct cardiac benefit from EPO administration [363, 364]. In addition, EPO administration can improve exercise tolerance either during cardiac or renal insufficiency in patients with anemia and congestive heart failure [170, 365], and that may be dependent upon improved pulmonary function [366]. Furthermore,

a randomized, concealed, multicenter trial of 1,460 patients who received 40,000 U of epoetin alfa up to a 3-week maximum after intensive care unit admission for trauma demonstrated a reduced mortality [367].

Yet, EPO is not well tolerated with comorbid conditions such as congestive heart failure, hypertension, and neoplasms. Some studies suggest that elevated plasma levels of EPO independent of hemoglobin concentration can be associated with increased severity of disease in individuals with congestive heart failure [368] and that EPO may contribute to vascular stenosis with intima hyperplasia [369]. Adverse effects during treatment with EPO are not uncommon, such as an increased incidence of thrombotic vascular effects [367] or the use of EPO in cancer patients receiving chemotherapy that has been associated with nonfatal myocardial infarction, pyrexia, vomiting, shortness of breath, paresthesias, and upper respiratory tract infection [370]. In addition, both acute and long-term administration of EPO can significantly elevate mean arterial pressure that may place patients with hypertension at risk [371].

Cancer progression has been another significant concern raised with EPO administration [259, 372]. The potential for the initiation or progression of cancer during EPO administration supports investigations that can elucidate the downstream mechanisms of this growth factor and cytokine to avoid unwanted clinical outcomes. In particular, the close association that EPO holds with FoxO proteins suggest potential avenues to limit or block tumor cell proliferation. EPO and its receptor can be found in tumor specimens and may block tumor cell apoptosis through Akt activation [373], enhance metastatic disease [374], and complicate radiotherapy by assisting with tumor angiogenesis [375]. The potential for EPO to lead to neoplastic growth is not well defined or understood at this time [376]. A number of competing factors must be considered and weighed that include the possible benefits of EPO administration in patients with cancer, the synergistic effects of EPO with chemotherapeutic modalities [377, 378], the potential protection against chemotherapy tissue injury [379], and the treatment of cancer-related anemia.

Additional considerations for EPO also exist. In addition to the problems associated with EPO abuse and gene doping [380–382], EPO has been correlated with the alteration of red cell membrane properties leading to a cognitive decrement in rodent animal models [75, 140, 255]. Development of potentially detrimental side effects during EPO therapy, such as for cerebral ischemia with increased metabolic rate and blood viscosity [383], could also severely limit the use of EPO for neurovascular diseases. As a result, alternate strategies have been suggested. New proposals examine the role of targeted bioavailability for EPO such as in bone marrow stromal cells genetically engineered to secrete EPO [384] and controlled release of EPO from encapsulated cells [385, 386]. The passage of EPO entry into the central nervous system continues to attract significant interest [387] as well as does the use of novel intranasal routes for EPO administration [210]. The development of derivations of EPO to reduce erythropoietic activity and the potential associated vascular complications [211] have also been put forth as new directions for treatment. Yet, these lines of investigation are not without limitations, as chemical derivatives of EPO can become absent of clinical efficacy [75, 140] as well as possibly lose the

ability to promote sustainable cytoprotective effects, such as neurogenesis [388] and angiogenesis [279, 280, 282, 389].

As combined therapeutic entities and biomarkers, FoxO proteins, EPO, and the pathways that they share offer new directions for neurovascular disorders during aging and oxidative stress. Future work that blends basic research with clinical trials is necessary to expand our understanding of the multiple pathways involved (such as with Akt, Wnt, cell cycle regulation, and apoptosis) that can affect disorders of the nervous, cardiovascular, and immune systems. As our knowledge increases regarding the cell biology and clinical outcome tied to FoxOs and EPO, new therapeutic strategies will become more focused to yield clinical success and eliminate unwanted complications for age-related disorders.

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Oxidative Stress in Vascular Disease

Isabelle Gorenne and Martin R. Bennett

Abstract The importance of oxidation in vascular disease was established in the mid-1980s with the emergence of the "LDL modification hypothesis" of atherosclerosis, initially referring to the vessel wall damage that occurs subsequent to oxidation of low-density lipoproteins deposited in the vascular wall. Oxygen radicals and derived reactive species not only accelerate the development or affect the stability of atherosclerotic plaque but are also involved in regulation many aspects of vascular diseases or conditions associated with hypertension, diabetes, ischemia-reperfusion or vascular injury. In recent years, the term *oxidative stress* has been used to describe the manifestations of changes in redox enzyme activity in vascular cells, as well as the process of oxidation of cellular components caused by the intracellular excess of free radicals. The cellular response attributed to these reactive species can be as diverse as cell dysfunction, alteration of cell proliferation or migration, modification of extracellular matrix composition or cell death by apoptosis. In addition, there is growing evidence showing that the long-term and insidious action of oxygen radicals may irreversibly damage genomic or mitochondrial DNA. Accumulation of nucleic acid lesions due to oxidant stress may accelerate the natural processes of vascular cells aging. In addition, DNA damage may interfere with the cell cycle and cell survival by activating ROS-sensitive transcription factors and activating DNA damage-dependent signalling pathways. This chapter will describe the evidence for oxidative stress in vascular disease, the sources of reactive species in the vascular wall, and their relevance in vascular pathologies Largely focusing on atherosclerosis.

Keywords Oxidative stress · Atherosclerosis · Hypertension · Vascular remodeling · Aging · Telomeres · DNA damage

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1 Introduction

Arteriosclerosis is the most common form of vascular disease. Arteriosclerosis is a generic term referring to the thickening and hardening of the arteries. Several forms exist, including arteriolosclerosis, the hardening of small arteries as seen in hypertension and diabetes; the Monckeberg's sclerosis, which involves vessel wall calcification; and atherosclerosis, a form of arteriosclerosis associated with lipid deposition. Atherosclerosis is by far the most prevalent type of arteriosclerosis, and underlies most cardiovascular diseases. By itself, atherosclerosis is the leading cause of morbidity and mortality in most industrialized countries and will be the focus of this chapter.

Atherosclerosis affects large and medium sized arteries by causing permanent narrowing of the vessel lumen (stenosis) or the sudden formation of a blood clot (thrombosis). As a result, partial or complete restriction of the blood supply to a specific organ will cause serious and often irreversible downstream ischemic injury. For example, atherosclerosis in coronary arteries leads to angina pectoris i.e. chest pain associated with heart muscle damage, and is the essential substrate for heart attacks. In carotid or cerebral arteries thrombosis may cause transient ischemia attack (TIA) and stroke. Renal artery stenosis may promote hypertension and renal failure. Femoral artery stenosis and thrombosis will be responsible for critical limb ischemia and possible gangrene of the limb as the most serious complication.

Atherosclerosis is often described as a response to chronic endothelial injury, and is promoted by multiple factors such hyperlipidemia, inflammation or hemodynamic forces. All of these factors have been associated in some extent with cells production of reactive oxygen species (ROS). Hypercholesterolemia is one of the initiating events of atherosclerosis, promoting high plasma levels of low-density lipoprotein (LDL) and its deposition in the vascular wall [1]. Lipoproteins deposited in the subendothelial space may become oxidized and act as chemoattractants for inflammatory cells. Infiltrated monocytes differentiate into macrophages and ingest the oxidized LDL (oxLDL), a phenomenon resulting in the formation of foam cells and the so-called "fatty streak". Oscillatory shear stress imposed by hemodynamic forces in areas of turbulent flow are an important component of the location of the fatty streak, which appears predominately at branch points and curvatures in large elastic arteries [2]. Shear stress and mechanical stretch have been shown to trigger oxidative damage in both endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) of the vessel wall [3], further contributing to lipid accumulation in the vessel wall and favoring the recruitment of inflammatory cells.

VSMC reaction to inflammation within atherosclerotic lesions promotes the transformation of the early fatty streak into a more complex fibrotic structure. For example, VSMC migration, proliferation, and extracellular matrix synthesis result in the formation of a fibrous cap. This structure protects the procoagulant lipidic and necrotic core from the flowing blood (see Fig. 1). Adventitial inflammation and subsequent reaction to inflammation result in adventitial thickening. Altogether, these events cause thickening of the vessel wall, which is accompanied over time by arterial wall remodeling. Remodeling in atherosclerosis describes changes in shape and

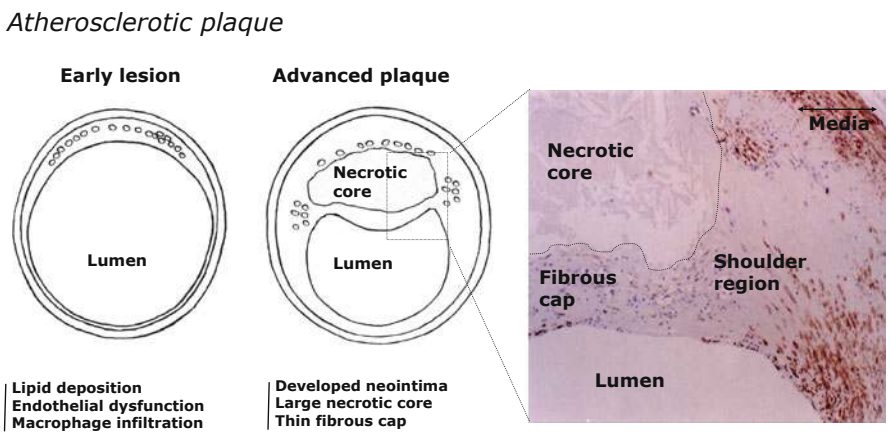

Fig. 1 Structure of early and advanced atherosclerotic plaques. (*Left panels*) Scheme of atherosclerosis development. Early atherosclerosis (grade I–II) is characterized by LDL deposition and formation of a fibrous plaque with local thickening of the vessel wall due to VSMC proliferation and migration. As the plaque progresses (grade III–IV), its morphology changes with expansion of the neointimal layer, the formation of an identifiable fibrotic cap, the presence of large amounts of inflammatory cells, and appearance of a necrotic core containing intracellular and extracellular lipid and necrotic debris. Oxidation of LDL will promote its own capture by vascular cells and macrophages, thereby leading to the formation of foam cells. The advanced plaque (grade IV) becomes vulnerable due to the thinning of the fibrous cap, which may rupture and cause thrombotic occlusion or become further complicated by healing and calcification (grade V–VI). (*Right panel*) Immunohistochemistry of a human coronary artery with features of an advanced plaque. Cells were labeled with an anti–smooth muscle α-actin antibody (*brown staining*) and counterstained with eosin

size of the vessel wall to adapt to the growth of the plaque and aims at effectively maintaining the lumen size and blood flow [4]. This process of arterial compensatory enlargement, also referred to as positive remodeling, is effective to the point where adaptation fails and stenosis takes place. Remodeling involves in particular VSMC proliferation, apoptosis, and inflammation, events that are all promoted by oxidative stress.

The thickness of the fibrous cap and its level of cellularity determine the stability of the atherosclerotic lesion. VSMCs are the sole producers of extracellular matrix proteins and must compensate for matrix degradation by metalloproteinases released by inflammatory cells and VSMCs. As plaques become advanced, the rate of cell division decreases while VSMC death by apoptosis increases [5]. The subsequent loss of VSMCs results in thinning of the fibrous cap increasing the risk of plaque rupture. Plaque rupture exposes the procoagulant necrotic core region composed of cellular debris and lipids to the bloodstream, causing thrombosis responsible for acute coronary or cerebrovascular events.

An increase in vessel wall thickness is not only present in atherosclerosis but can also be seen in the normal aging of the vascular system, even in the absence of atherosclerotic plaque [6]. In particular, the intima and media can change over

time in response to a variety of factors not necessarily related to atherosclerotic plaque formation and progression. Medial thickening caused by smooth muscle cell hypertrophy is closely associated with arterial hypertension and is part of the process of vessel remodeling [7]. Intimal hyperplasia and intimal fibrocellular hypertrophy are compensatory reactions of the arterial wall to changes in luminal pressure and shear stress. The arterial wall thickening, in addition to the enhanced reactivity to contractile agents and impaired endothelial-dependent vasorelaxation, are hallmark of hypertension. Abnormal thickening of the intima (formation of neointima) also underlies vascular occlusive diseases associated with in-stent stenosis or vein graft stenosis and is, in general, associated with endothelial injury and oxidative stress.

2 Lipoprotein Oxidation in Atherosclerosis

As mentioned above, the oxidative modification of serum LDL, lipoproteins dedicated to cholesterol export, plays an important role in the atherogenic process. The LDL particle consists of an outer layer of phospholipids, apolipoprotein B, free cholesterol, and a core of cholesterol bound into long esters by fatty acid chains [8]. LDL modification involves unsaturated fatty acid peroxidation and conversion into reactive aldehydes, including malondialdehyde and 4-hydroxynonenal [9, 10]. Interaction of aldehydes with lysine residues in apolipoprotein B100 increases the LDL negative charge. As a result, oxLDL particles have a decreased affinity for the LDL receptor compared with that of native LDL and an increased affinity for scavenger receptors present on macrophages and VSMCs, resulting in the formation of the so-called foam cells [11, 12]. As oxidation does not occur in the presence of serum and requires metal ions, which are not present in the circulation, LDL oxidation is thought to occur in the vessel wall, specifically in the subendothelial space. In vitro, macrophages, lymphocytes, ECs, or VSMCs are all capable of enhancing the rate of oxidation of LDL [13–15], and direct evidence of the presence of oxLDL has been reported in plaques [16, 17].

LDL oxidation is an important process promoting the transition of fatty streaks toward inflammatory and fibrotic plaques. Among all of its proatherogenic properties, oxLDL is a chemoattractant to monocytes, induces VSMC migration and proliferation, enhances platelet adhesion and aggregation causing thrombosis, and interferes with NO signaling in ECs, impairing vasodilation in various vascular beds [8]. Oxidized lipids are also well known to be proapoptotic for all cell types present in atherosclerotic plaque, partly by promoting further reactive oxygen species (ROS) production [18–20].

High levels of oxLDL are also found in patients with acute coronary syndromes [21, 22]. In particular, unstable atherosclerotic lesions contain a significantly higher percentage of oxLDL-positive macrophages suggesting that increased levels of oxLDL relate to plaque instability in human coronary atherosclerotic lesions. In contrast, lower levels of oxLDL-positive macrophages are present in patients with stable coronary heart disease [21]. Elevated serum LDL cholesterol in patients correlates with elevated lipid oxidation products such as urinary levels of F_2-isoprostanes [23, 24]. Elevated titers of serum autoantibodies against MDA-modified LDL also

constitute an independent predictor of the progression of atherosclerosis [25]. Taken together, these data suggest that oxLDL is both a tissue marker of an unstable phenotype and a serum marker of plaque progression.

3 Oxidants in the Vascular Wall

Oxidant damage is attributed to cellular overproduction of reactive oxygen or nitrogen species, a group of radical and nonradical entities essentially released by the mitochondrial respiratory chain or generated by cellular enzymatic activities including oxidases, peroxidases, dehydrogenases, or oxygenases. Among the major reactive species relevant to vascular diseases (summarized in Fig. 2), the superoxide anion ($O_2^{-\bullet}$) plays a central role and can be produced by mitochondria, NADPH oxidases, xanthine oxidase (XO), endothelial NO synthase (eNOS), and endoplasmic reticulum oxidases. The $O_2^{-\bullet}$ radicals can readily be converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs) but, if not, will generate the powerful pro-oxidant hydroxyl group ($HO^{\bullet}$) in the presence of transition metal ions.

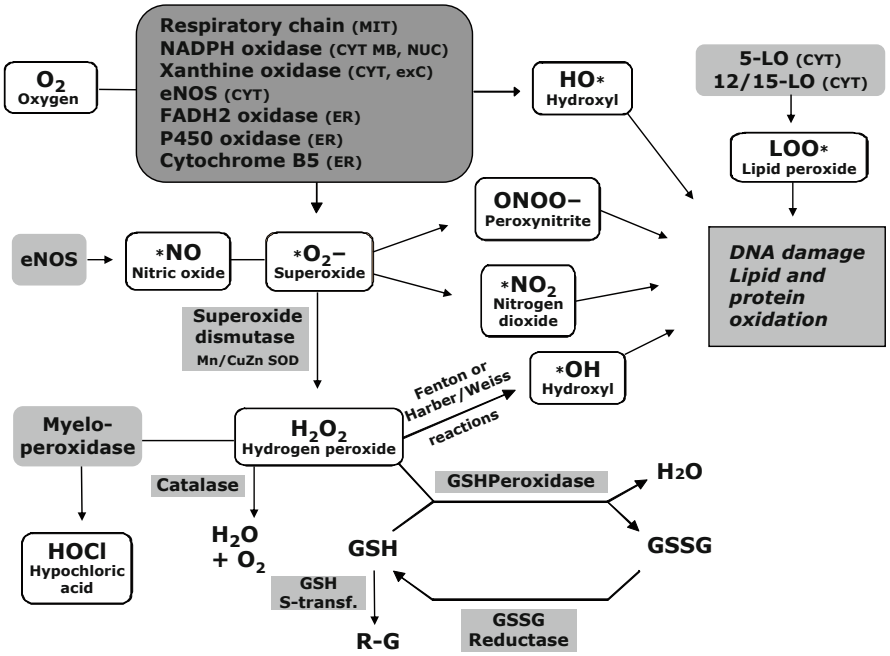

Fig. 2 Pathways of production and inactivation of reactive oxygen/nitrogen species. All cells within the vascular wall can produce ROS, from cytosolic enzymes or from mitochondria. ROS may also be produced in the nuclear compartment where the NADPH oxidase isoform NOX4 has been detected in ECs. Abbreviations: CYT, cytoplasmic; eNOS, endothelial nitric oxide synthase; exC, extracellular; ER, endoplasmic reticulum; GSH, reduced glutathione; GSH S-transf., GSH *S*-transferase; GSSG, oxidized glutathione; 5-LO, lipoxygenase; MB, membrane-associated; MIT, mitochondria; NUC, nuclear; R-G, glutathione conjugate

Peroxynitrites ($ONOO^-$) and nitrogen dioxide ($^\bullet NO_2$) are derived from the nitric oxide radical ($^\bullet NO$) released by constitutive or inducible NO synthases in ECs and macrophages. Peroxynitrites are stable but can be converted to peroxynitrous acid, which is highly reactive yielding oxidizing and nitrating species. The phagocytic myeloperoxidase can produce hypochloric acid (HOCl), a product of H_2O_2 reaction with Cl– atoms, which is also a strong oxidant species released in the extracellular space. By-products of lipoxygenases, derived from nonspecific arachidonic acid peroxidation, are also very reactive radicals in ECs and macrophages. Virtually all cell types of the vascular wall (i.e., ECs, VSMCs, adventitial fibroblasts, or infiltrated phagocytes) have potential enzymatic equipment for producing harmful oxidants. However, in order to be oxidizing, reactive species have to overcome the cellular antioxidant systems, both enzymatic (mitochondrial and cytosolic SODs, catalase, GSH peroxidase) and small chemical antioxidants (glutathione system, ascorbate).

4 Role of Redox Enzymes in Vascular Disease

4.1 NADPH Oxidase

NADPH oxidase is one of the most studied redox enzymes in the vasculature. This multimeric enzyme is present in all cell types of the vascular wall, although selected isoforms of the catalytic gp91phox-like subunits (Nox1, Nox3, Nox4, and Nox5) are expressed differently (see Fig. 3) and are distinct from the gp91phox/Nox2 of phagocytic cells [26]. In both VSMCs and ECs, its activation is required in the signaling pathway of numerous growth factors and cytokines, and mediates their proliferative action via activation of intracellular pathways such as JAK/STAT, ERK1/2, p38 MAP kinase, and JNK [27]. NADPH oxidase is also induced in activated monocytes/macrophages, and its activation contributes to oxidant stress in inflammatory tissue environments [28].

In atherosclerosis, several studies have demonstrated an upregulation of NADPH oxidase isoforms and increased enzyme activity. The mRNA and/or protein levels of Nox2 and Nox4 subunits are increased in coronary artery disease and indicative of increased phagocytic (Nox2) and nonphagocytic (Nox4) NADPH oxidase function [29, 30]. Atherosclerosis-prone apoE null mice lacking the ubiquitous p47phox subunit of the NADPH oxidase develop smaller aortic plaques [31], suggesting that the enzyme promotes atherosclerosis, although gp91phox-deficient mice exhibit no reduction in lesion size [32].

NADPH oxidase subunits are also involved in hypertension of diverse etiology. For example, in salt-sensitive hypertension with activation of the renin-angiotensin system, angiotensin receptor blockade [33] or inhibition of gp91phox [34] attenuates blood pressure. The gp91phox subunit is essential for the production of $O_2^{-\bullet}$ and contributes to increased blood pressure in renal-induced hypertension [35]. In contrast, genetic modification of p47phox or Nox1 isoforms has a negative impact on blood pressure [36–39]. Vascular NADPH oxidase is also involved in spontaneously

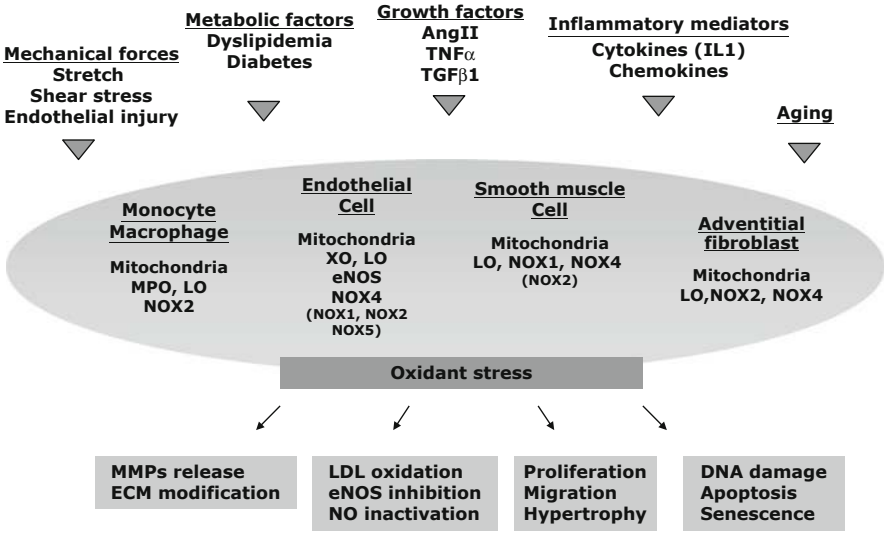

Fig. 3 Factors promoting ROS production on vascular cells and consequences. NADPH oxidase activity is found in all cells of the vascular wall, but the distribution of its subunits is cell-specific. Five isoforms of NADPH oxidase exist, which contain at least p47phox, p22phox, and the gp91phox/Nox2 in macrophages or a gp91phox homologue (Nox1, Nox3, Nox4, and Nox5) in other cells. The phagocytic NADPH oxidases contain additional p67phox regulatory subunits. In ECs, the abundant expression of XO and the uncoupled eNOS are powerful sources of ROS. Extracellular ROS can be generated by the XO or myeloperoxidase released by ECs and phagocytic cells, respectively. Abbreviations: AngII, angiotensin II; ECM, extracellular matrix; eNOS, endothelial nitric oxide synthase; IL1, interleukin 1; LDL, low-density lipoprotein; LO, lipoxygenase; MMPs, matrix metalloproteinases; MPO, myeloperoxidase; NOX, NADPH oxidase; TNF-α, tumor necrosis factor-α, TGF-β1, transforming growth factor-β1

hypertensive rats [40] and in DOCA salt-induced or mineralocorticoid-induced hypertension. These models show increased vascular $O_2^{-\bullet}$ due to NADPH oxidase but independently of the influence of angiotensin II (AngII). Endothelin has also been shown to raise $O_2^{-\bullet}$ and blood pressure through NADPH oxidase [41].

4.2 Xanthine Oxidase

The flavoprotein xanthine oxidase (XO), which can also generate significant amounts of $O_2^{-\bullet}$, is highly expressed in ECs, suggesting a major role for this oxidase in these cells. Indeed, endothelium-derived XO activity is increased in tissues of hypercholesterolemic animals [42] or tissues from patients with coronary artery disease or carotid stenosis [29, 43]. The intracellular enzyme can be released and bind the extracellular surface of ECs and can also be released into the plasma after heparin administration [44]. XO activity measured ex vivo inversely correlates with the extent of endothelium-dependent vasodilation [43], suggesting a role

for this oxidase in blood pressure homeostasis. Indeed, the participation of XO in AngII-induced endothelial oxidant stress has been reported [45], and in patients with coronary disease, losartan therapy reduces endothelium-bound XO activity and may contribute to improved endothelial function. XO in vascular endothelium has also been involved in hypertension as well as in ischemia reperfusion [46].

4.3 Endothelial Nitric Oxide Synthase

Redox active compounds can also be produced by the uncoupled activity of endothelial nitric oxide synthase (eNOS). In absence of the required cofactors tetrabiopterin (BH_4) or L-arginine, eNOS produces significant amounts of $O_2^{-\bullet}$ instead of $^{\bullet}NO$. In addition, the interaction between $O_2^{-\bullet}$ and nitric oxide can produce harmful peroxynitrites ($ONOO^-$/ONOOH), which are themselves capable of oxidizing BH_4 and amplifying eNOS uncoupling while decreasing NO bioavailability. Consistent with this, rats treated with BH_4 show an improvement of hypertension [47–49].

4.4 Myeloperoxidase

The myeloperoxidase produced by invading macrophages is the only enzyme known to generate the reactive entity HOCl. This enzyme is present in atherosclerotic plaques and colocalizes with macrophages in the lipid core. HOCl is capable of oxidizing LDL in vitro and is thought to be responsible for its oxidation in plaques [50]. HOCl interacts with protein tyrosine residues, producing 3-chlorotyrosine derivatives that are markers of macrophage-induced oxidative stress [51]. The formation of tyrosyl radicals may also be involved in LDL oxidation [52]. HOCl also converts α-amino acids to aldehydes [53] and L-serine to advanced glycation end products [54]. By oxidizing nitrites, HOCl promotes the formation of harmful secondary products such as the $^{\bullet}NO_2$ and NO_2Cl. These latter compounds participate in protein nitration by converting tyrosine into 3-nitrotyrosines [55]. The measurement of nitrotyrosines has been used as a marker of NOS activity [56].

4.5 Lipoxygenases

The eicosanoids derived from the 12/15-lipoxygenase (LO) activity promote lipoprotein oxidation and are suspected to contribute to inflammation and foam cell formation. For example, lipid peroxidation products measured in stimulated macrophages incubated with LDL were not observed in 12-LO–deficient macrophages [57]. In addition, 12/15-LO null mice bred on an apoE null background and fed a high-fat diet demonstrate reduced aortic atherosclerosis when compared with 12/15-LO–expressing controls [58]. Areas of lipid deposition in the lesser curvature of the aortic arch, branch points, and in the abdominal aorta were reduced although serum lipid profiles were similar. Atherosclerosis-prone

mice lacking 5-LO in their monocyte/macrophage population also showed reduced atherosclerosis [59].

4.6 Antioxidants

All cells harbor a number of protection mechanisms against oxidative damage, including direct interaction of radicals with antioxidant molecules such as glutathione, ascorbic acid, or α-tocopherol. Antioxidant enzymes are present within cells or in the extracellular space. Among them, the superoxide dismutases (cytosolic CuZn-SOD, mitochondrial Mn-SOD, or extracellular EC-SOD) convert the $O_2^{-\bullet}$ radical to H_2O_2 and water. Glutathione peroxidase (GPx-1) buffers radicals by oxidizing glutathione, and the glutathione *S*-transferase conjugates reduce GSH to highly reactive lipid peroxides. Interestingly, as pro-oxidant enzymes in vascular cells tend to accelerate or aggravate atherosclerotic lesions, antioxidant enzymes, on the contrary, have protective effects. For example, $apoE^{-/-}$ mice with overexpression of both CuZn-SOD and catalase had delayed onset of atherosclerotic plaques and smaller lesions, as well as reduced F_2-isoprostanes levels in plasma and aortic tissues [60]. These results are suggestive of decreased lipid peroxidation in cells protected against H_2O_2 overload, underlying the importance of ROS effects and antioxidant mechanisms in atherogenesis. Similarly, mice with GPx-1 deficiency demonstrate accelerated progression of atherosclerosis [61]. Similarly, the lack of functional GPx-1 accelerates diabetes-associated atherosclerosis via upregulation of proinflammatory and profibrotic pathways in $apoE^{-/-}$ mice [62]. Low levels of GPx-1 activity in human plasma are an independent risk factor for cardiovascular events in patients with coronary artery disease [63].

5 Role of Reactive Species in Mechanical and Shear Stress

Local and systemic hemodynamic changes, including shear stress and mechanical stretch, have been associated with an oxidative response [64]. Laminar shear stress, i.e. pulsatile forces acting parallel to the blood flow, exert antiatherogenic and potent antiapoptotic effects on ECs [65, 66]. In contrast, shear stress due to turbulent flow is proatherogenic. Although acting by different mechanisms, both laminar and oscillatory shear stress promote eNOS expression and activity as well as an increase in $O_2^{-\bullet}$ and H_2O_2 production by ECs in vitro. Shear stress causes transient NADPH oxidase activation but is associated with a long-term induction of redox sensitive genes, such as the protective CuZn-SOD, which can inactivate $O_2^{-\bullet}$ [67]. Oscillatory shear stress caused by disturbed flow also triggers the expression of gp91phox and Nox4 NADPH oxidase subunits [68] and the production of $O_2^{-\bullet}$ and H_2O_2 but has a more dramatic effect than laminar shear stress [69]. As a result of the $O_2^{-\bullet}$ release and associated peroxynitrite formation, oscillatory shear stress is thought to promote LDL oxidation, its uptake by scavenger receptors [70], and increased expression of adhesion molecules VCAM and ICAM.

In addition to shear stress, mechanical stretch and its consequent stimulation of vascular oxidant stress may contribute to hypertension. Stretch caused by the luminal pressure of blood flow is required to maintain VSMC differentiation [71], however, excessive mechanical stretch induces remodeling of the vascular wall, particularly in hypertension and diabetes, by stimulating VSMC proliferation [72]. Vessel wall stretch can also trigger overproduction of $O_2^{-\bullet}$ by ECs in vivo [73], and the increase in ROS caused by stretch has been implicated in the activation of NF-κB [74, 75], the activation of metalloproteinases [76], MAP kinase activation [77], angiogenesis [78], and altered vasomotion [79].

6 Role of Mitochondria in Vascular Disease

Mitochondrial electron transport accounts for most $O_2^{-\bullet}$ produced by mammalian cells. Mitochondrial respiration produces ATP and simultaneously generates $O_2^{-\bullet}$ as electrons at complex I and III of the respiratory chain react with oxygen. Although most of $O_2^{-\bullet}$ is inactivated by the mitochondrial SOD (Mn-SOD or SOD2), radical leaks from the respiratory chain can cause cumulative damage to mitochondrial DNA (mtDNA) as mutations, deletions, or nucleotide oxidation [80]. Because mtDNA encodes genes essential for oxidative phosphorylation, mtDNA damage will affect the efficiency of the respiratory chain by preventing the regeneration of its complexes and amplifying the oxidative stress. As a result, a decline in oxidative phosphorylation may hinder cell energy production efficiency or cell viability [80].

Links between accumulated mitochondrial defects and susceptibility to cardiovascular diseases have been demonstrated [81, 82]. Hypercholesterolemia in apoE$^{-/-}$ mice can induce significant mtDNA damage in aortic cells. In addition, the extent of atherosclerotic lesions and mtDNA damage is dramatically potentiated by the exposure of mice to tobacco smoke, also a promoter of mtDNA damage and protein nitration [82]. The fact that mtDNA damage was found to be a marker for ROS effects [83] strongly supports the idea that mtDNA damage in vivo can be attributed to the detrimental effects of reactive species. This study also provided evidence for an effect of peroxynitrites, formed from nitric oxide and mitochondrial ROS, on the intramitochondrial SOD2, resulting in its decreased activity [82]. Interestingly, altered function in SOD2$^{+/-}$ mice correlates with increased oxidative damage, as indicated by high carbonyl group content in proteins or increased incidence of 8-hydroxydeoxyguanosine in mtDNA of liver cells [84]. Lowering mitochondrial antioxidant capacity of apoE$^{-/-}$ mice also seems to promote mtDNA damage in aortic cells. In fact, mice with decreased SOD2 activity show higher mtDNA damage than control mice and increased atherosclerotic lesions [60]. mtDNA damage appears prematurely with hypercholesterolemia and precedes atherosclerosis, suggesting causality in the development of the disease. In addition, mtDNA damage was found more prominent in human atherosclerotic aortic samples than in control tissues and may be a consequence of accumulated oxidative damage [85]. Taken

together, this evidence indicates that mtDNA damage and dysfunction is an early event in atherosclerosis, with the resultant increase in ROS accelerating further both mtDNA damage and atherosclerosis.

Mitochondrial superoxide production can be regulated by mitochondrial uncoupling proteins (UCPs), which may play a significant role in vascular disease. For example, UCPs are activated by superoxide radicals, which, by dissipating the proton motive force, results in a feedback mechanism aimed at preventing excess $O_2^{-\bullet}$ production. Uncoupling the respiratory chain therefore increases oxidative stress. Consistent with a role for macrophage mitochondrial-associated oxidative stress in atherosclerosis, UCP2 knockout in macrophages is associated with increased oxidative stress markers and atherosclerotic plaque size [86]. This effect is possibly due to increased secretion of matrix metalloproteinases and enhanced macrophage apoptotic rates, leading to a larger necrotic core in the plaque. On the other hand, the overexpression of the UCP1 isoform in smooth muscle cells was unexpectedly promoting oxidative stress, resulting in increased blood pressure and exacerbated atherosclerosis [87].

There is also extensive cross-talk between intracellular signals and mitochondrial ROS formation. For example, activation of NADPH oxidase leads to increased ROS formation by mitochondria [88]. iNOS-dependent NO production may also modulate mitochondrial production of ROS. Through its binding to respiratory complexes (in particular cytochrome c oxidase), NO can reduce the mitochondrial electron transport and promote superoxide production [89, 90]. In conjunction with NADPH oxidases, mitochondria also contribute to the production of ROS induced by growth factors such as TGF-β [91] or EGF [92]. Mitochondrial ROS also participates in the activation of MAP kinase and downstream signaling [93, 94], thereby providing an alternate source of radical and oxidative stress in vascular disease.

7 Oxidative Stress, Telomere Length, and Senescence

Recent studies have examined the long-term consequences of ROS overload in the cell including the cumulative effects of oxidative damage to DNA, and in particular to the telomeric regions. Due to their high content of G nucleotides, telomeres are particularly susceptible to oxidative damage and DNA strand breaks caused by radicals. In vitro, oxidant stress induced by chronic H_2O_2 treatment [95], hyperoxic culture conditions [96], or alterations of the cellular antioxidant properties [97] can cause telomere single-strand breaks that promote telomere shortening [98].

Short telomeres are a hallmark of the senescent state. Cellular senescence is defined as the state of growth arrest accompanying the exhaustion of the cell's replicative potential and is by definition irreversible [99]. Telomere shortening consequently induces premature cell cycle arrest by triggering pathways converging toward activation of the G_1-associated cell cycle inhibitors ($p21^{Cip1}$, $p16^{ink4}$) often via activation of the tumor suppressor p53 (see Fig. 4). Telomere shortening with

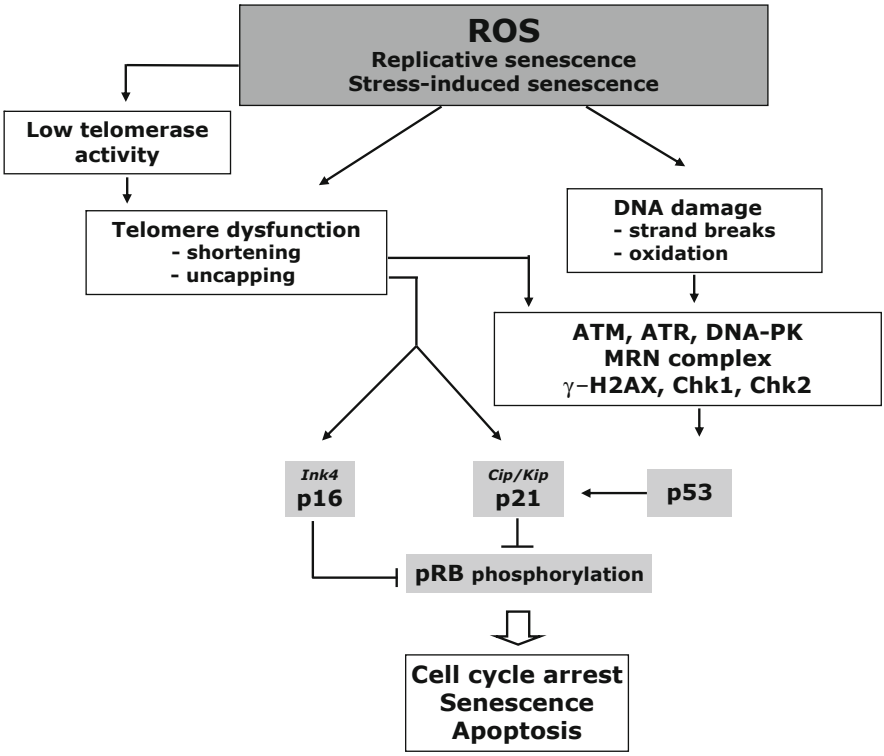

Fig. 4 Pathways of induction of oxidative stress–induced senescence and apoptosis. ROS are known to cause oxidative damage to DNA (oxidation, DNA strand breaks) including damage to telomeric repeats. DNA breaks as well as short/damaged telomeres trigger the induction of a DNA damage response involving damage sensors, transducers, and repair enzymes, leading to the activation of p53 and, according to the degree of damage, cell cycle arrest, apoptosis, or senescence. Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ATM-related protein; Chk, checkpoint kinase; DNA-PK, DNA-activated protein kinase; γ-H2AX; phosphorylated histone 2AX; MRN complex, MRE11-Rad50-NBS1 protein complex; pRB, retinoblastoma protein

activation of DNA damage response using this pathway has been documented in both ECs and VSMCs [100, 101].

There is direct evidence of telomere loss in cells of the vessel wall in atherosclerosis. VSMC and EC telomere size is not uniform throughout the vasculature, and differences between the intima and media have been observed [102]. In particular, vessels subject to high hemodynamic stress have significantly shorter telomeres in their intima when compared with that of arteries with low hemodynamic stress [103], suggesting that high cell turnover or radicals produced at sites of high stress cause telomere erosion. In addition, telomere length in the media and intima of distal and proximal abdominal aorta has been shown to negatively correlate with predisposition to atherosclerosis [104]. The atherosclerosis grade inversely correlated with telomere length, but the correlation was lost after adjustment for age [104]. In

addition, fibrous cap VSMCs exhibited markedly shorter telomeres compared with those of normal medial VSMCs [101]. In this study as well, telomere shortening was closely associated with increasing severity of atherosclerosis. Although these data suggest that atherosclerosis is associated with telomere loss, they do not prove that telomere loss promotes atherosclerosis rather than just being a marker of increased cell turnover or stress damage. However, it is conceivable that within the context of atherosclerotic plaque, senescence may have dramatic consequences. Loss of proliferative capability among VSMCs coupled to ongoing apoptosis may account for cell loss in the fibrous cap, which then becomes highly susceptible to the action of metalloproteinases degrading the extracellular matrix. Thinning of the cap in advanced lesions is a major feature of the vulnerable plaque (i.e., a plaque that is prone to rupture). EC senescence inhibits endothelial vasoactive function [105], and may reduce the regenerative or angiogenic capacity of the endothelium [106, 107]. Atherosclerotic plaque VSMCs isolated from human endarterectomy specimens exhibit features of senescent cells [108], including slow rates of proliferation, premature growth arrest at early passage in culture, and the characteristic expression of the senescence marker SAβG [109]. Their profile of expression of cell cycle regulators is analogous to the one of senescent cells arrested in G_1 of the cell cycle, including increased expression of $p21^{Cip1}$ and p16, reduction of cyclins $D_{1–3}$ and E, and dephosphorylation of the tumor suppressor pRB [101, 110, 111]. In histologic sections, SAβG activity localizes with VSMCs in atherosclerotic lesions, and particularly within the fibrous cap of advanced plaques [101]. SAβG also colocalizes with $p21^{Cip1}$ and p16 and is not found in cells from the media. Senescence markers have also been reported among ECs in the plaque [100, 112] and in the endothelium of rabbit carotid artery submitted to repeated injury [106].

8 DNA Damage in Vascular Disease

Oxidative stress can induce premature senescence both by inducing telomere shortening but also independently of telomere length. For example, pulse treatments of fibroblasts with low concentrations of H_2O_2 can cause irreversible cell cycle arrest accompanied by an increase in SAβG staining without concomitant changes in telomeres [113]. The term SIPS (stress-induced premature senescence) has been coined to describe these telomere-independent processes of cell growth arrest, which are driven by increased expression of $p21^{Cip1}$ [113]. The mechanism underlying SIPS is unclear, but activation of p53 and $p21^{Cip1}$ can be direct consequences of oxidative DNA damage.

In fact, one of the major consequences of long-term ROS action is DNA damage. It has been estimated that approximately 2×10^4 DNA damaging events occur in every cell/day [114], a major portion of these occurring via ROS [115]. Although superoxide and hydrogen peroxide released from the mitochondria are normally not reactive to DNA, both these diffusible factors can be converted to the extremely reactive hydroxyl radical. Hydroxyl radicals can induce a vast array of damage to

both nuclear and mitochondrial DNA [116]. In particular, hydroxyl radicals can interact with pyrimidine (T, C) double bonds causing glycolytic damage that can cause mispairing, transcriptional interrupts, and, if left unrepaired, induce cell cycle arrest. Thymidine oxidation, and the addition of methyl and alkyl groups have been attributed to ROS of any kind. Dimerization of adjacent pyrimidines in the DNA also occurs. Similarly, interaction of $HO^{\bullet}$ radicals with purines (A, G) will generate formamidopyrimidines and other purine oxidative products. For example, unrepaired 8-hydroxy-guanine or 8-oxo-guanine (8-oxo-G) will mispair with dA, leading to an increase in G to T transition mutations that will irreversibly affect DNA integrity. The creation of apurinic/apyrimidinic sites after base loss are also common lesions in DNA; these sites can cause cell death and are mutagenic. Another major type of DNA damage is DNA strand breaks. Measurement of DNA strand breaks in vascular cells by single-cell DNA electrophoresis has shown an increased number of DNA strand breaks in plaque VSMCs compared with that in normal cells [117]. Reactive nitrogen species are also potent inducers of DNA damage, The effects of the peroxynitrite $ONOO^{-}$, a product of interaction of NO and superoxide anion, on DNA include the formation of abasic sites in treated DNA due to the presence of depurinating lesions, most likely 8-nitro-guanine [118]. In addition, in macrophages and VSMCs, $ONOO^{-}$ can rapidly induce DNA strand breaks, whereas NO donors did not produce any effects [119].

In all cases and if not repaired, the base modifications within expressed genes or DNA strand breaks will affect the integrity of the genome in dividing cells and irreversibly interfere with the fidelity of the transcription. Low concentrations of oxidant will cause repairable damage; however, overwhelming the repair system with high oxidative burden will trigger irreversible growth arrest or apoptosis. Repair of these lesions is important in preventing apoptosis. Both ROS and telomere dysfunction elicit a general DNA damage response (described in Fig. 4) and growth arrest via the activation of a protein kinase cascade including the kinases ataxia telangiectasia mutated (ATM) and ATM-related kinase (ATR) [120, 121]. The DNA damage response involves the phosphorylation and recruitment of the kinases ATM, ATR, and DNA protein kinase (DNA-PK) [122] at the site of DNA damage and, sequentially, the phosphorylation of histones H2AX, the association of DNA repair enzymes and cofactors (53BP1, MDC1/NFBD1, and NBS1), and activation of the transducer proteins Chk1 and Chk2 [116]. Targeted damage of either telomeres or genomic DNA finally converges on to a common pathway characterized by the activation of at least one of the cell cycle inhibitors $p21^{cip1}$ (via p53) and $p16^{ink4}$ [123, 124] responsible for inducing growth arrest and senescence (Fig. 4).

The evidence for DNA damage accumulation in nondividing mammalian cells and the possible biological consequences of damage accumulation in aging cells have been reviewed [125]. Within the vasculature, signs of DNA damage and chromosome instability can be observed in both circulating cells and the vessel wall of atherosclerotic vessels (coronary, aorta). For example, patients with coronary artery disease have a higher micronucleus index (a marker of genetic instability) than that in healthy controls, which correlates with disease severity [126, 127]. There

is also evidence of DNA damage within human plaques, including loss of the Y chromosome, deletions, and trisomy [128]. Unstable carotid plaques also show trisomy and tetrasomy of chromosome 7 and monosomy of chromosome 11 [129]. VSMCs demonstrate microsatellite instability, which may affect gene expression, and loss of heterozygosity [130, 131], suggesting that genomic destabilization may play a direct role in atherosclerosis. DNA damage can also occur in the mitochondria. Indeed, mtDNA damage correlates with the extent of atherosclerosis in human specimens and aortas from apoE$^{-/-}$ mice [85].

The DNA damage in atherosclerosis is likely caused by ROS, due to the nature of some of the damage observed. There is a higher incidence of oxidized pyrimidines and altered purines and damaged DNA in circulating cells in patients with coronary artery disease compared with that in controls [126]. In addition, human plaques show markers of oxidative damage, including DNA strand breaks, expression of 8-oxo-G (an oxidative modification of guanine residues in DNA), and activation of DNA repair enzymes [101, 117, 132]. Adducts of 8-oxo-G are detected in plaque VSMCs, macrophages, and ECs but not in VSMCs of adjacent normal media or normal arteries [132]. DNA damage is also a direct correlate of extent of atherosclerosis in experimental animals. For example, cholesterol feeding of rabbits induces oxidative damage in plaques, manifested by 8-oxo-G staining, DNA strand breaks, and apoptosis [133]. In human carotid plaques, increased phosphorylation of ATM/ATR substrates and H2AX phosphorylation were detected in advanced plaques [117]. Altogether, these studies directly implicate oxidative DNA damage, DNA repair, and cell senescence in atherosclerosis.

In cells where the DNA damage is too much to repair, or in cells that are driven to proliferate, DNA damage induces apoptosis. Apoptosis is evident in ECs, macrophages, and VSMCs in atherosclerotic plaques. EC death is implicated in both atherogenesis and plaque erosion [134], whereas VSMC death may promote thinning of the fibrous cap and plaque rupture [135]. Indeed, increased levels of VSMC apoptosis are seen in mature plaques compared with control vessels [136], and in unstable versus stable plaques or patients [137, 138]. Cell death can be induced by ROS and oxidized lipids in vascular cells, whereas p53 expression in an experimental neointima can induce apoptosis and promote plaque rupture [139]. The p53 tumor suppressor gene is increased with DNA damage in response to oxidative stress. Increased p53 expression and phosphorylation are seen in atherosclerosis, associated with markers of DNA damage and activation of DNA repair pathways, suggesting that p53 expression is ultimately triggered by DNA damage in cells from the vessel wall.

9 Antioxidants and Cardiovascular Diseases

The protective properties of antioxidants have been extensively studied. In particular, vitamin supplements (vitamin C, vitamin E, or β-carotene) were assessed in large-scale randomized trials analyzing coronary artery disease, the incidence of

cardiovascular events, or morbidity linked to cardiovascular disease. These studies involved either healthy subjects or cohorts of patients with risk factors or a history of cardiovascular events. A recent meta-analysis efficiently summarized the conclusions [140]. From more than 20 controlled randomized trials, antioxidant administration showed no conclusive benefit, and two long-term studies involving healthy subjects showed negative results [141, 142]. In addition, in most cohorts of subjects with established cardiovascular disease or with single or combined risk factors, including subjects with hyperlipidemia or diabetic patients, dietary antioxidants did not show any benefit [143–151]. No effects on prevention of cardiovascular events, vascular thickness, or risks of re-stenosis after angioplasty were observed. These results were corroborated by a recent analysis [152] where long-term cardiovascular mortality and morbidity where not shown to be decreased after antioxidant administration. In some studies, positive results were associated with the use of higher concentrations of supplements or combinations or in smaller numbers of patients [142, 148, 153, 154], although any beneficial effect of a vitamin combination did not appear to be consistent [142, 147]. Most surprising is the fact that supplementation of antioxidant vitamins can be associated with an increased risk for all-cause mortality, including increase of fatal coronary heart disease [146–148, 151, 152, 155–157]. Indeed, the meta-analysis indicating a significant relationship between antioxidant dosage and all-cause mortality raises suspicions that certain vitamins such as vitamin E may act as a pro-oxidant [158]. It has been suggested that cells that may benefit from the action of antioxidants in the plaque may not be affected by low levels of vitamins, their bioavailability, or the possibility that the high oxidative stress environment converts antioxidants into inactive molecules and affects their local efficiency [159]. Adverse effects by inhibition of natural vitamins by racemic compositions could also be an issue. Whatever the reason, at present there is no consistent evidence from clinical trials to support the use of antioxidant vitamins to either prevent or treat atherosclerosis.

10 Conclusions

There is extensive evidence that implicates free radicals in atherosclerosis and other vascular diseases. Plaques that demonstrate oxidative damage and its effects including cellular senescence and apoptosis in multiple cell types support this notion. Senescence and apoptosis in advanced atherosclerotic plaques are deleterious and may promote plaque rupture. In contrast, clinical trials have shown that there is, at present, no strong consensus on the beneficial effects of antioxidant supplements in cardiovascular disease, and that strategies that reduce free radicals indirectly, for example by lowering cholesterol levels, are more successful. In contrast, recent experiments showing the protective effects of antioxidant enzymes in animal models strongly support the role of intracellular antioxidant systems in protecting against vascular disease. The study of intracellular pathways that counteract the effects of oxidative stress may prove beneficial in the future.

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The Role of Mitochondrial Reactive Oxygen Species Formation for Age-Induced Vascular Dysfunction

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Abstract Aging is an important risk factor for the development of cardiovascular diseases, which can be accelerated by atherosclerosis, diabetes, hypercholesterolemia, or obesity. Vascular aging is mainly characterized by endothelial dysfunction, an alteration of endothelium-dependent signaling processes, and vascular remodeling. The underlying mechanisms include increased production of reactive oxygen species (ROS), inactivation of nitric oxide (•NO), and subsequent formation of reactive nitrogen and oxygen species (RNOS). Elevated RNOS may exhibit new messenger functions by posttranslational oxidative modification of intracellular regulatory proteins or lead to irreversible alterations of biological macromolecules. Various cellular sources may contribute to radical formation and are discussed in the context of the free radical hypothesis of aging. Clinically, endothelial dysfunction can be assessed by plethysmography, which may serve as an independent predictor for the risk of cardiovascular events. Current concepts in vascular aging, consequences for the development of cardiovascular events, and the particular role mitochondria may play in the development of RNOS-induced pathologic processes are discussed.

Keywords Aging · Mitochondrial oxidative stress · Vascular oxidative stress · Vascular dysfunction · Endothelial dysfunction · Antioxidant proteins

1 Introduction

The proportion of people more than 65 years old will dramatically increase within the next few decades in Western societies [1]. Consequently, the prevalence of cardiovascular, cerebrovascular, and neurodegenerative diseases will balloon,

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critically reduce the quality of life, and further burden the budget for health care and assistance. Therefore, the major objective of research into aging should be to better understand molecular processes involved in the pathogenesis of age-dependent diseases. This could ultimately result in earlier diagnostic procedures and preventive measures to improve the quality of the eventide and reduce health care costs. Cardiovascular diseases and their acute and chronic manifestations such as myocardial infarction, stroke, and peripheral arterial occlusive disease increase in frequency with age and are further aggravated by the onset of other risk factors [2], such as adiposity, hyperlipidemia, diabetes, or hypertension. Pathophysiologically, vascular aging is mainly characterized by endothelial dysfunction, which is considered a precursor of arteriosclerosis. The aging vasculature displays typical morphologic and molecular alterations leading to increased vascular stiffness, reduced compliance, and the loss of vascular homeostasis. Current research focuses on the regulation of vascular tone and vessel remodeling mediated by free radicals, particularly the role of superoxide ($^{\bullet}O_2^-$) and nitric oxide (•NO). Since the discovery of vascular NADPH-oxidases, it has become clear that reactive nitrogen and oxygen species (RNOS) play a pivotal role in cell signaling. Besides the classic function of •NO activating soluble guanylyl cyclase and increasing the second messenger cGMP, •NO can lead to S-nitros(yl)ation, tyrosine nitration, and glutathiolation of important enzymes involved in vascular signaling via secondary species [3–6]. These processes and novel signaling cascades are part of the emerging field termed *redox regulation* or *redox signaling*. Therefore, enhanced age-dependent free radical formation will not only cause nonspecific oxidation of biological macromolecules but will also interfere with vascular redox regulation. Current knowledge about vascular redox signaling and clinical consequences for age-dependent cardiovascular disease will be discussed.

2 The Cardiovascular System

Historically, the circulatory system has been regarded as a blood distribution network that moves nutrients, gases, and wastes to and from cells to maintain tissue homeostasis. However, vascular and endothelial function is as vital as every heartbeat because it regulates blood pressure, prevents blood coagulation, and modulates the innate immune response. A well-balanced or homeostatic vascular system is protected from hypertension, vascular hypertrophy, atherosclerosis, stenosis, vascular occlusions and thrombosis, and endothelial dysfunction in the long term [7, 8]. The endothelium plays a crucial role as a specialized monolayered squamous epithelium that lines the interior surface of blood vessels and acts as the interface between circulating blood and the vessel wall or tissue. Preserving the blood barrier function and thereby preventing immune cells from infiltration [9, 10] is an important step in controlling the innate immune response and prevents monocytes from entering lesion-prone areas of the endothelium, which promotes

plaque formation in atherosclerosis. Vascular tone is controlled by a complex network of regulatory systems that consists of catecholamines, vasoactive peptides such as angiotensin or vasopressin, vasoactive prostaglandins, and the water retention/excretion system. The concerted synthesis of endothelium-derived mediators such as •NO, endothelium-derived hyperpolarizing factor, prostacyclin, and natriuretic peptides makes the endothelium one of the most important cell types within the vasculature and, in turn, deems the vasculature one of the most important "organs" within the organism.

Importantly, vascular function is directly related to the risk for heart attacks or stroke because the endothelium controls the release of antiaggregatory mediators that suppress thrombus formation, vascular stenosis [11], and cardiac hypertrophy.

One of the central regulatory systems of the vascular system is the synthesis of the endothelium-derived relaxing factor (EDRF) nitric oxide (•NO) by endothelial NO synthase (eNOS or NOSIII). •NO is a free radical and exerts potent vascular messaging properties such as vasodilation and antiaggregatory and antiadhesive effects [12–14]. The signaling function of this potent messenger molecule can be chemically modulated by another radical, superoxide ($O_2^{-\bullet}$), and builds a novel signaling network, which involves vascular NADPH-oxidases and oxidative posttranslational protein modifications. These emerging signaling cascades are summarized as *redox signaling* or *redox regulation*. In the past 10 years, knowledge about redox regulation and the role of free radicals in regulating endothelial and vascular function has vastly increased and the cardiovascular system is one of the best studied organ systems. As a result, it is well established that RNOS interfere in central endothelial functions and significantly affect vascular tone and homeostasis. Various targets of oxidants such as the •NO/cGMP signaling cascade [15], calcium homeostasis by sarcoplasmic reticulum calcium ATPase (SERCA), p21Ras, or prostacyclin synthase have been reported.

2.1 Vascular Function and Oxidative Stress

Recent research informs us that an increase in RNOS is not solely a consequence but plays a crucial role in the development and progression of cardiovascular diseases [8, 16, 17]. Acutely, reactive oxygen species (ROS) overproduction can interfere in important signaling cascades such as inactivation of bioavailable •NO [18], alterations in prostaglandin metabolism, or dysregulation of calcium and phosphorylation cascades. Chronically, ROS lead to irreversible oxidations and accumulation of oxidized biological macromolecules (e.g., increase in DNA mutations) [19]. The accepted concept of oxidant-induced vascular dysfunction is based on the oxidative scavenging of the vasodilator •NO by enhanced $O_2^{-\bullet}$ production. In addition, various essential enzymes for maintenance of vascular homeostasis such as prostacyclin synthase, soluble guanylyl cyclase, and endothelial NO synthase (Fig. 1) are oxidatively inactivated [20, 21]. Meanwhile, the contribution of oxidative stress toward

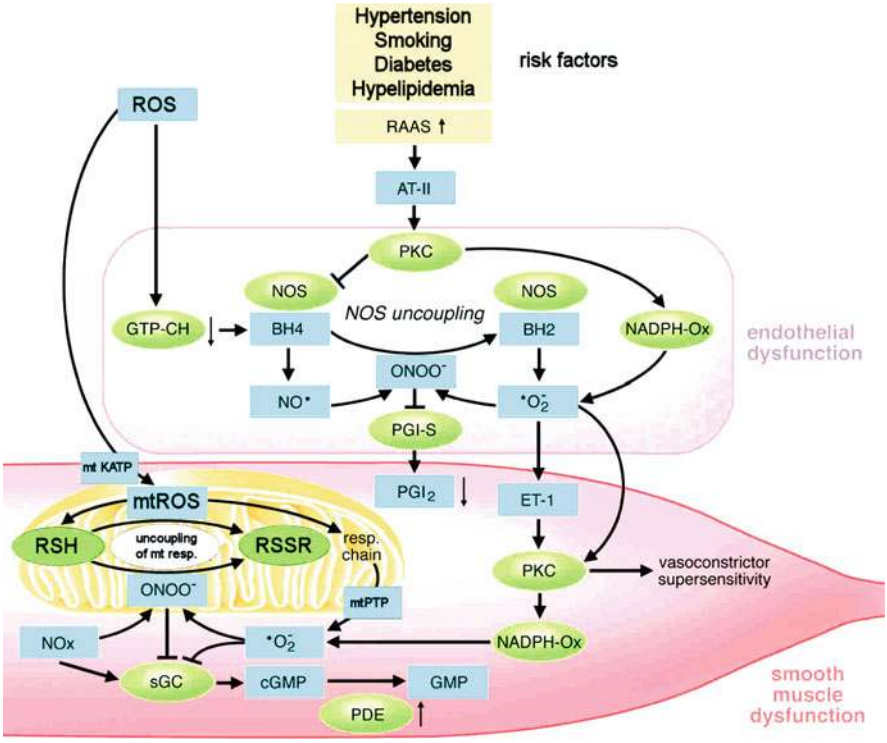

Fig. 1 Scheme illustrating the mechanisms underlying vascular (endothelial) dysfunction by oxidative stress. Known cardiovascular risk factors (e.g., smoking, hypertension, hyperlipidemia, diabetes) activate the renin–angiotensin–aldosterone system (RAAS) leading to elevated angiotensin II levels as well as increased endothelial and smooth muscle superoxide ($O_2^{-\bullet}$) formation from NADPH oxidase activation by protein kinase C (PKC) and from the mitochondria. Superoxide reacts with •NO, thereby decreasing •NO bioavailability in favor of peroxynitrite ($ONOO^-$) formation. Peroxynitrite causes uncoupling of endothelial NOS due to oxidation of tetrahydrobiopterin (BH_4) to BH_2 and nitration/inactivation of prostacyclin synthase (PGI_2S). Direct proteasome-dependent degradation of the BH_4 synthase GTP-cyclohydrolase (GTP-CH) further contributes to eNOS uncoupling. Uncoupled NOS produces superoxide instead of •NO, and nitrated PGI_2S produces no prostacyclin (PGI_2), but activated cyclooxygenase-2 (due to increased peroxide tone [92, 93]) generates vasoconstrictive prostaglandin H_2. Inhibition of smooth muscle soluble guanylyl cyclase (sGC) by superoxide and peroxynitrite contributes to vascular dysfunction as well as increased inactivation of cyclic GMP (cGMP) by phosphodiesterases (PDE), and oxidative stress increases the sensitivity to vasoconstrictors such as endothelin-1 (ET-1). Mitochondrial ROS formation is modulated by oxidative activation of ATP-dependent potassium channels (K_{ATP}) leading to altered mitochondrial membrane potential and permeability. Upon uncoupling of mitochondrial respiratory complexes, the mitochondrial permeability transition pore (mPTP) may be oxidatively opened allowing mtROS to escape to the cytosol activating the PKC-NADPH-oxidase system. Modified from Münzel et al. [94]. Reproduced with the permission of the American Heart Association

the pathogenesis of cardiovascular diseases [16, 17] is well established, and molecular mechanisms have been further elucidated in the past few years. RNOS were attributed to distinct enzymatic sources such as NADPH oxidases, mitochondria, xanthine oxidase, and uncoupled NO synthases [15] as illustrated by the following examples.

Deoxycorticosterone acetate (DOCA)-salt–induced hypertension was dramatically improved by genetic deletion of the NADPH oxidase subunit $p47^{phox}$ or by supplementation with the cofactor of NO synthase, tetrahydrobiopterin (BH_4) [22]. In both cases, improved •NO liberation was observed, suggesting that ROS derived from NADPH oxidase and uncoupled NOS play a central contributory role in the pathogenesis of hypertension and associated endothelial dysfunction. Moreover, these results indicate that repression of a major ROS source and restoration of eNOS function can normalize experimental hypertension.

In mice with myocardial infarction (MI), genetic deletion of the NADPH oxidase subunit $p47^{phox}$ almost normalized vascular •NO bioavailability [23], reduced ROS formation, improved heart function, and improved the survival rate by 20% after MI.

Furthermore, the deletion of the NADPH oxidase subunit $p47^{phox}$ and Nox1 exerted protective effects on blood pressure and endothelial function in angiotensin II–induced hypertension in mice [24, 25]. Conversely, overexpression of Nox1 raised blood pressure in transgenic mice [26]. Therefore, oxidative stress is a key contributor in the pathogenesis of cardiovascular disease and should be included in pharmacological strategies to prevent and treat cardiovascular disorders.

2.2 The Nitric Oxide/Superoxide System

Figure 1 summarizes the most important mechanisms by which reactive oxygen and nitrogen species impair vascular •NO/cGMP signaling [27]. Typical risk factors for endothelial dysfunction such as chronic smoking, hypertension, diabetes, and hypercholesterolemia lead to an induction and/or (PKC-dependent) activation of vascular NADPH oxidases. Superoxide, which is generated by the NADPH oxidases, reacts with the endothelial NO synthase–derived •NO to form peroxynitrite ($ONOO^-$) within the endothelium. This reaction per se leads to a decrease in •NO bioavailability (a depletion of •NO in favor of $ONOO^-$). Peroxynitrite is a very potent oxidant, which can react directly with sulfhydryl moieties or decay to hydroxyl (•OH) and nitrogen dioxide ($•NO_2$) radicals affecting almost all known biomolecules [28, 29]. Peroxynitrite can nitrate and inactivate prostacyclin synthase thereby removing the potent vasodilator and antiaggregatory compound prostacyclin [30, 31]. In addition, peroxynitrite oxidizes BH_4, an essential cofactor of eNOS, and thereby causes uncoupling of eNOS [22]. Uncoupled NOS produces superoxide instead of •NO (a semi-uncoupled NOS was reported to function as a peroxynitrite synthase) converting a highly protective enzyme to a harmful one. Because of this possible switch of NOS enzymes from good to evil, they are also termed “Janus-headed” enzymes. In vascular smooth muscle, the aforementioned described risk factors also lead to

an activation of NADPH oxidases, which may lead to a desensitization of soluble guanylyl cyclase (sGC) as well as increased activity of phosphodiesterases (PDEs) catalyzing the breakdown of cGMP.

Mitochondria represent an additional major source of RNOS whose role has been neglected in the pathogenesis of cardiovascular disease and may play a crucial role in aging. It is currently unknown whether mitochondrial RNOS, derived from electron leaks in the respiratory chain, are accidental or a means of active redox-signaling. However, their role in regulating apoptosis, which is triggered by opening of the mitochondrial permeability transition pore (mPTP), the release of mitochondrial proteins such as cytochrome c or apoptosis-inducing factor (AIF) into the cytoplasm, and activation of caspase-3, is evident and described elsewhere. Caspase-3 is one of the known targets of redox regulation via nitrosation and glutathiolation of the active site cysteine. The conversion of xanthine dehydrogenase into xanthine oxidase is another potent cellular source of superoxide and has been implicated in mediating oxidative stress during the reperfusion phase after ischemia. As the conversion of xanthine dehydrogenase into the oxidase form is also controlled by intramolecular disulfide formation, xanthine oxidase may play a role as an amplifier in the cellular RNOS response. Infiltrating neutrophils and monocytes are also a major source of RNOS and contribute to vascular dysfunction [6] as observed in arteriosclerosis.

2.3 Cross-Talk Between Mitochondrial and NADPH Oxidase–Generated Reactive Nitrogen and Oxygen Species

We have recently reported on the cross-talk between mitochondrial reactive oxygen species (mtROS) and cytosolic RNOS in a model of increased mitochondrial oxidative stress (nitroglycerin-induced tolerance). In this system, endothelial dysfunction (sensitive to NADPH oxidases) and vascular dysfunction (sensitive to mitochondria) were dependent on the activation of distinct oxidant sources [32]. This cross-talk was blocked by in vivo and ex vivo administration of the mitochondrial permeability pore inhibitor cyclosporin A, which selectively improved endothelial dysfunction, whereas nitrite tolerance was not affected. In contrast, the respiratory complex I inhibitor rotenone improved endothelial dysfunction and tolerance. Conversely, in vivo or ex vivo treatment with the K_{ATP} opener diazoxide caused a nitrate tolerance-like phenomenon in control animals, whereas the K_{ATP} inhibitor glibenclamide improved tolerance in nitroglycerin-treated animals. Very similar effects of rotenone (Rot), cyclosporin A (CsA), diazoxide (Diaz), and glibenclamide (Glib) have been recently demonstrated by another group in an experimental model of angiotensin II–induced hypertension [33]. A role of K_{ATP} channels for NADPH oxidase–driven activation of mitochondrial ROS formation via changes in the membrane potential was previously proposed [34]. $gp91^{phox-/-}$ and $p47^{phox-/-}$ mice developed tolerance but no endothelial dysfunction in response to nitroglycerin treatment. The findings of this study are summarized in Fig. 2. The

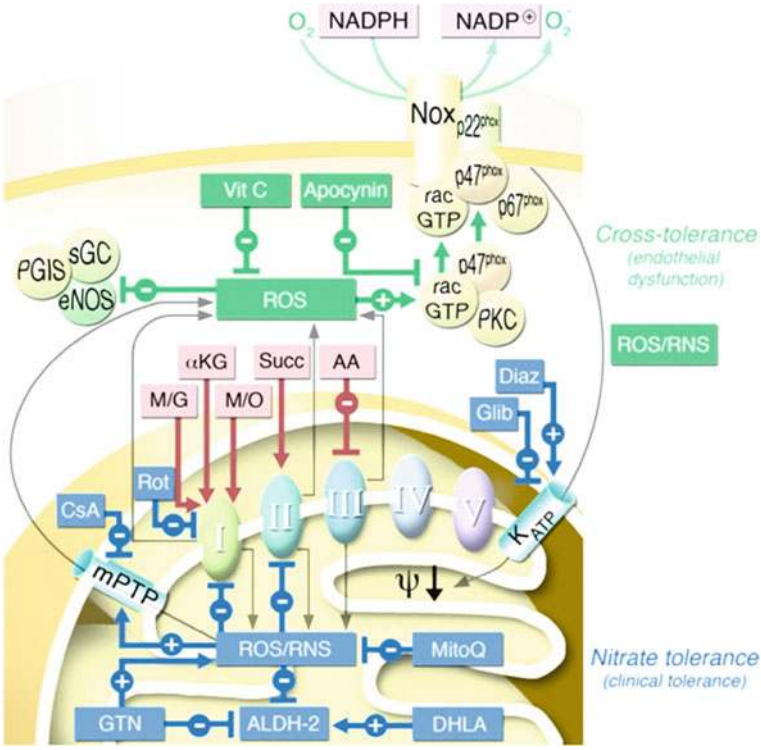

Fig. 2 Proposed hypothetical scheme of the cross-talk between mitochondrial and cytosolic (NADPH oxidase–derived) reactive oxygen and nitrogen species. CsA, cyclosporin A; Rot, rotenone; mPTP, mitochondrial permeability transition pore; Glib, glibenclamide; Diaz, diazoxide; DHLA, dihydrolipoic acid; M/G, malate/glutamate; αKG, α-ketoglutarate; M/O, malate/oxaloacetate (which decays to pyruvate); Succ, succinate; AA, antimycin A; PGIS, prostacyclin synthase; sGC, soluble guanylyl cyclase; eNOS, endothelial NO synthase; Vit C, vitamin C; PKC, protein kinase C; MitoQ, mitochondria-targeted quinone; K_{ATP}, ATP-dependent potassium channel. Adopted from Wenzel et al. [32]. Reproduction was granted by Rightslink® by Copyright Clearance Center

mechanism underlying this concept is based on mtROS-driven PKC activation, which in turn will activate NADPH oxidases. The NADPH oxidase–dependent cytosolic ROS and reactive nitrogen species (RNS) formation will then uncouple eNOS, nitrate prostacyclin synthase, and desensitize sGC. Previous experimental studies have shown that increased oxidative stress in cellular tissue per se is able to activate NADPH oxidase in a positive feedback fashion [35]. Thus, nitroglycerin-induced mitochondrial superoxide production may cause secondary activation of Nox. One may also speculate that via its hypotensive action, nitroglycerin may cause an activation of the renin–angiotensin–aldosterone system [36], leading to increased circulating levels of angiotensin II and aldosterone and therefore to an activation of the NADPH oxidase. This concept is further corroborated by the demonstration that in vivo treatment with an AT_1 receptor blocker was

able to prevent the development of nitroglycerin-induced endothelial dysfunction in an animal model of nitrate tolerance [37]. In addition, our current findings could explain why treatment with an AT_1 receptor blocker was not able to prevent the development of nitroglycerin-induced nitrate tolerance in human subjects [38, 39].

We propose that a similar cross-talk exists in the aging vasculature and that age-induced mtROS can activate cytosolic ROS/RNS sources leading to age-related vascular dysfunction [40]. This proposal is based on the finding that mtROS formation increases with age (and is higher in Mn-SOD$^{+/-}$ mice), and endothelial function is impaired with age (to a higher extent in Mn-SOD$^{+/-}$ mice).

3 Clinical Background

Pathophysiologic conditions such as hypertension, hyperlipidemia, atherosclerosis, and diabetes mellitus are associated with chronic RNOS formation, which causes oxidative damage and impairs essential redox-signaling pathways within the vasculature [41]. As a consequence, endothelial function deteriorates irreversibly (= endothelial dysfunction) [7], which is a well-known marker for cardiovascular events with high prognostic value [42, 43]. Therefore, the prognostic importance of evaluating endothelial function by diagnostic procedures such as the noninvasive flow-mediated dilation (FMD) of the brachial artery in the forearm, invasive plethysmography (acetylcholine-dependent dilation in the forearm), or coronary vasoreactivity (acetylcholine infusion into the coronaries by a catheter) [44] increases as a person ages. For example, patients with well-defined risk factors such as chronic smoking or hypercholesterolemia showed a significantly impaired acetylcholine-triggered endothelium-dependent vasodilation during plethysmography [45]. These risk factors are known to increase vascular free radical formation leading to endothelial dysfunction. Correspondingly, co-infusion of vitamin C (ascorbate) as a potent antioxidant during plethysmography significantly improved endothelial function by increasing the NO bioavailability. Furthermore, this study demonstrated that the vitamin C effects on acetylcholine-induced vasodilation may be used as a surrogate parameter reflecting vascular oxidative stress. A higher proportion of patients in the group with a pronounced vitamin C in a 4.5-year follow-up period suffered from cardiovascular events such as ischemic stroke or MI and suggests that this diagnostic procedure may serve as an independent predictor for the risk of cardiovascular events (Fig. 3b) [43].

Endothelial dysfunction of coronary arteries is directly associated with an increased risk of MI [44]. Deterioration of endothelial homeostasis in the vasculature of the heart increase susceptibility to atherosclerosis, stenosis, and occlusive events [46]. Coronary stenosis leads to angina pectoris in which the heart muscle undergoes ischemic events caused by reduced blood perfusion and oxygen supply due to obstruction or spasm of the coronary arteries [47]. The pathophysiology of

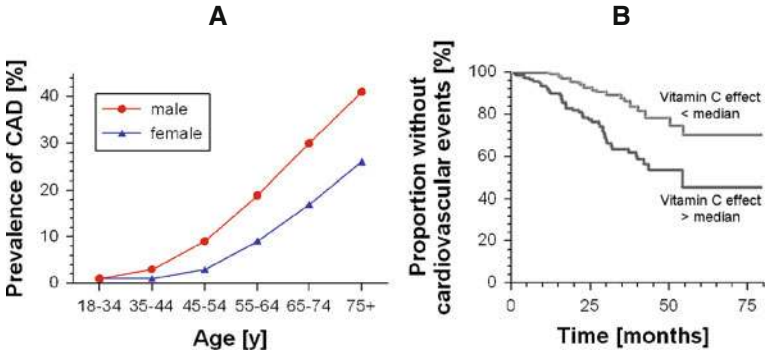

Fig. 3 (**a**) Prevalence of coronary artery diseases (CAD) in dependence on age and gender in Germany. Drawn from results of the Detect Study. (**b**) Kaplan–Meier analysis for the effect of vitamin C on endothelial function (measured by forearm plethysmography after infusion of acetylcholine) and the cardiovascular event rate. The take-home message is: Higher levels of vascular oxidative stress (free radicals) are associated with a more pronounced effect of the radical scavenger vitamin C and an increased cardiovascular event rate. Drawn from Heitzer et al. [43]

atherosclerosis differentiates stable angina from unstable angina. In stable angina, the developing atheroma is protected with a fibrous cap forming the atherosclerotic plaque. This cap may rupture in unstable angina, promoting blood clot formation that encroaches upon the lumen of the coronary vessel, which may lead to total closure and MI.

The basic principals underlying oxidative stress–induced endothelial dysfunction can also clinically be observed in patients who suffer from angina pectoris and require regular administration of nitroglycerin. These patients can develop nitroglycerin-triggered nitrate tolerance leading to serious vascular oxidative stress and dysfunction characterized by reduced bioavailable •NO and likely inactivation of prostacyclin synthase and mitochondrial aldehyde dehyrdogenase-2 [a mitochondrial enzyme that is involved in the detoxification of reactive aldehydes such as 4-hydroxynonenal (HNE) and mediates the biological activation of organic nitrates].

These examples illustrate that endothelial dysfunction has been identified as a characteristic of cardiovascular disease [7] and is always associated with vascular oxidative stress, decreased •NO bioavailability, and/or impaired activity (uncoupling) of endothelial NO synthase [20, 48]. As shown in Fig. 3a, the risk and frequency of cardiovascular diseases such as hypertension, diabetes, and atherosclerosis increases with age [49]. In parallel, oxidative stress from mitochondria and other enzymatic sources as well as endothelial dysfunction manifest in aged tissues [50]. This suggests a strong correlation between aging, oxidative stress, and deterioration of endothelial function. Therefore, age-induced oxidative stress originating from mitochondria may gain clinical importance and serve as a valuable diagnostic tool. Mitochondria-derived ROS may cause endothelial dysfunction, which

promotes the development of cardiovascular diseases such as hypertension and atherosclerosis. The presence of additional risk factors such as smoking, obesity, and diabetes may significantly accelerate this process.

4 Aging

4.1 Aging and Oxidative Stress

In 1954, Harman expressed for the first time the free radical theory of aging: "the reaction of active free radicals, normally produced in the organism, with cellular constituents" [51]. The participation of ROS in cardiovascular disorders and aging is well-documented. This hypothesis was extended to mitochondria as the most abundant cellular source of ROS and the resulting increase in the mutation rate of the mitochondrial genome. In general, the biogenesis of respiratory chain components requires concerted contributions from two physically separated genomes: the nuclear DNA and the maternally inherited mtDNA. Mutations of the mitochondrial genome are assumed to impair mitochondrial physiology and ATP synthesis, which is accompanied by enhanced ROS formation and increased apoptosis [52]. Age-dependent interference in vascular redox regulation is best demonstrated by •NO bioavailability in various rodent models [53] and in humans [54, 55]. With age, •NO is gradually reduced and therefore serves as a useful biomarker for age-dependent endothelial dysfunction. The prevailing hypothesis is that an age-dependent increase in $O_2^{-\bullet}$ reacts with •NO and consequently reduces endothelium-derived free •NO, thus impairing vasorelaxation.

4.2 Aging and Mitochondrial DNA

Mitochondria represent the most potent source of ROS, caused by electron leakage in the respiratory chain that results in univalent reduction of oxygen into $O_2^{-\bullet}$. The free radical and mitochondrial hypothesis of aging suggests that ROS over an organism's life span lead to accumulation of altered biological macromolecules such as DNA mutations. In particular, mitochondrial DNA (mtDNA) is regarded as a highly susceptible target for oxidant-induced mutations and deletions, which cause progressive deterioration of mitochondrial function over time. mtDNA deterioration takes part in a vicious cycle in which mitochondrial dysfunction further increases oxidative stress resulting in loss of cell function and finally cell death. One of the major oxidative alterations of mtDNA is 8-oxodeoxyguanosine (8-oxodG) [56, 57]. 8-OxodG is highly mutagenic, and it seems that there appears to be a direct correlation between 8-oxodG accumulation and pathologic processes [58]. The correlation of life span and oxidant-induced mtDNA damage was demonstrated for the first time by Barja and co-workers [19] who showed that in short-lived organisms, 8-oxodG content was increased.

This hypothesis was further substantiated in transgenic mice with a proofreading-deficient mitochondrial polymerase γ. These mice accumulated severe mtDNA mutations, leading to mitochondrial dysfunction, increase in apoptosis, and premature aging [59, 60]. A recent study showed that a transgenic mouse expressing cardiac-targeted mutated human Polγ [61] developed early aging symptoms combined with enhanced ROS formation and severe cardiomyopathy similar to observations in the "mtDNA-mutator mouse." Several maternally inherited human diseases [62], for example, DAD syndrome (Leu-UUR tRNA = diabetes mellitus and deafness), MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like syndrome) [63–65], or KSS (Kearns–Sayre syndrome) [63, 65], stress the importance of mtDNA. Point mutations or deletions, particularly in tissues with high oxygen and energy demand such as the myocardium, increase the rate of apoptosis, free radical formation, and energy depletion, leading to impairment of tissue function [63, 66]. Therefore, mutated mtDNA is commonly regarded as a major contributor to vascular aging and various cardiovascular disorders [63, 66].

4.3 Aging, Mitochondrial Oxidative Stress, and Endothelial Dysfunction

In a recent study, we provided evidence that mitochondrial oxidative stress increases with age and contributes to age-related vascular dysfunction [50]. Importantly, two knockout mouse models with increased mitochondrial oxidative stress (ALDH-2$^{-/-}$ and Mn-SOD$^{+/-}$ mice) demonstrated that mitochondrial ROS generation and oxidative mtDNA lesions are an important determinant for age-associated vascular dysfunction. Moreover, mitochondrial ALDH-2 exerts crucial vasoprotective effects and reduces age-dependent dysfunction, as middle-aged ALDH-2$^{-/-}$ mice display premature age-dependent endothelial dysfunction. Indeed, we could establish a correlation between mitochondrial ROS formation and endothelial function (as measured by acetylcholine-dependent relaxation of isolated murine aortic ring segments by isometric tension recording in organ chambers) clearly indicating that increased oxidative stress impairs endothelial function and that this process is more pronounced with age as well as in mice lacking essential antioxidative proteins (Fig. 4a). This also correlated with mtROS and mtDNA strand breaks (Fig. 4b). Regarding the fact that 70% of the mtDNA encodes for enzymes of the respiratory chain complexes [67], one may assume that chronic cumulative oxidative damage to mtDNA will ultimately lead to dysfunction of mitochondrial respiration and likely further aggravation of mitochondrial uncoupling. This will finally result in a vicious cycle of ROS-triggered ROS formation and a mitochondrial oxidative burst that may directly hit cytosolic structures upon opening of the mitochondrial permeability transition pore. Besides age-induced endothelial dysfunction, mtROS formation, and mtDNA strand breaks, there was also increased protein tyrosine nitration as well as decreased levels of reduced peroxiredoxin-3, the mitochondrial isoform of this antioxidant protein (Fig. 5).

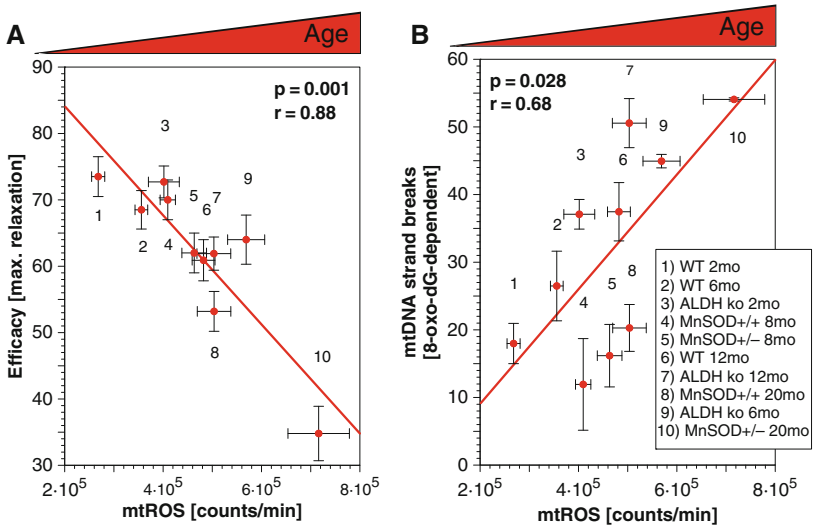

Fig. 4 Correlations between mitochondrial oxidative stress (mtROS), mitochondrial DNA (mtDNA) damage, and vascular (endothelial) function (ACh-induced maximal relaxation). (**a**) mtROS formation was plotted for all age groups and mouse strains versus the corresponding maximal efficacy in response to acetylcholine (ACh). (**b**) mtROS was plotted for all age groups and mouse strains versus the corresponding mtDNA damage. r is the correlation coefficient. The groups are as follows: 1 = B6 WT, 2 months; 2 = B6 WT, 6 months; 3 = ALDH-2$^{-/-}$, 2 months; 4 = Mn-SOD$^{+/+}$, 7 months; 5 = Mn-SOD$^{+/-}$, 7 months; 6 = WT B6, 12 months; 7 = ALDH-2$^{-/-}$, 12 months; 8 = Mn-SOD$^{+/+}$, 16 months; 9 = ALDH-2$^{-/-}$, 6 months; 10 = Mn-SOD$^{+/-}$, 16 months. Adopted from Wenzel et al. [50]. Reproduced with the permission of the Oxford University Press

4.4 Futile Counterregulation by Enhanced Gene Expression of Oxidant Defense Mechanisms

Expression of selected genes involved in antioxidant defense such as SOD-2, glutathione peroxidase-1 (GPX-1), heme oxygenase-1 (HO-1), and mitochondrial ferritin were assessed with quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR). Cellular $O_2^{-\bullet}$ is a normal by-product of the mitochondrial electron transport chain that increases with age. $O_2^{-\bullet}$ dismutates to the relatively stable hydrogen peroxide (H_2O_2), but via the Fenton reaction, which is catalyzed by transition metals such as iron, H_2O_2 can produce the highly reactive hydroxyl radical (•OH). Sequestering intracellular and mitochondrial iron by ferritin and maintaining low H_2O_2 levels by glutathione peroxidase prevents this reaction. The mRNA expression profile of these antioxidant enzymes significantly differed between B6 WT and ALDH-2$^{-/-}$ mice (Fig. 6). The B6 WT mice showed no age-related alteration in gene expression of Mn-SOD (SOD-2) or GPX-1, but a significant increase in HO-1 expression was observed in the aged animals (Fig. 6a). In contrast, ALDH-2$^{-/-}$ mice demonstrated an age-dependent increase in Mn-SOD and

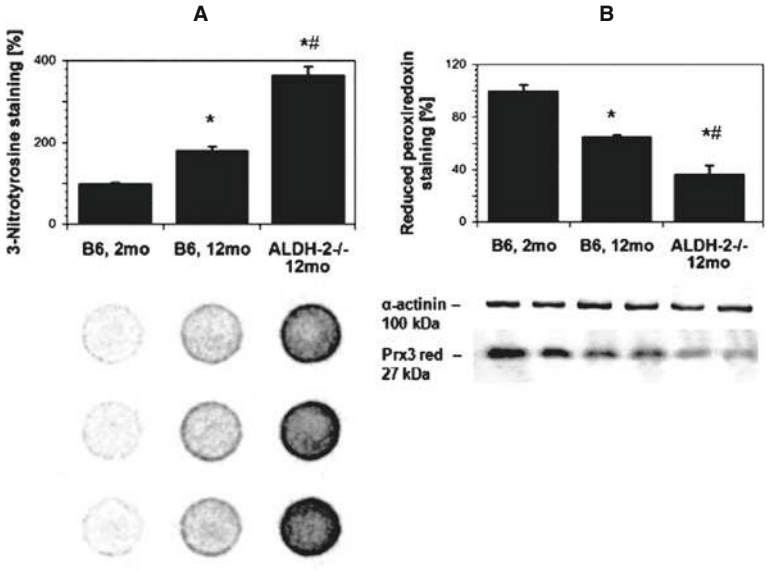

Fig. 5 (**a**) 3-Nitrotyrosine staining in cardiac tissue from young and old C57/Bl6 wild-type mice as well as old ALDH-2 knockout mice. Cardiac homogenates were transferred to the membrane and stained by the dot-blot technique using a 3-nitrotyrosine specific antibody. The original blots show stainings from three animals/group. Data are mean ± SEM: $^{*}p < 0.05$ versus B6, 2 months; $^{\#}p < 0.05$ versus B6, 12 months. (**b**) Peroxyredoxin-3 monomer staining (Prx-3, reduced form) in aortic tissue from young and old C57/Bl6 wild-type mice as well as old ALDH-2 knockout mice. Aortic homogenates were transferred to the membrane and stained by the dot-blot technique using a Prx-3 specific antibody. The original blots show stainings for samples pooled from three animals/group. Data are mean ± SEM: $^{*}p < 0.05$ versus B6, 2 months; $^{\#}p < 0.05$ versus B6, 12 months. Adopted from Wenzel et al. [50]. Reproduced with the permission of the Oxford University Press

HO-1 mRNA but a significant decrease in GPX-1 expression (Fig. 6b). The HO-1 profile in the ALDH-$2^{-/-}$ groups of different age may reflect severe endothelial dysfunction in the middle-aged group (see also Fig. 4a) and the trend for compensation in the old group with HO-1 being a marker of oxidative stress and vascular dysfunction. Even more pronounced were the differences for mitochondrial ferritin, which increased with age in the B6 WT groups but decreased in middle-aged ALDH-$2^{-/-}$ mice and increased in aged animals (Fig. 6c). Finally, RT-PCR detection of mRNA revealed approximately 50% deficiency in Mn-SOD mRNA in the Mn-$SOD^{+/-}$ mice and an increase in ALDH-2 mRNA compared with that in young B6 WT mice (Fig. 6d). Probably such counterregulatory mechanisms partially compensate for the decreased plasma and tissue levels of vitamin C and vitamin A in a rat model of aging [68, 69]. Vitamin E increase may be another rescue mechanism to prevent cardiovascular aging [70].

Studies by van der Loo et al. have shown that expression of SOD-1 decreased in an age-dependent manner [71]. Interestingly, SOD-1 loses its membrane association

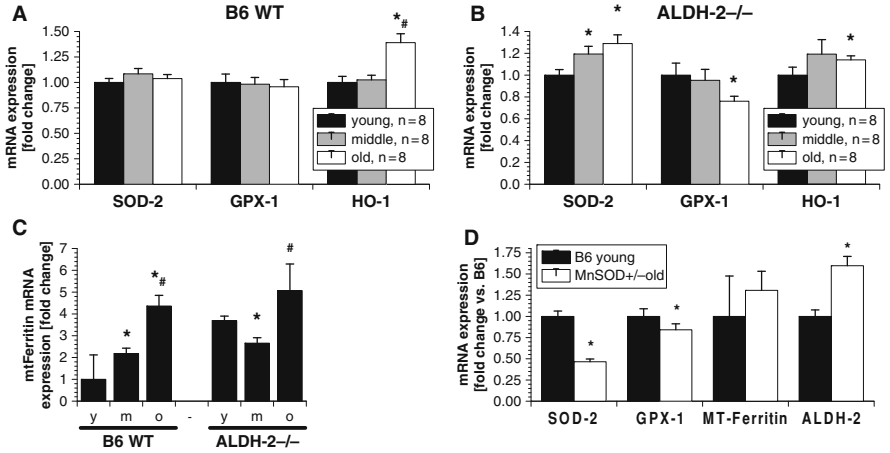

Fig. 6 Effects of aging in ALDH-2 or Mn-SOD deficient mice on gene expression of antioxidant enzymes in the myocardial mRNA expression of antioxidant enzymes in (**a**) B6 WT, (**b**) ALDH-$2^{-/-}$, (**c**) B6 WT versus ALDH-$2^{-/-}$, and (**d**) B6 WT versus Mn-$SOD^{+/-}$ mice. Data are mean ± SEM of 8 (**a** and **b**), 6–8 (**c**), or 5–8 (**d**) murine samples. $^{*}p < 0.05$ versus young group and $^{\#}p < 0.05$ versus middle-aged group

and is also detached from the caveolae with increasing age. Instead, a relocation of SOD-1 to the mitochondria occurs, presumably in an attempt to maintain mitochondrial integrity and to counterbalance age-associated oxidative stress. Unlike SOD-2, which is constitutively expressed in mitochondria controlling $O_2^{-\bullet}$ radical fluxes, SOD-1 is not inactivated by peroxynitrite and is not nitrated as a function of age. These novel insights into oxidative stress–associated vascular aging and the understanding about how redox systems are regulated in old age may identify new targets to ameliorate aging as the greatest cardiovascular risk factor.

Substantial evidence was acquired over the past 35 years that supports the concept of oxidant-induced damage to the mitochondrial respiratory chain and mtDNA as a determinant of mammalian life span. A recent review by Jang and Remmen [72] summarized recent studies using transgenic and knockout mouse models with altered expression of mitochondrial antioxidant enzymes such as Mn-SOD (SOD-2*Tg* and SOD-$2^{+/-}$), thioredoxin-2 (Trx-$2^{+/-}$), and mitochondrial targeted catalase (mCAT) or mice expressing a proofreading-deficient mitochondrial DNA polymerase, Polγ(D257A/D257A). The majority of these studies were inconclusive regarding the role for mitochondrial oxidative stress or a vicious cycle of oxidative damage in determining life span of mice or supporting the free radical theory of aging. However, several key questions remain to be addressed, and clearly more studies are required to fully understand the role of mitochondria in age-related disease and aging.

5 Recent Developments in Aging Concepts

In a very recent article, Moosmann and co-workers have shown that intramembrane accumulation of methionine exhibits antioxidant and cytoprotective properties in living cells [73]. Their results uncloak methionine as an evolutionarily selected antioxidant building block of respiratory chain complexes. Moreover, they provide evidence that methionine is an important antioxidant, and redox cycling between oxidized and reduced forms is a vital antioxidant defense system. These authors conclude that oxidative stress has shaped the mitochondrial genetic code. This concept is well in line with previous observations indicating that knockout mice for methionine sulfoxide reductase suffer from a decreased life span and several additional pathologies [74, 75]. In another report, Moosmann and Behl provided data from a meta-examination of genome sequences from 248 animal species with known maximum life span, including mammals, birds, fish, insects, and helminths [76]. This analysis indicated that the frequency with which cysteine is encoded by mitochondrial DNA is a specific and phylogenetically ubiquitous molecular indicator of aerobic longevity: long-lived species synthesize respiratory chain complexes that are depleted of cysteine. These results provide distinct support for the free radical theory of aging.

Another hypothesis for age-related deterioration is based on an important role of the mitochondrial enzyme p66Shc as an adaptor protein that plays an important role as a redox enzyme implicated in mitochondrial ROS generation and translation of oxidative signals into apoptosis [77]. Mice lacking p66Shc$^{-/-}$ gene display reduced production of intracellular oxidants and a 30% prolonged life span. For this reason, a series of studies conceived to elucidate the function of p66Shc and its possible implication in age-associated cardiovascular diseases has been carried out. Indeed, p66Shc$^{-/-}$ mice have been shown to be protected from age-dependent endothelial dysfunction [78] as well as age-related risk factors such as diabetes and hypercholesterolemia. The review of Camici et al. focused on delineating the role of the p66Shc adaptor protein and its potential implication in the pathophysiology of aging and age-related cardiovascular disease. Even more exiting was the finding that that the p66Shc N-terminus forms a redox module responsible for apoptosis initiation, and that this module can be activated through reversible tetramerization by forming two disulfide bonds [79]. Glutathione and thioredoxins can reduce and inactivate p66(Shc), resulting in a thiol-based redox sensor system that initiates apoptosis once cellular protection systems cannot cope anymore with cellular stress. But also, protein kinase C beta and prolyl isomerase-1 regulate mitochondrial effects of the life-span determinant p66Shc [80]. The specific signaling route leading to mitochondrial import of p66Shc, which accounts for its proapoptotic activity upon oxidative stress, was reviewed [81]. Recently, a study was published that claims to have revealed the final common molecular pathways of aging and cardiovascular disease with a key role of p66Shc [82]. But also, benefits for diabetic cardiovascular complications [83] and age-related endothelial •NO formation [84] were reported for genetic deletion of p66Shc. Finally, it was demonstrated that a p53-p66Shc signaling pathway controls intracellular redox status and accounts

for increased levels of oxidation-damaged DNA [85] and mitochondrial oxidative stress [86].

Finally, the mitochondrial and cytosolic thioredoxin system plays an important role in the aging muscle [87] and the endothelium [88]. Both genes have also been demonstrated to be essential for viability of *Drosophila* flies [89]. The thioredoxin system is closely linked to the telomerase reverse transcriptase, another important player in the progression of senescence [90].

6 Perspective

In the current chapter, we have provided strong evidence from our own and others' studies that mitochondrial oxidative stress is key determinant for age-induced perturbance of cellular signaling and, as a consequence, cell death. There is a large body of evidence for the association of cellular aging with mitochondrial dysfunction based on genetic animal models with increased mitochondrial ROS formation (e.g. Mn-SOD or Trx-2 deficiency). Interestingly, overexpression of catalase, another enzyme crucially involved in the antioxidative defense, enhanced protection of mitochondria from ROS and led to an extended life span in mice [91]. The increase in life span was much more pronounced when catalase was targeted to mitochondria compared with overexpression in peroxisomes or in the nucleus. There is also evidence for an interaction (cross-talk) between mitochondrial oxidative stress and cytosolic sources of oxidative stress providing a direct link between aging and vascular dysfunction. Therefore, a better understanding of the role of mitochondria in the aging process may lead to specifically designed therapies to interfere with mitochondrial dysfunction and to delay the aging process for longevity. As regulation of the vascular tone largely depends on a redox balance in favor of a more reductive milieu, increased oxidative stress impairs vascular function and leads to endothelial dysfunction, atherosclerosis, and other cardiovascular complications. Therefore, therapeutic intervention at the level of mitochondrial dysfunction would not only beneficially influence the aging process but also most kinds of cardiovascular diseases. As cardiovascular diseases are the main reason for mortality in the Western world and as the prevalence of these increases with age, development of therapeutic interventions not only promises a cure for a large part of the world population but also represents a large pharmaceutical market.

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Aging, Oxidative Stress, and Cardiovascular Disorders

Yi Shi, Giovanni G. Camici, and Thomas F. Lüscher

Abstract Aging is a progressive decay of the physiologic efficiency of an organism as well as a major risk factor for the development of cardiovascular disease. Due to constant birth rates and increasing life expectancy, the proportion of elderly people is steadily growing in most developed countries, thus the need to better understand aging and its molecular basis is becoming a central issue. Accumulation of oxidative stress with age is hypothesized to be the primary causative mediator of age-associated diseases. Among different tissues, aging vessels are known to accumulate oxidative damage and undergo functional impairment. Oxidative stress affects the availability and/or balance of key regulators of vascular homeostasis and favors the development of cardiovascular disease. Reactive oxygen species are generated by different intracellular molecular pathways principally located in the cytoplasm and in the mitochondria. The mitochondrial protein $p66^{Shc}$ and the deacetylase enzyme Sirt1 were shown to be involved in different aspects of aging and age-dependent disease. This chapter focuses on the latest scientific advances in understanding aging and age-related disease and delineates the possible therapeutic implications of $p66^{Shc}$ and Sirt 1 in this process.

Keywords Aging · Free radical · $p66^{Shc}$ · Sirt-1

1 Background

Aging is a physiologic process as well as a major risk factor for the development of cardiovascular disease. Due to increasing life expectancy, the proportion of elderly people is steadily growing in most developed countries, thus the need

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to better understand aging and its molecular mechanisms is becoming a central issue [1]. With aging, some physiologic functions are altered and resemble those occurring in disease conditions such as hypertension, chronic coronary disease, and diabetes [2, 3]. Moreover, cardiovascular disease increases in frequency with aging even in the absence of established risk factors, suggesting that aging per se is an independent risk factor for the development of cardiovascular disorders such as atherosclerosis and hypertension and their complications [4, 5]. Therefore, understanding the mechanisms of aging and age-related cardiovascular disorders emerges as an important scientific goal for this century and is critical for the development of new therapies aimed at improving quality of life of the elderly and prolonging life span.

2 Free Radical Theory of Aging

The free radical theory of aging was first proposed by Dr. Harman in the 1950s [6]. Reactive oxygen species (ROS) are generated as part of normal cellular metabolism, but at supraphysiologic levels they can cause DNA mutation, protein oxidation, and lipid peroxidation (Fig. 1; [7–9]). Mitochondria are the main source of oxygen-derived free radicals. Furthermore, lifelong exposure to active and abundant ROS in turn induces mitochondrial damage and enhanced ROS production, which ultimately and negatively affects life span [6, 10, 11]. In early studies of the free radical theory, antioxidant treatments indeed increased both average and maximum life span of *Drosophila* [12, 13].

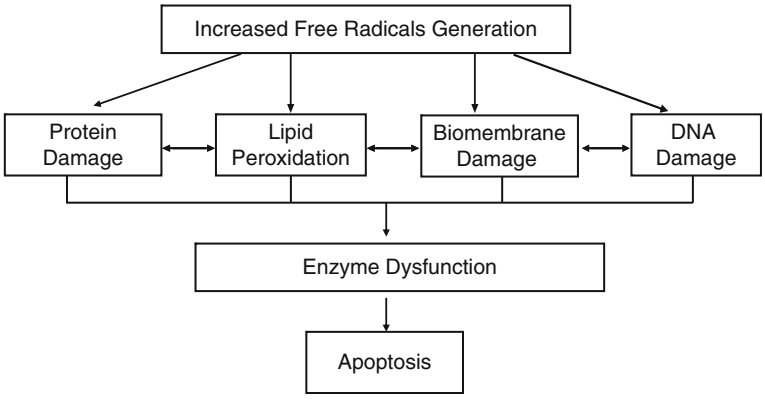

Fig. 1 Increased production of free radicals such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and peroxynitrite ($ONOO^-$) cause oxidation of proteins, lipids, biomembranes, as well as DNA damage. Substrate oxidation causes enzymatic dysfunction and ultimately leads to apoptosis

3 Imbalance Between Oxygen-Derived Free Radicals and Antioxidative Defense in Aging

Oxygen-derived free radical species originate from molecular oxygen. Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and peroxynitrite ($ONOO^-$) fall into this group. The special feature of these species is their high oxidative reactivity creating a significant threat for redox-sensitive components such as primary proteins, lipids, and nucleic acids. O_2^- is the primary oxygen-derived free radical. It has a short half-life and is an intracellular-restrained molecule. O_2^- is dimutased to H_2O_2 either spontaneously or by superoxide dismutase. Unlike O_2^-, H_2O_2 is more stable and diffuses freely within tissues as it is not charged. The metal-catalyzed interaction between H_2O_2 and O_2^- produces OH^-.

The burden of free radical production is largely counteracted by the antioxidant defense system, including the enzymatic scavenger superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). SOD mediates the conversion of O_2^- to H_2O_2, whereas catalase and GPx convert H_2O_2 to water. A variety of other nonenzymatic, low-molecular-mass molecules are important in scavenging ROS. These include ascorbate, pyruvate, flavonoids, carotenoids, and, perhaps most importantly, glutathione, which is present in millimolar concentrations within the cell.

A large body of evidence indicates that aging increases the production of ROS in several organisms including humans [14–16]. ROS are associated with upregulated expression of induced nitric oxide synthase (iNOS) [17] and NAD(P)H oxidase [18] and/or downregulation of antioxidants, such as extracellular SOD (ecSOD) [19], and decreased catalase activity [20]. Therefore, with aging there is a global shift toward an enhanced generation of ROS caused by an increased activity of prooxidant species and a concomitant decrease in the activity of antioxidant defense systems.

4 Age-Induced Oxidative Stress and Vascular Dysfunction

The increased production of oxygen-derived free radicals is an important characteristic of aging [14–16].

With advanced age, blunted endothelium-dependent relaxations are consistently observed in different species including human (Fig. 2; [21–24]) and are associated with a reduced nitric oxide bioavailability [25] and/or enhanced endothelium-dependent contractions [20, 26, 27]. O_2^- inactivates the production of nitric oxide in a few seconds [28], leading to the impairment of vasodilation, while the activity of the endothelial isoform of nitric oxide synthase is either declined [17] or preserved [20].

Moreover, O_2^- per se has been proposed as an endothelium-derived contracting factor, as SOD prevents the endothelium-dependent contraction in canine basilar artery [29].

Uncoupled eNOS is an important source of ROS under pathologic conditions [30, 31]. The production of $ONOO^-$ results from the combination of nitric oxide

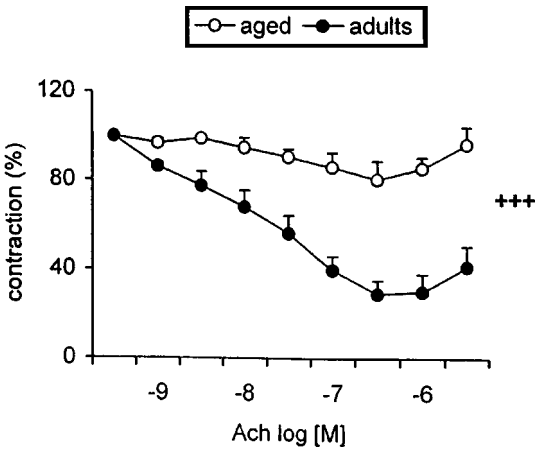

Fig. 2 Influence of aging on acetylcholine (Ach)-induced relaxation in mesenteric artery of aged and adult rats [24]

and O_2^- [32–34]; $ONOO^-$ is a strong cytotoxic oxidant, as it easily penetrates across the phospholipid membrane and produces substrate nitration, thereby inactivating important regulatory receptors and enzymes [14, 35]. The inactivation of manganese superoxide dismutase (Mn-SOD) by peroxynitrition leads to increased levels of superoxide and ultimately mitochondrial dysfunction and cell death [36, 37]. Moreover, prostacyclin-dependent vasodilation is replaced by prostaglandin-dependent vasoconstriction when prostacyclin synthase is isolated from arteries and is inactivated by peroxynitrition [25].

In addition, the increased production of ROS with aging is also implicated in proinflammatory processes by directly acting as second messengers. For instance, NF-κB is a redox-sensitive transcription factor, expressed by both endothelial and smooth muscle cells, and can be activated by ROS [38]. Activation of NF-κB by increased ROS levels promotes the transcription of several genes implicated in inflammation and thus critical for atherogenesis; such genes include cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6), chemokines, and adhesion molecules. In line with the above, it is generally believed that chronic activation of NF-κB predisposes arteries to atherosclerosis [38].

5 Aging-Related Genes: $p66^{Shc}$

The mitochondrial adaptor protein $p66^{Shc}$ is an important aging-related protein and is currently considered as a potential therapeutic target for reducing age-dependent cardiovascular disease (Fig. 3; [39]). Genetic ablation of $p66^{Shc}$ in the mouse was shown to reduce production of intracellular oxidants and consequently prolong life span by 30% [40]. The mammalian Shc locus encodes for three different adaptor proteins with respective molecular masses of 46, 52, and 66 kDa. The three isoforms share a Src-homology 2 domain, a collagen-homology region, and

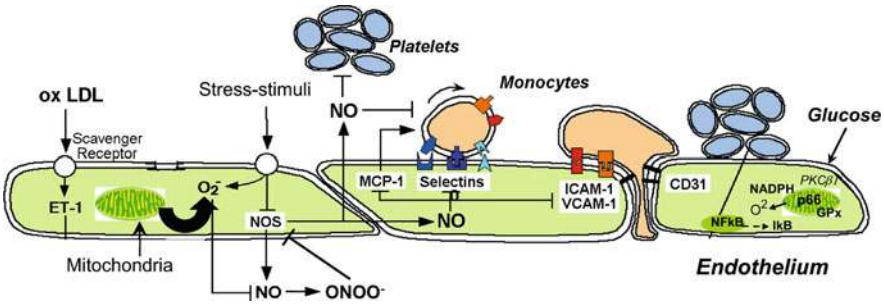

Fig. 3 Schematic representation of the endothelium and some of its key derived vasoactive substances. The $p66^{Shc}$ is principally located in the mitochondria, where it contributes to the production of superoxide anion (O_2^-) radicals, which scavenge NO to form peroxynitrite ($ONOO^-$). Different stress stimuli (e.g., glucose) increase in number and occurrence with age and enhance the production of O_2^-, thus exacerbating endothelial dysfunction. oxLDL, oxidized low-density lipoprotein; ET1, endothelin 1; NOS, NO synthase, ONOO, peroxynitrite; MCP-1, monocyte chemotactic protein-1; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; NF-κB, nuclear factor kappa B; IκB, inhibitor to NF-κB; NADPH, nicotinamide adenine dinucleotide phosphate oxidase; PKC1, protein kinase C-1; GPx, glutathione peroxidase [39]

a phosphotyrosine-binding domain. However, the splice variant $p66^{Shc}$, which contains a unique N-terminal region, is the only isoform to play an important role as a redox enzyme implicated in mitochondrial ROS generation and translation of oxidative signals into apoptosis [41]. $p66^{Shc}$ has been proposed to regulate ROS production by controlling the partition of ATP generation in the cell and by participating to the electron flow chain in the mitochondria [42]. In fact, in the absence of $p66^{Shc}$, mitochondrial oxidative phosphorylation is reduced in favor of glycolysis. As mitochondrial electron flow, besides being the major source of ATP, also represents the major producer of cellular ROS, this may provide an explanation for the decreased production of ROS observed in $p66^{Shc-/-}$ cells and organisms [42]. In light of its pivotal role in ROS generation, many efforts have been made to investigate the biochemical and pathophysiologic role of $p66^{Shc}$ in age-dependent and ROS-mediated cardiovascular disease.

6 $p66^{Shc}$: Role in Aging and Age-Related Cardiovascular Diseases

In view of its role in determining the redox state of cells and their responses to free radicals [40], $p66^{Shc}$ adaptor protein has been regarded as a key player involved in mediating age-dependent loss of endothelial integrity. In line with this hypothesis, $p66^{Shc}$ mice are protected from age-dependent endothelial dysfunction (Fig. 4; [43]). Wild-type mice display age-dependent loss of acetylcholine-induced, NO-mediated vasorelaxation, whereas $p66^{Shc-/-}$ mice do not [43]. In line with this, aged $p66^{Shc-/-}$ mice, unlike age-matched wild types, show increased endothelial

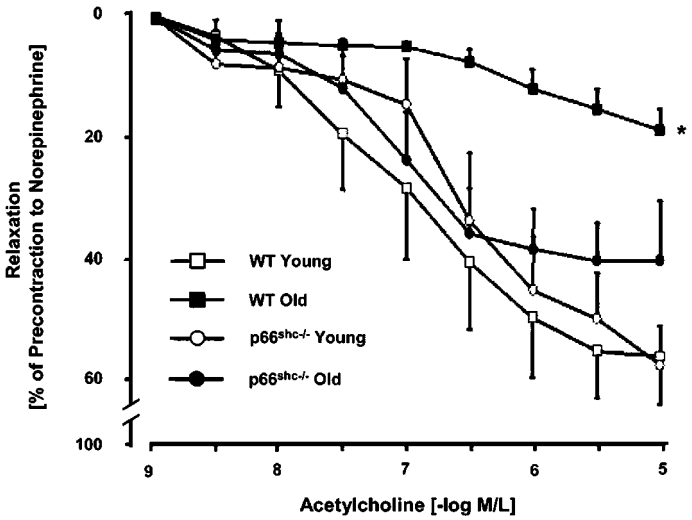

Fig. 4 Age-dependent changes in endothelium-dependent relaxation of WT and $p66^{Shc-/-}$ aortas. *Line graphs* show concentration–response curves to acetylcholine [43]

bioavailability of NO, lower aortic O_2^- levels, and reduced aortic 3-nitrotyrosine content [43], thus suggesting a potential mechanism by which NO availability and vasorelaxant responses are preserved in aged $p66^{Shc-/-}$ mice. Based on these findings, one could also speculate that this is one of the mechanisms involved in the extended life span observed in $p66^{Shc-/-}$ mice.

Increased ROS production not only determines a steady loss of NO bioavailability but also damages endothelial cells throughout the lifetime. In normal conditions, a balance between ROS damage and endothelial progenitor cell (EPC)-mediated repair exists [44] and guarantees endothelial integrity. This balance becomes disturbed with age due to an increased production of ROS, which in turn causes (a) additional cellular damage by protein oxidation and nitration and (b) decreased EPC function eventually leading to an impaired repair system and organ dysfunction [45, 46]. In keeping with the theory, aged mice display enhanced endothelial mitochondrial ROS compared with young mice. The increased ROS production observed in aged mice could be the explanation for (a) the decreased endothelial cell function and (b) the decreased EPC-mediated repair ultimately leading to vascular dysfunction. These changes, however, are not observed in age-matched $p66^{Shc-/-}$ mice, which present lower levels of ROS and a preserved endothelial function [43].

The expression pattern of $p66^{Shc}$ expression in aged humans was recently investigated [47]. $p66^{Shc}$ protein and messenger RNA levels were assessed in young people, elderly, and centenarians. In this study, the expression of $p66^{Shc}$ was shown to increase with age [47], suggesting that the $p66^{Shc}$ protein concurs with the process of aging. However, it is rather difficult to extrapolate the real role of $p66^{Shc}$ in aged people, as this study was performed as a one-point evaluation, rather than

a long-term, age-matched follow-up. Similar Shc genomic organization, transcript assembly, as well as a high degree of amino acid identity have been observed in mice and in humans [48], therefore $p66^{Shc}$-knockout mice are a promising tool to investigate the role of $p66^{Shc}$ in aging and to observe an adaptive response to age-dependent cellular damage in vascular tissue.

The initial theory that $p66^{Shc}$ may sit at the crossroads between ROS production and arterial dysfunction drove one of the earliest investigations meant for elucidating its role in arterial atherogenesis [49]. Wild-type and $p66^{Shc-/-}$ mice were fed a normocholesterolemic diet or a high-fat diet, and then systemic versus vascular levels of oxidative stress, as well as the extent of atherogenesis, were investigated. In this experimental setting, much enhanced early aortic lesion formation was found in wild-type mice fed a high-fat diet compared with $p66^{Shc-/-}$ mice. In addition, $p66^{Shc-/-}$ mice exhibited a decrease in intimal foam cell formation, arterial oxidized low-density lipoproteins (LDLs), as well as systemic plasma isoprostanes [49]. A later report confirmed a tight correlation between plasma levels of LDL-cholesterol, early markers of arterial dysfunction, and $p66^{Shc}$ in pacemaker-implanted patients [50], thus underscoring that $p66^{Shc}$ plays a role in translating the effects of different age-related risk factors into arterial dysfunction and early atherosclerotic lesions.

Abnormal glucose metabolism predominately affects older individuals; indeed, 35% of the aged population presents to some degree abnormal glucose tolerance and shows signs of insulin resistance [51, 52]. Hyperglycemic states frequently encountered in such conditions are thought to play a central role in generation of ROS leading to arterial endothelial dysfunction and later to atherosclerosis [53, 54]. Indeed, high levels of glucose induce a cascade of cellular events that increase the production of free radicals thus decreasing NO bioavailability and eventually leading to vascular dysfunction [55, 56]. In conditions of raised glucose plasma levels, $p66^{Shc}$ is known to oxidize cytochrome c and in turn to generate proapoptotic ROS through a PKC-β–dependent pathway [42, 57]. In line with this concept, peripheral blood monocytes from patients with diabetes mellitus display increased $p66^{Shc}$ mRNA expression compared with that in healthy subjects [58]. The putative role of $p66^{Shc}$ in hyperglycemia-induced, ROS-mediated vascular dysfunction was investigated further by separate studies. Likewise, $p66^{Shc}$ protein expression is increased in aortas in type 1 diabetic mice [57], and $p66^{Shc-/-}$ mice have comparable glucose level to that of control mice [57, 59], indicating that knocking out $p66^{Shc}$ protein does not alter glycemic levels. Additionally, $p66^{Shc-/-}$ mice are protected from diabetic glomerulopathy, a leading cause of chronic renal failure (Fig. 5; [60]). Indeed, $p66^{Shc-/-}$ mice did not show high glucose-induced, ROS-dependent increase in glomerular cell apoptosis and extracellular matrix deposition, thus indicating the pivotal role of $p66^{Shc}$ in translating ROS-mediated insults into apoptosis. Furthermore, in the aortas of streptozotocin-treated diabetic mice, impaired endothelium-dependent relaxation was restored in the $p66^{Shc-/-}$ hyperglycemic group due to an unaltered ROS production resulting in a preserved NO bioavailability, thus underlining once more a causal relationship between high glucose, ROS, $p66^{Shc}$, and vascular dysfunction [57].

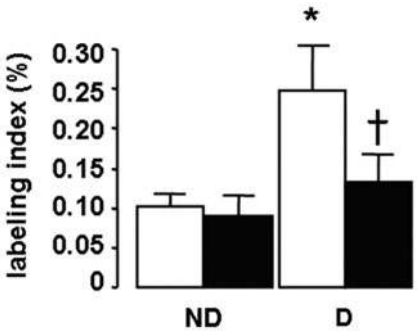

Fig. 5 Quantification of glomerular cell death rate in nondiabetic (ND) and diabetic (D) wild-type (*white bars*) and p66^{Shc} KO (*black bars*) mice [60]

7 Aging-Related Gene: Sirt1

Protein acetylation is an important posttranslational modification in cell signaling pathways. Numerous proteins such as transcription factors, cytoskeletal proteins, and metabolic proteins are activated via acetylation. Deacetylases reverse the process of acetylation to promote stability of DNA and play an important role in cell metabolism [61]. Sirtuins, a class III histone deacetylase (HDAC), possess distinctive nicotinamide adenine dinucleotide (NAD^+)-dependent enzymatic activity (Fig. 6). The unique NAD^+ dependent characteristic sheds light on their critical role

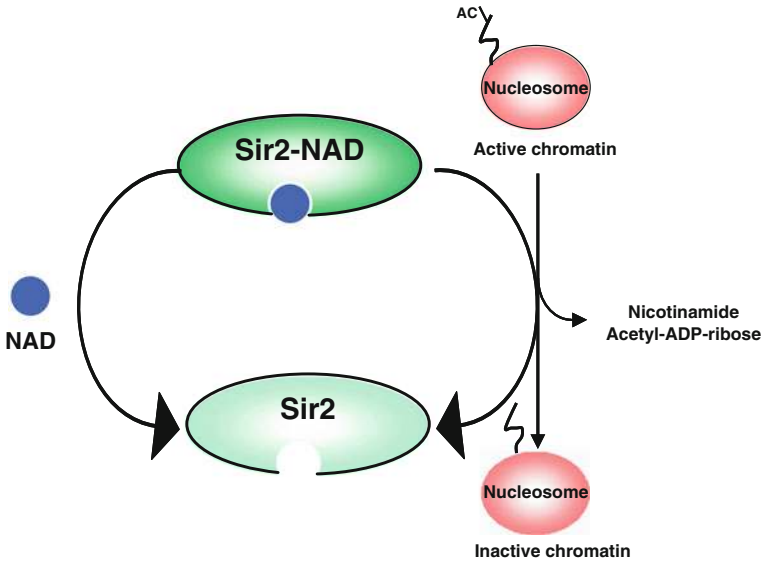

Fig. 6 The sirtuin Sir2 protein requires NAD for its enzymatic activity. It couples NAD breakdown to nicotinamide and ADP-ribose with the removal of acetyl groups from histone and other proteins. The acetyl moiety is transferred to ADP-ribose, which creates the chemical acetyl-ADP ribose. Deacetylated nucleosome are packed up to silenced chromatin structure and involved in silencing gene transcription. (Adapted from Jennifer Fisher Wilson, Enzyme role found for aging gene. The Scientist. 2002;16(22):36)

in energy state and longevity. In mammalian cells, seven Sirt proteins have been identified. Sirt1, the most important and well-characterized protein of this family, emerges as a promising therapeutic approach for aging and age-associated diseases.

Sirt1 deacetylates a variety of substrates, depending on the experimental cell types and/or stimulations. When cancer cells are exposed to oxidative insults such as H_2O_2, UV radiation, lysophosphatidic acid, or heat shock stimulation, Sirt1 deacetylates forkhead box (FoxO) transcription factor and by doing so positively affects cell viability and prevents cell death [62–64]. In fibroblast cells exposed to ionizing radiation, Sirt1 prevents cell apoptosis by deacetylating p53 protein on lysine 382 [65, 66]. Moreover, Sirt1 inhibits the transcriptional activity of RelA/p65 NF-κB at lysine 310 in human embryonic kidney 296 cells (HEK 296) treated with TNF-α [67].

8 Sirt1: Role in Aging and Age-Related Cardiovascular Diseases

Caloric intake was shown to affect life span in many organisms. In mouse liver, the expression of Sirt1 protein is induced after 24-h starvation and returns to normal level upon feeding [68]. Similar results are also observed in p12 cells, a human mammalian peripheral blood mononuclear cell line, suggesting that Sirt1 protein takes part in nutrient control and energy homeostasis [69]. The upregulated Sirt1 protein deacetylates PGC-1 α in an NAD^+-dependent manner in both hepatic cells and mice livers, indicating a regulatory role of Sirt1 in the gluconeogenic/glycolytic pathway [68]. Moreover, in a chronic caloric control model, which extends life span, the expression of Sirt1 protein is enhanced in the calorie-restricted group [70]. Likewise, Sirt1 overexpressing mice share a similar phenotype to that of calorie-restricted mice; such phenotype includes lower body weight, lower cholesterol level, improved glucose homeostasis, and increased metabolic rate [71]. Thus, Sirt1 is an important mediator of the beneficial effects of caloric restriction and is probably involved in life-span control.

Additionally, Sirt1 protein is expressed in pancreatic β-cell, indicating a possible role of this deacetylase in pancreatic endocrine function [72]. In cell culture experiments, inhibition of Sirt1 reduced the secretion of insulin. These results were confirmed in Sirt1 knockout mice where insulin levels are reduced in both normoglycemic and glucose tolerance experiments. Inhibition of Sirt1 is also coupled to enhanced expression of uncoupled protein (UCP2), which uncouples oxygen consumption during respiration, leading to an increased production of ROS [71]. Consistently, upregulation of Sirt1 in transgenic mice [71] or mice treated with a small-molecule activator of Sirt1 [73], restores UCP2 protein expression, enhances insulin secretion, and hence improves glucose tolerance in type 2 diabetes. These data suggest that activators of Sirt1 are a potentially interesting therapeutic principle for diabetes.

In a heart-specific Sirt1 transgenic model, beneficial effects are observed in mice with slight or moderate upregulation of Sirt1 due to an increased catalase expression through FoxO-dependent signal pathway. In addition, Sirt 1 overexpression protects

the heart from paraquat-induced oxidative stress and reduces ROS generation in the hearts of aged mice [74].

In rat aorta, inhibition of Sirt1 ex vivo causes a blunted acetylcholine-induced, endothelium-dependent relaxation. In turn, restoration of Sirt1 increases eNOS activity by deacetylation of eNOS on both lysine 496 and 506, underscoring that Sirt1 regulates endothelial nitric oxide synthase (eNOS) [75]. On the other hand, supply with nitric oxide increases Sirt1 expression and delays cell senescence in human endothelial cells [76]. Further, in nitric oxide knockout mice, caloric restriction fails to upregulate Sirt1 expression thus suggesting that nitric oxide modulates Sirt1 expression as well [70]. These data demonstrate a strong link between nitric oxide and Sirt1 and confirm a crucial role of Sirt1 in regulation of life span.

Further, in a mouse model of atherosclerosis, high-fat diet reduces Sirt1 expression accompanied by impaired endothelium-dependent relaxations. Differently, endothelial cell–specific Sirt1 transgenic mice fed a high-fat diet display upregulated eNOS expression, improved relaxation, and a decreased atherogenesis. These data confirm that Sirt1 plays a role in arterial dysfunction and early atherosclerotic lesion formation [77].

9 Environmental Factors Affecting Life Span

9.1 Caloric Restriction

The concept that caloric restriction extends life span was first introduced early in the past century. This concept was proved in several organisms including rodents and possibly primates [78–80]. Strict control of food and caloric intake increases average and maximum life span, reduces the incidence of age-related disease, and maintains organ function later in life [80]. However, the precise mechanisms by which caloric restriction mediates beneficial effects remain unclear. It is thought that caloric restriction reduces mitochondria-derived free radicals and decelerates the rate of aging. However, caloric restriction also decreases the formation of ATP, which seriously limits possible clinical applications of this concept. Hence, it is generally believed that the caloric intake should be taken down as much as possible but not below a comfortable level [81].

9.2 Physical Exercise

Exercise training enhances efficiency of the antioxidant system and improves physical dysfunction in aging [82–88]. Recently, it was reported that regular physical activity restores Sirt1 protein in both heart and adipose tissue of aged mice, supporting the concept of beneficial effects of exercise training in aging [87].

9.3 Antioxidant Treatment

A large body of scientific evidence supports the free radical theory of aging. However, antioxidant treatments did not prove successful in reducing the risk of cardiovascular events in large, long-term clinical trials [89–91]. This could be accounted for by several factors including the following: (a) antioxidant supplements are commonly found in normal human daily diet; (b) additional antioxidant treatment may depress endogenous antioxidant defense systems thus preventing the expected positive effects of antioxidant therapy [10]. Therefore, as an alternative to antioxidants treatment, inhibition of endogenous oxidant proteins such as $p66^{Shc}$ seems a more attractive therapeutic option.

9.4 Resveratrol

Resveratrol, one of the polyphenolic compounds found in grapes and wine, has multiple beneficial effects on the cardiovascular system. Resveratrol enhances the expression of nitric oxide synthase [92, 93] and improves vasodilation in an endothelium-dependent manner [94–96]. Resveratrol also induces endothelium-independent vasodilation [96–98]. Furthermore, resveratrol downregulates tissue factor expression (a key regulator of thrombosis) through TNF-α–induced NF-κB activation in human endothelial cells [99] and prevents adhesiveness of monocytes and platelets upon inflammation [100, 101]. Moreover, resveratrol delays endothelial progenitor cell senescence via PI3K-Akt pathway [102]. Most strikingly, resveratrol is a potential activator of Sirt1 [103, 104]. However, the effects of resveratrol on life-span extension are not consistent, hence care should be taken before drawing conclusions as to the effectiveness of resveratrol on longevity.

10 Summary and Conclusions

There is no doubt that elucidating the mechanisms of aging and age-related morbidity and mortality is one of the biggest challenges of this century. $p66^{Shc}$ is a mitochondrial protein and a newly discovered mediator of oxidative stress. Inhibition of $p66^{Shc}$ reduces oxidative stress in aging and age-related cardiovascular disease, which indicates that $p66^{Shc}$ is a promising target to retard the process of aging. Sirt1 improves cell survival and functions by deacetylating regulatory proteins in metabolism. Upregulation of Sirt1 exhibits multiple beneficial effects on extension of life span.

In the years to come, additional studies should be undertaken to fully elucidate the mechanisms of aging and age-related disease to offer an improved quality of life to the elderly and possibly to extend life span.

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Oxidative Stress, Aging, and Cardiovascular Disease

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Abstract Coronary artery disease (CAD) represents the primary cause of death in Western countries and has a great impact on human health and high social costs. Oxidative stress induced by reactive oxygen species (ROS) plays an important role in the etiology of this disease. In particular, low-density lipoprotein (LDL) oxidization has a key role in the pathogenesis of atherosclerosis and cardiovascular heart diseases through initiation of the plaque formation process. Dietary phytochemical products such as antioxidant vitamins (A, C, E) and bioactive food components (α- and β-carotene) have shown an antioxidant effect in reducing both oxidative markers of stress and the LDL-oxidization process. Scientific evidence supports the beneficial roles of phytochemicals in the prevention of some chronic diseases. Many vitamins and carotenoids with great antioxidant properties have shown both in epidemiologic studies and human supplementation trials a reduction of cardiovascular risk. However, controlled clinical trials and dietary intervention studies using a well-defined subject population have not provided clear evidence of the efficacy of these substances in the prevention of cardiovascular diseases. The current chapter aims to evaluate the beneficial effect of antioxidant substances in the prevention of cardiovascular disease.

Keywords Oxidative stress · Vitamins · Carotenoids · Aging · Atherosclerosis · Cardiovascular risk · Oxidized LDL

1 Introduction

Heart diseases and stroke represent an epidemic cause of death and disability in developed countries [1], responsible for about 30% of all deaths worldwide each year. The term cardiovascular disease (CVD) includes coronary heart disease

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(CHD; myocardial infarction, angina pectoris, coronary insufficiency, and coronary death), cerebrovascular diseases (stroke and transient ischemic attacks), peripheral vascular disease (PVD), congestive heart failure (CHF), hypertension, and valvular and congenital heart disease [2].

Numerous epidemiologic investigations have characterized the risk pattern for CHD. In particular, age, male sex, elevated low-density lipoprotein cholesterol (LDL-C) levels, low high-density lipoprotein cholesterol (HDL-C) levels, diabetes mellitus, and cigarette smoking are key risk factors for CHD [3].

Along with genetic factors and age, lifestyle and diet are also considered important risk factors. In particular, carotenoids, a group of phytochemical substances responsible for different colors of foods, play an important role in the prevention of human diseases and maintenance good health [4–7].

It is estimated that nearly one third of all cancer deaths in the Occidental developed countries could be prevented through appropriate dietary modification. Various dietary antioxidants have shown considerable promise as effective agents for cancer prevention by reducing oxidative stress, which has been implicated in the development of many diseases, including cancer. Therefore, for reducing the incidence of cancer, modifications in dietary habits, especially by increasing consumption of fruits and vegetables rich in antioxidants, are increasingly advocated. Accumulating research evidence suggests that antioxidant nutrients are believed to slow the progression of atherosclerosis because of their ability to inhibit the tissue damage deriving from the oxidative process [8–10].

2 Oxidative Stress

Advances in pathophysiologic research suggested that CVD represents a *continuum pathophysiologic process* that includes oxidative stress, endothelial dysfunction, inflammatory process, and vascular remodeling [12]. Normal endothelial function appears to depend greatly on the homeostatic balance between nitric oxide (NO) and reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide [13].

ROS are highly reactive oxidant molecules generated endogenously through regular metabolism, lifestyle activity, and diet. They react with cellular components, causing oxidative damage to such critical cellular biomolecules as lipids, proteins, and DNA. This damage may play a significant role in the pathogenesis of several chronic diseases [14–16].

Oxidative stress induced by ROS is characterized by production of oxidized-LDL (oxLDL), which plays a key role in the pathogenesis of atherosclerosis and represents an underlying disorder leading to heart attack and ischemic stroke [17].

In particular, the endothelial cells actively participate in the development of inflammatory reactions. The recruitment of leukocytes to sites of inflammation is initiated by endothelial secretion of chemotactic molecules and enhanced expression of adhesion molecules that interact with surface proteins and leukocytes [18].

Cytokines and arachidonic acid metabolites of the leukocyte pathway derived from cells of vessel walls stimulates endothelial secretion of many of these molecules [19].

Inflammation characterizes all phases of atherothrombosis and provides a critical pathophysiologic link between plaque formation and acute rupture, leading to occlusion and infarction. An inflammation complex of novel risk factors, including high-sensitivity C-reactive protein (hsCRP), lipoprotein(a), and homocysteine, and markers of fibrinolytic and hemostatic function such as fibrinogen, D-dimer, tissue plasminogen activator (t-PA), and plasminogen activator inhibitor (PAI-1) antigens characterize the several phases of atherothrombosis. In particular, hsCRP, a circulating member of the pentraxin family, plays a major role in the human immune response. Although CRP is primarily derived from the liver, recent data indicate that cells within human coronary arteries, particularly in the atherosclerotic intima, can elaborate CRP [20, 21]. More than simply a marker of inflammation, CRP may influence directly vascular vulnerability through several mechanisms, including enhanced expression of local adhesion molecules, increased expression of endothelial PAI-1, reduced endothelial nitric oxide bioactivity, altered LDL uptake by macrophages, and colocalization with complement within atherosclerotic lesions [22].

Large series of prospective epidemiologic studies have demonstrated that CRP, when measured with high-sensitivity assays (hsCRP), strongly and independently predict risk of myocardial infarction, stroke, peripheral arterial disease, and sudden cardiac death even among apparently healthy individuals [23, 24]. The hsCRP levels correlate only modestly with underlying atherosclerotic disease as measured by carotid intima–media thickness or by coronary calcification. This observation suggests that hsCRP does not simply reflect the presence of subclinical disease but rather indicates an increased propensity for plaque disruption and/or thrombosis [25].

3 Free Radicals

The role of free radicals in human pathology is quite a puzzling issue, and the benefits of oxidative stress drug therapy in terms of disease prevention or therapy are still debated.

On the other hand, new inputs are coming from the market about the diagnostic methods and instruments, with special attention to the point-of-care philosophy, making easier, cheaper, and quicker epidemiologic and clinical investigations.

In the marketplace, ROS-level detection in plasma in the upcoming years is supposed to be competitive with the erythrocyte sedimentation rate (ESR) test, the latter being an aspecific marker, a bystander of inflammation or other degenerative or proliferative diseases, enclosed the cancer, and the former a sensitive monitor of a dangerous, unsteady background of pathogenetic processes and illnesses. ESR reflects in fact plasma proteins and fibrinogen increased turnover and concentration,

and ROS reflect an imbalance of oxidative stressors often not adequately controlled by physiologic antioxidant activity.

A free radical can be defined as a chemical species possessing an unpaired electron. It can also be considered as a fragment of a molecule. As such, free radicals can be formed in three ways:

1. by the homolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining one of the paired electrons ($X{:}Y \rightarrow X^{\bullet} + Y^{\bullet}$);
2. by the loss of a single electron from a normal molecule ($X{:}Y \rightarrow X^{-} + Y^{+}$);
3. by the addition of a single electron to a normal molecule ($A + e^{-} \rightarrow A^{-\bullet}$).

The latter, electron transfer, is a far more common process in biological systems than is homolytic fission, which generally requires high energy input from either high temperatures, UV light, or ionizing radiation. Heterolytic fission, in which the electrons of the covalent bond are retained by only one of the fragments of the parent molecule, does not result in free radicals but in ions, which are charged or electrically neutral. The unpaired electron and the radical nature of a species are conventionally indicated by writing it with a heavy superscript dot.

It can be a source of confusion that the electrons in one of the most relevant molecules in free radical biochemistry, oxygen, are distributed in such a way that two of the electrons are "unpaired." Thus, oxygen is sometimes considered a di-radical. Although the di-radical nature of oxygen does enable it to react readily with many other free radicals, generally it reacts relatively slowly with nonradical species. When considering its reactions in the context of free radical biochemistry, it is usually easiest to simply consider it as a normal molecule that can readily add to free radicals or accept a single electron from them, while not itself being a free radical.

The most relevant free radicals in biological systems are radical derivatives of oxygen. Reduction of oxygen by the transfer to it of a single electron will generate the superoxide free radical anion (superoxide): $O_2 + e^{-} \rightarrow O_2^{-\bullet}$.

A two-electron reduction of oxygen would yield hydrogen peroxide: $O_2 + 2e^{-} + 2H^{+} \rightarrow H_2O_2$.

Hydrogen peroxide is often produced in biological systems via the generation of superoxide: two superoxide molecules can react together to form hydrogen peroxide and oxygen:

$$2O_2^{-\bullet} + 2H^{+} \rightarrow H_2O_2 + O_2.$$

Because the free radical reactants generate nonradical products, this is known as a dismutation reaction. It can take place spontaneously (even if rather slowly) or can be catalyzed by the enzyme superoxide dismutase. Hydrogen peroxide is not a free radical but falls into the category of ROS, which includes not only oxygen free radicals but also nonradical oxygen derivatives that are involved in oxygen radical production.

Hydrogen peroxide is a relevant molecule in free radical biochemistry because it can rather easily break down, particularly in the presence of transition metal ions, to generate the most reactive and damaging of the oxygen free radicals, the hydroxyl radical (•OH): $H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+}$. This reaction is often referred to as the iron-catalyzed Haber–Weiss reaction. The noncatalyzed Haber–Weiss reaction is the reaction of superoxide directly with hydrogen peroxide:

$$O_2^{-\bullet} + H_2O_2 \rightarrow OH + OH^- + O_2.$$

The spontaneous reaction is less likely in biological systems due to low steady-state concentrations of the reactants. The iron (or copper) catalyzed reaction can still be considered to be dependent on superoxide as both the source of the hydrogen peroxide and as the reductant of the transition metal ion:

$$O_2^{-\bullet} + Fe^{3+} \rightarrow Fe^{2+} + O_2^{-\bullet}$$

$$O_2^{-\bullet} + Cu^{2+} \rightarrow Cu^{+} + O_2.$$

Ferrous (Fe^{2+}) iron and cuprous (Cu^{+}) copper are much more reactive with hydrogen peroxide than their oxidized counterparts, ferric (Fe^{3+}) and cupric (Cu^{2+}), respectively.
The autoxidation of reduced transition metals can also produce superoxide:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\bullet}; Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{-\bullet}.$$

Thus, the reactions of the transition metal ions with oxygen can be considered reversible redox reactions and are extremely relevant in the promotion of free radical reactions. The "key players" in the biochemistry of oxygen free radicals are oxygen itself, superoxide, hydrogen peroxide, transition metal ions, and the hydroxyl radical, the first four of which conspire by a variety of reactions to produce the last [26].

Superoxide, although a free radical, is not a particularly damaging species: it is mostly reductive in nature, and its main significance is probably as a source of hydrogen hydroperoxide and as a reductant of transition metal ions. Its reaction with NO•, which is believed to be the identity of endothelium-derived relaxing factor, may also prove to be physiologically relevant [27]. At low pH values, superoxide will protonate to form the perhydroxyl radical ($HO_2^{\bullet}$), a more reactive, oxidizing species, but at physiologic pH less than 1% will be in the protonated form.

Hydrogen peroxide is an oxidizing agent but not especially reactive, and its main significance lies in it being a source of hydroxyl radicals in the presence of reactive transition metal ions. In the absence of metal catalysts, superoxide and hydrogen peroxide are readily removed and are virtually harmless.

The *hydroxyl radical* is an extremely reactive oxidizing radical that will react with most biomolecules at diffusion-controlled rates. Is therefore will not diffuse a significant distance within a cell before reacting and has an extremely short half-life but is able to cause great damage within a small radius of its site of production.

Singlet oxygen is another nonradical, reactive oxygen species often related to oxygen free radicals; it can lead and be produced by free radical reactions. Oxygen free radicals are not the only relevant free radicals in biochemistry, even if they are often the first species formed. Other free radicals of importance are the wide range of carbon-centered radicals ($R^{\bullet}$) that arise from the attack of an oxidizing radical (e.g. •OH) on a biomolecule (RH) such as carbohydrate, lipid, nucleic acid, or protein. These react very slowly with oxygen to form the corresponding peroxyl radicals ($ROO^{\bullet}$). In turn, these peroxyl radicals can participate in reactions that produce alkoxyl radicals ($RO^{\bullet}$). Sulfur atoms can also be the center for free radicals (thiyl radicals, $RS^{\bullet}$) generated, for example, in the oxidation of glutathione.

3.1 Production

With the exception of unusual circumstances such as the influence of ionizing radiation, free radicals are generally generated in cells by electron transfer reactions. These can be mediated by the action of enzymes or nonenzymatically, often through the redox chemistry of transition metal ions.

Free radical production in animal cells can either be accidental or deliberate. Free radicals are produced deliberately by animal cells in some special conditions because they can be useful entities if constrained and targeted. Some enzymes utilize a free radical at their active site in the process of catalysis; for example, ribonucleotide reductase [28, 29]. In these situations, the free radical is not really "free" at all, and its reactivity is targeted toward a specific reaction. Activated phagocytes also deliberately produce superoxide as part of their bactericidal role [30]. Although the free radicals are generated only at the interface of the phagocyte plasma membrane and bacterium, some leakage of superoxide, hydrogen peroxide, and other ROS is inevitable.

Under normal conditions, the major source of free radicals in cells is electron "leakage" from electron transport chains, such as those in mitochondria and in endoplasmic reticulum, to molecular oxygen, producing superoxide. Other enzymes can also generate superoxide or hydrogen peroxide, such as the range of flavin oxidases situated in peroxisomes. Another source of superoxide in animal cells is the auto-oxidation of some compounds including ascorbic acid (vitamin C), thiols (e.g. glutathione, cysteine), adrenaline, and flavin coenzymes. These auto-oxidation reactions can be greatly enhanced by the involvement of transition metal ions. This accidental production of free radicals is kept to a minimum by the high efficiency of enzyme-mediated electron transfer and by keeping metal ions tightly sequestered; these are fundamental means of preventive antioxidant defense. Such precautions cannot be completely efficient, and animals have evolved enzymic and nonenzymic

antioxidant defenses to deal with the inevitable low-level generation of free radicals during normal metabolic activity.

Free radical generation in cells can be greatly increased by some toxic foreign compounds. The typical example is carbon tetrachloride, which was the first such compound to be shown to exert its toxicity through a free radical mechanism, being metabolized to the trichloromethyl free radical by the action of cytochrome P-450 in the liver [31, 32]. The production of reactive free radicals overwhelms the antioxidant defenses in the liver and results in the oxidative destruction of cellular membranes and serious tissue damage. Other examples of toxic compounds exerting their toxicity via the generation of free radicals are "redox-cycling" compounds that readily accept an electron to form a free radical and then transfer it to oxygen, producing superoxide and thence hydrogen peroxide. The efforts of glutathione peroxidase to remove the continuously produced hydrogen peroxide results in the depletion of glutathione (GSH) and allows oxidative damage to the cell [33, 34]. It is possible to postulate the involvement of the free radical mechanism in the toxicity of many compounds, but caution must be exercised. In many conditions, the free radical generation may be secondary to the initial toxic mechanism, a consequence rather than the cause of cell damage.

3.2 Damaging Reactions

All of the most important classes of biomolecules may be attacked by free radicals, but lipids are probably the most sensitive. Cell membranes are rich sources of polyunsaturated fatty acids (PUFAs), which are readily attacked by oxidizing radicals. The oxidative destruction of PUFAs, kown as lipid peroxidation, is particularly damaging because it proceeds as a self-perpetuating chain reaction [35]. The general process of lipid peroxidation can be envisaged as in the scheme below, where LH is the target PUFA and $R^{\bullet}$ the initiating, oxidizing radical. Oxidation of PUFA produces a fatty acid radical ($L^{\bullet}$) that rapidly adds oxygen to form a fatty acid peroxyl radical ($LOO^{\bullet}$). The peroxyl radicals are the carriers of the chain reaction, they can oxidize further PUFA molecules and initiate new chains, generating lipid hydroperoxides (LOOH) that can break down to yet more radical species and to a wide range of compounds, notably aldehydes [36, 37]:

$$LH + R^{\bullet} \rightarrow L^{\bullet} + RH$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + L'H \rightarrow LOOH + L'^{\bullet}$$

$$LOOH \rightarrow LO^{\bullet}, LOO^{\bullet}, \text{aldehydes.}$$

The breakdown of lipid hydroperoxides often involves transition metal ion catalysis in reactions analogous to that with hydrogen peroxide yielding lipid peroxyl and lipid alkoxyl radicals. Aldehydes are always formed when lipid hydroperoxides break down, and many of them are biologically active, particularly a class known as the hydroxyalkenals, whose best known member is 4-hydroxynonenal [38, 39]. These molecules can diffuse from the original site of attack and spread the damage to other parts of the cell. In summary, lipid peroxidation is of particular significance as a damaging reaction consequent to free radical generation in cells because:

- it is a very likely occurrence, given the availability and sensitivity of PUFA in membranes;
- it is a very destructive chain reaction that can directly damage the membrane's structure and indirectly damage other cell components by the production of reactive aldehydes.

Lipid peroxidation has been implicated in a wide range of tissue injures and diseases. It has been established to be casually involved in such tissue injuries as carbon tetrachloride hepatotoxicity [40] and may be involved in the pathogenesis of atherosclerosis [41].

Proteins and nucleic acids appear less sensitive than PUFAs to free radical attack in that there seems to be less possibility of rapidly progressing, destructive chain reactions being initiated. Random attack of radicals on proteins is unlikely to be very damaging unless very extensive. Free radical damage to proteins is only likely to be important to the viability of the cell if the damage is allowed to accumulate, which in most cells is not likely, or if the damage is somehow focused on specific sites of particular proteins. One way that damage may be focused on specific sites of particular proteins is if the protein binds a transition metal ion at a particular site (e.g., the binding of copper by a histidine residue). In this case, the reaction of the transition metal with hydrogen peroxide can produce hydroxyl radical that will react at or near the metal-binding site; this concept is known as "site-specific" damage [42, 43]. A wide range of residue modifications can occur such as the formation of peroxides [44] and carbonyls [45], the latter of which may be a useful measure of oxidative damage to proteins.

DNA is readily attacked by oxidizing radicals if they are formed in its vicinity as has been clearly demonstrated by radiation biologists. It must therefore be considered a susceptible and relevant target. As with proteins, there appears little possibility of rapid chain reactions occurring, and again it is important that it must be either "site-specific" such that damage is focused and of high intensity, leading to strand breaks, or must elude the repair systems before replication occurs, leading to mutations. The detection of oxidized nucleobases in human urine has been taken as evidence for a continual oxidative attack on DNA [46, 47]. Even with a very high level of efficiency of repair, sufficient damage may accumulate over a lifetime to lead to mutations and thence cancer.

3.3 Defense: Antioxidants

Antioxidants are protective agents that inactivate ROS and therefore significantly delay or prevent oxidative damage. In particular, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH_{Px}) are endogenous naturally occurring antioxidants present within human cells. In addition, antioxidants such as vitamin E, vitamin C, polyphenols, and carotenoids are available from foods. Current dietary guidelines to combat chronic diseases, including cancer and CHD, recommend increased intake of plant foods, including fruits and vegetables, which are rich sources of antioxidants [48, 49]. The role of dietary antioxidants, including vitamin C, vitamin E, carotenoids, and polyphenols, in disease prevention has received much attention recently and appears to have a wide range of antiatherogenic properties [50–53]. These observations may explain the epidemiologic data indicating that diets rich in fruits and vegetables are associated with a reduced risk of numerous chronic diseases [54, 55].

Because some free radical generation in animal cells is inevitable and because they can be very damaging, defenses against the severe actions of free radicals have evolved. These are known as antioxidant defenses, and the two main categories are those whose role is to prevent the production of free radicals and those that intercept any that are produced [56]. They exist in both the aqueous and membrane compartments of cells and can be enzymes or nonenzymes.

The preventive defenses include efficiency of electron transfer and sequestration of transition metal ions. Iron, for example, is held tightly bound to special proteins such as transferrin and ferritin [57]. Some iron, however, is postulated to exist in a more reactive low-molecular-weight pool and, moreover, the production of free radicals may really release free transition metal ions [58]. Another form of preventive antioxidant defense is the removal of peroxides that react with transition metal ions to generate reactive free radicals. This includes both hydrogen peroxide and also the lipid hydroperoxides that are generated during lipid peroxidation. Catalase and glutathione peroxidase are enzymes whose role is to safely decompose peroxides. The former is mainly located in peroxisomes and acts upon hydrogen peroxide; the latter is found in the cytosol of most cells and is active toward both hydrogen peroxide and, if first cleaved from membrane phospholipids by a phospholipase, fatty acid hydroperoxides [59]:

$$2H_2O_2 \rightarrow \text{catalase} \rightarrow 2H_2O + O_2$$

$$ROOH + 2GSH \rightarrow \text{GSH peroxidase} \rightarrow ROH + GSSG.$$

Other defenses exist to intercept, or “scavenge,” free radicals. One of these is the only known enzyme whose substrate is a free radical, SOD. Most free radical scavengers are not enzymes, however. In cell membranes, the best characterized and perhaps the most relevant is α-tocopherol, the major member of the vitamin E family [60–62]. This compound is known as a “chain-breaking antioxidant”

because it functions to intercept lipid peroxyl radicals ($LOO^{\bullet}$) and so terminate lipid peroxidation chain reactions:

$$LOO^{\bullet} + \alpha\text{-tocopherol-OH} \rightarrow LOOH + \alpha\text{-tocopherol-O}^{\bullet}.$$

The resultant tocopheroxyl radical is relatively stable and, in physiologic conditions, insufficiently reactive to initiate lipid peroxidation itself; an essential criterion of a good antioxidant. Other lipid-soluble chain-breaking antioxidants, such as ubiquinol [63], are as yet insufficiently characterized to establish their physiologic relevance.

In the aqueous phase, other molecules act as free radical scavengers. Ascorbic acid is a relevant antioxidant both within cells and in the plasma [64]. It has been shown to produce α-tocopherol from the tocopheroxyl radical in vitro [65].

Uric acid in plasma and glutathione in cell cytosol also possess strong radical scavenging properties. It is readily apparent that cells have evolved an array of antioxidant defenses designed to severely limit damage caused by free radicals wherever and whenever it might occur. This condition is perhaps the most persuasive argument that exists for the significance of free radicals as a threat to the viability of cells and organisms.

Many biological molecules will react with oxidizing free radicals, and it might be thought that they are all antioxidants, but to qualify as such they must not be converted to a reactive radical themselves, and the proposed target that they are supposed to be defending should also be identified [66]. Many putative biological antioxidants are really just innocent bystanders caught up in the action.

A third group of natural antioxidant defense is repair processes, which remove damaged biomolecules before they can accumulate and before their presence results in altered cell metabolism or viability. Oxidatively damaged nucleic acids are repaired by specific enzymes, oxidized proteins are removed by proteolytic systems, and oxidized membrane lipids are acted upon by lipases, peroxidases, and acyl transferases.

Finally, there is a great deal of effort being expended currently on finding effective antioxidant drugs for treatment or prevention of free radical–mediated tissue damage. Such compounds include metal-chelating agents and radical scavengers. Very few have as yet been proved to be useful. A notable exception is probucol, used clinically as a lipid-lowering drug and since found to be an effective antioxidant that may help protect against atherosclerosis by preventing oxidation of low-density lipoprotein [67].

One of the major problems related to efforts toward antioxidant intervention regimens is that reactive free radicals cannot easily be specifically targeted and scavenged in biological systems. The hydroxyl radical, for example, will react readily with practically any compound, so the concept of a "specific $^{\bullet}OH$ scavenger" in a biological system is nonsense. To compete with the cell components for reaction, the $^{\bullet}OH$ radical scavenger would have to be present at concentrations much higher than the biomolecules, which is not feasible.

With the increasing acceptance of free radicals as commonplace and relevant biochemical intermediates, they have been implicated in a very great number of human diseases. Free radicals have lifetimes measured in microseconds and are extremely difficult to measure per se, not least in the clinical condition. The researcher must generally rely on the measurement of products of free radical reactions, referred to as their "footprints," and these are often transitory in nature. The technique available for studying free radicals and their reactions is improving all the time, but it must be applied with scientific rigor to elucidate the true role of free radicals in human diseases, if the field is not to be brought into disrepute. Sensitive and specific techniques should be applied to prove the presence of free radicals at the site of injury. It is essential that the role of free radicals in the causation of disorders and their generation as a consequence of disorders be clearly distinguished. To do this, it is necessary to understand the time course of the free radical generation and injury. Preventing free radical generation, possibly by the enhancement of the natural antioxidant defenses, should decrease both the detectable free radicals and the severity of the injury.

4 Free Radicals and Aging

The aging process is very complex: It depends on genetic disposal but also on lifestyle and environmental conditions at the same time.

Harman suggested 50 years ago that the accumulation of oxidants could explain the alteration of physical and cognitive functions of aging. Oxygen metabolism leads to reactive species, including free radicals, which tend to oxidize the surrounding molecules such as DNA, proteins, and lipids. Oxidative stress is an adaptive process that is triggered upon oxidant accumulation and that comprises the induction of protective and survival functions. Experimental evidence suggests that the aging organism is in a state of oxidative stress, which supports the free radical theory. The free radical theory is not consistent with programmed senescence theories involving the cell division–dependent decrease in telomere length; however, oxidants are known to alter telomere structure. An appealing view of the role of oxidative stress in aging is the trade-off principle, which states that a phenotypic trait can be evolutionarily conserved because of its positive effects on development, growth, or fertility and despite its negative effect on somatic functions and aging. It is likely that most cellular stresses that comprise adaptive and toxic functions follow such a rule [68].

Emerging pathologic evidence indicates that major chronic aging-related diseases such as atherosclerosis, arthritis, dementia, osteoporosis, and cardiovascular diseases are inflammation-related. A proposal for the molecular inflammation hypothesis of aging views the redox derangement that occurs during aging as the major factor for increased risk for age-related inflammation. Accumulated data strongly indicate that the activation of redox-sensitive transcription factors and the dysregulated gene expression under age-related oxidative stress seem to be the major culprits. Key players involved in the inflammatory process are the

age-related upregulation of NF-κB, IL-1β, IL-6, TNF-α, cyclooxygenase-2, adhesion molecules, and inducible NO synthase. Furthermore, data are presented on the molecular events involved in age-related NF-κB activation and phosphorylation by IκB kinase/NIK and MAPKs. Also, another superfamily of transcription factors, PPARs (PPARα, PPARγ), regulators of proinflammatory responses and NF-κB signaling pathway, is involved in the physiologic significance of a well-maintained balance between NF-κB and PPARs [69].

Some strategies aimed at reducing oxidative stress–related pathology have been performed in animals. However, only a few can be used and are efficient in humans, such as avoidance of unfavorable environmental conditions (radiation, dietary carcinogens, smoking, etc.) and antioxidant dietary supplementation. Epidemiologic data suggest that antioxidants may have a beneficial effect on many age-related diseases: atherosclerosis, cancer, and some neurodegenerative and ocular diseases. However, the widespread use of supplements is hampered by several factors: the lack of prospective and controlled studies; insufficient knowledge of the pro-oxidant, oxidant, and antioxidant properties of the various supplements; and growing evidence that free radicals are not only by-products but also play an important role in cell signal transduction, apoptosis, and infection control. Although current data indicate that antioxidants cannot prolong maximal life span, the beneficial impact of antioxidants on various age-related degenerative diseases may forecast an improvement in life span and enhance quality of life. The current lack of sufficient data does not permit the systematic recommendation of antioxidants. Nevertheless, antioxidant-rich diets with fruits and vegetables should be recommended [70].

Even if the scientific discussion upon the OS hypothesis of aging is still open, on the basis of the existing correlative information, it seems reasonable to state the involvement of OS as a causative factor in aging.

5 Oxidative Stress and Cardiovascular Diseases

Common vascular risk factors, including hyperlipidemia (cholesterol, low-density lipoproteins, etc.), hypertension, cigarette smoking, diabetes, overweight, physical inactivity, age, male sex, and familial predisposition, only partially explain the excess risk of developing cerebrovascular disease and CHD, and a lot of studies support today the role of OS in their pathogenesis.

Paradoxically, even if moderate exercise develops an acute oxidant stress, regular endurance exercise is associated with improved cardiovascular function and a reduction in traditional CHD risk factors. These findings are consistent with the theory that adaptations induced by acute exposures to exercise-induced oxidative stress lead to long-term vascular protection. This occurs through activation of signaling pathways that lead to increased synthesis of intracellular antioxidants and antioxidant enzymes and decreased ROS production during exercise [71].

As nitric oxide (NO) is a cell-signaling molecule that plays a relevant role in the regulation of vasomotor tone and blood flow, NO is interesting with respect to

vascular diseases, and a decrease in NO bioactivity has been related to impaired endothelial function. Endothelial cells produce NO in response to various stimuli, including shear stress, acetylcholine, bradykinin, and circulating factors in plasma such as estrogen, insulin, and lipids. NO, in turn, diffuses to neighboring smooth muscle cells and platelets, where NO induces vasorelaxation, inhibits platelet activation and adhesiveness, and modulate inflammatory responses. An increase in ROS results in a decrease in NO bioactivity by diverting NO away from anti-inflammatory reactions to those that promote injury [72].

Previous clinical studies with prostaglandin I [2] (PGI [2]) analogue beraprost sodium suggested the potential effects on protection of cardiovascular events in patients with peripheral artery disease. Although the mechanism is not well known, experimental studies have shown protective effects of endothelial cells. Ohata et al. [73] evaluated the effects of beraprost sodium on vascular endothelial function in the forearms of patients with coronary artery disease. Beraprost sodium (120 μg/d) was orally administered to 14 coronary artery disease patients for 4 weeks and then stopped for 4 weeks. Eleven control patients did not receive beraprost sodium treatment. Reactive hyperemia was induced in the forearm, endothelium-dependent vasodilation was assessed by plethysmography, and urinary 8-iso-prostaglandin F(2α) (8-iso-PGF(2α)) was measured at baseline, 4 and 8 weeks. Both groups had similar reactive hyperemic responses at baseline. In the control group, reactive hyperemic response and urinary 8-iso-PGF(2α) remained unchanged for 8 weeks. In the beraprost group, maximum forearm blood flow increased significantly ($p = 0.01$) after 4 weeks of treatment and returned to baseline at 8 weeks. Duration of hyperemia increased significantly ($p = 0.003$) after 4 weeks and remained greater than baseline at 8 weeks ($p = 0.02$). Urinary 8-iso-PGF(2α) decreased significantly ($p = 0.03$) after 4 weeks and tended to be lower at 8 weeks ($p = 0.07$). Changes in reactive hyperemia correlated weakly but significantly with changes in 8-iso-PGF(2α) ($p < 0.001$). Beraprost sodium decreased oxidative stress and improved forearm endothelium-dependent vasodilation in coronary artery disease patients. The favorable effects on vascular endothelium could potentially lead to a decrease in vascular events.

Cocoa and chocolate have recently been found to be rich, plant-derived sources of antioxidant flavonoids with beneficial cardiovascular properties. These favorable physiologic effects include antioxidant activity, vasodilation and blood pressure reduction, inhibition of platelet activity, and decreased inflammation. Increasing evidence from experimental and clinical studies using cocoa-derived products and chocolate suggest an important role for these high-flavanol-containing foods in heart and vascular protection [74].

6 Atherosclerosis

Atherosclerosis is a chronic pathology involving the deposition of plasma lipoproteins and the proliferation of cellular components in the artery wall that provide a barrier to arterial blood flow. Relevant evidence has supported the theory that

free radical–mediated oxidative processes and specific products arising thereby play a key role in atherogenesis [75]. At the base of this hypothesis are low-density lipoproteins (LDLs), which as part of the normal circulation occasionally leave the antioxidant-replete plasma, entering the subendothelial space of arteries: here LDL lipids are oxidized. The oxidized form of LDL (oxLDL) is able to initiate mechanisms leading to the formation of atherosclerotic lesions: It is taken up by macrophages and induces the release of factors that recruit other cells and stimulate smooth muscle cell proliferation. oxLDL may also upregulate expression of cellular adhesion molecules that facilitate leukocyte binding. High levels of oxLDL can also downregulate the expression of endothelial nitric oxide synthase (eNOS), an enzyme synthesizing most of vascular NO.

Oxidative modification of LDL caused by ROS triggers initiation of endothelial inflammation, which is the initial lesion of atherogenesis leading to atherosclerosis and vascular thrombosis. Moreover, oxidative mechanisms are proposed to play a role in lesion maturation and degeneration, causing heart attack and stroke. The oxidation theory is supported by the presence of oxLDL within atherosclerotic lesions and the correlation between the sensitivity of LDL to oxidation and risk of CVD.

Moreover, LDL oxidation can be inhibited by nutritional antioxidants. Various epidemiologic evidence and interventional studies correlate higher level of antioxidant-rich food uptake with lower incidence of CHD [76].

LDL is protected from oxidation by antioxidants, as well as by a second line of defense–paraoxonase 1 (PON1), which is a high-density lipoprotein–associated esterase that can hydrolyze and reduce lipid peroxides in lipoproteins and in arterial cells. Cellular paraoxonases (PON2 and PON3) may also play a relevant protective role against oxidative stress at the cellular level. Many epidemiologic studies have indicated a protective role for a diet rich in fruits and vegetables against the development and progression of cardiovascular disease. Basic research provides plausible mechanisms by which dietary antioxidants might reduce the development of atherosclerosis. These mechanisms include inhibition of LDL oxidation, inhibition of cellular lipid peroxidation, and consequently attenuation of cell-mediated oxidation of LDL. An additional possible mechanism is preservation/increment of paraoxonase activity by dietary antioxidants (vitamin E, carotenoids, and polyphenolic flavonoids) [77].

Evidence is now emerging that some dietary “antioxidants” influence signaling pathways and the expression of genes relevant in atherosclerosis by mechanisms other than antioxidative ones. By concrete examples, Brigelius-Flohe et al. [78] show that [1] vitamin E has gene regulatory functions that might be more important than acting as an antioxidant in vivo [2]; selenium itself is not an antioxidant at all, and even not in general when incorporated into glutathione peroxidases; and [3] a moderate oxidative stress is beneficial rather than detrimental as it can induce defense mechanisms counteracting xenobiotic and oxidative stress.

Consumption of soy protein is associated with a lower risk of cardiovascular disease in man and reduced atherosclerosis in a variety of experimental animals. Although a portion of the cardiovascular protective effects appears to be due to reductions in plasma lipoprotein concentration, in most people the magnitude of

this effect is relatively small. In many, but not all studies using animal models, the reduction in atherosclerosis is in part independent of changes in plasma lipids and lipoproteins. This implies that there may be a direct effect on the arterial wall of one or more of the components in soy protein that reduces susceptibility to atherosclerosis. The most actively studied components of soy protein that may be responsible for these antiatherogenic effects are the isoflavones and various protein fractions. Extraction of isoflavones and other alcohol-soluble components from soy protein lowers but does not eliminate its ability to reduce atherosclerosis. Surprisingly, in most studies, adding back the isoflavone-rich alcohol extract to the previously extracted soy protein, or to another protein, does not restore its lipoprotein lowering or antiatherogenic properties. This implies that alcohol extraction either destroys an active component of soy, alters the structural integrity of the soy proteins, or disassociates a required isoflavone–soy protein complex. The sites of action on the arterial wall and the mechanisms by which various soy components act to reduce atherosclerosis are just now being studied. The recent demonstration that expression of estrogen receptor alpha is required for atheroprotection by soy protein provides important new mechanistic insight. Other properties of soy, including antioxidant, anti-inflammatory, and potentially antithrombogenic properties need to be explored more mechanistically before the full potential of dietary soy protein for protection from cardiovascular disease will be known [79].

Leptin, a 167-amino-acid peptide hormone produced by white adipose tissue, is primarily involved in the regulation of food intake and energy expenditure. Leptin receptors are expressed in many tissues including the cardiovascular system. Recent studies suggest that hyperleptinemia may play a relevant role in obesity-associated cardiovascular diseases including atherosclerosis. Leptin exerts many potentially atherogenic effects such as induction of endothelial dysfunction, stimulation of inflammatory reaction, oxidative stress, decrease in paraoxonase activity, platelet aggregation, migration, and hypertrophy and proliferation of vascular smooth muscle cells. Leptin-deficient and leptin receptor–deficient mice are protected from arterial thrombosis and neointimal hyperplasia in response to arterial wall injury. Several clinical studies have demonstrated that high leptin level predicts acute cardiovascular events, restenosis after coronary angioplasty, and cerebral stroke independently of traditional risk factors. In addition, plasma leptin correlates with markers of subclinical atherosclerosis such as carotid artery intima–media thickness and coronary artery calcifications. Inhibition of leptin signaling may be a promising strategy to slow the progression of atherosclerosis in hyperleptinemic obese subjects [80].

Treatment of hypertension (HT) can reduce the risk for cardiovascular diseases. Tomato extract contains carotenoids such as lycopene, β-carotene, and vitamin E, which are known as effective antioxidants, to inactivate free radicals and to slow the progression of atherosclerosis. Engelhard et al. [81] wanted to evaluate the effect of tomato extract on systolic and diastolic blood pressure in grade 1 HT, on serum lipoproteins, plasma homocysteine, and oxidative stress markers. Their study is a single-blind, placebo-controlled trial. Thirty-one subjects with grade 1 HT, without concomitant diseases, who required no antihypertensive or lipid-lowering

drug therapy, and who were recruited from primary-care clinics, completed the trial. Subjects entered a 4-week placebo period, then an 8-week treatment period with tomato extract, 250 mg Lyc-O-Mato, and a 4-week control period with placebo. Systolic blood pressure decreased from 144 (SE ± 1.1) to 134 mmHg (SE ± 2, $p < 0.001$), and diastolic blood pressure decreased from 87.4 (SE ± 1.2) to 83.4 mmHg (SE ± 1.2, $p < 0.05$). No changes in blood pressure were demonstrated during placebo periods. Thiobarbituric acid–reactive substances (TBARS), a lipid peroxidation products marker, decreased from 4.58 (SE ± 0.27) to 3.81 nmol/mg (SE ± 0.32, $p < 0.05$). No significant changes were found in lipid parameters. A short-term treatment with antioxidant-rich tomato extract can reduce blood pressure in patients with grade 1 HT, naive to drug therapy.

Hatzigeorgiou et al. [82] studied 865 consecutive patients, 39–45 years of age, without known coronary artery disease and presenting for a periodic physical examination. Antioxidant intake was assessed with the Block Dietary Questionnaire, and coronary atherosclerosis was identified by measuring coronary artery calcification using electron beam computed tomography. The mean age was 42 years (±2), 83% were male, and the prevalence of coronary artery calcification was 20%. Vitamin supplements were used by 56% of the participants, and the mean (±SD) daily intake (dietary plus supplemental) of vitamins A, C, and E were 1,683 mg (±1,245), 371 mg (±375), and 97 mg (±165), respectively. There was no significant correlation between coronary artery calcification score and individual vitamin or total antioxidant vitamin intake, even after adjusting for traditional cardiac risk factors. The highest quartile of vitamin E was positively associated with calcification (odds ratio = 1.77; 95% confidence interval, 1.02–3.06). Antioxidant vitamin intake is not significantly related to coronary artery calcification, implying that there is no effect on the development of early coronary atherosclerosis. High doses of vitamin E may confer an increased risk of calcified atherosclerosis.

7 Conclusions

Free radicals and oxidative stress are involved in the process of atherosclerosis and thrombosis. There is a growing interest in identifying noninvasive markers of oxidative stress involved in the atherosclerotic process to assess correct and effective antioxidant therapies, which represents a key goal to control the oxidative process.

The antioxidant therapies provide a logical and scientific treatment of chronic atherosclerotic disease process. Although published studies on the association between antioxidant intake supplementation and cardiovascular disease have been inconclusive, there is noteworthy evidence that low plasma concentrations of certain antioxidants (vitamin A, vitamin E, β-carotene, lycopene) are associated with early atherosclerotic carotid lesions. Atherosclerosis remains clinically undetectable for a long time and frequently manifests itself with an acute cardiovascular event; therefore, the possibility of detecting the disease in a subclinical phase and reducing or reversing its progression is an issue of great public health relevance.

Several therapeutic strategies have been adopted to slow the early atherosclerotic process in asymptomatic persons in order to reduce the risk of cardiovascular events. Prospective trials employing multifactor nonpharmacological interventions (diet, exercise, smoking cessation) have demonstrated favorable effects on progression of atherosclerosis and represent the first-line, low-cost treatment. Further reductions in cardiovascular risk require more intensive intervention, including the addition of drugs that reduce LDL-C levels (statins), blood pressure values, and platelet aggregation. These interventions are also very effective and scientifically validated. An additional step to slow the atherosclerotic process may include interventions to decrease newly emerging coronary risk factors, such as oxidative stress and inflammation. Consuming a diet rich in fruits and vegetables will provide antioxidant vitamins and carotenoids and may slow the progression of early atherosclerosis and consequently reduce cardiovascular events.

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Antioxidation in Prevention of Cardiovascular Diseases – An Effect of Polyphenols

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Abstract Oxidative stress plays a critical role in the development of cardiovascular diseases. Catechins are major components of green tea with many biological functions, including antioxidative, anti-inflammatory, and anticarcinogenic effects. Antioxidative effects of tea catechins are characterized by the ability to inhibit free radical generation and to scavenge free radicals, among other effects. They also influence activation of transcription factors such as nuclear factor kappa B, a multipotential promoter of inducible nitric oxide synthase and adhesion molecules. Although these characteristics of catechins have been well documented, antioxidative effects of catechins on cardiovascular diseases have not been well investigated. In this chapter, we review recent clinical and experimental papers to reveal the antioxidative effects of catechins in cardiovascular diseases. We performed oral administration of catechins in murine and rat models of cardiac transplantation, myocarditis, and myocardial ischemia to reveal the effects of catechins on the oxidative stress–induced ventricular and arterial remodeling. From our results and those of other investigations, we conclude that catechins are potent agents for the treatment and prevention of oxidative stress–related cardiovascular diseases because they are critically involved in the suppression of the stress. In this chapter, we review these reports and other investigations.

Keywords Tea · Catechin · Oxidative stress · Free radical · Inflammation

1 Introduction

Green tea has favorable effects to prevent cardiovascular diseases; tea consumption is known to be associated with lower mortality of clinical myocardial infarction [1, 2]. Recently, it has been reported that green tea consumption reduced

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cardiovascular disease mortality in the Japanese population [3–5]. Catechins are key components of green tea with many biological functions, including antioxidative, anti-inflammatory, and anticarcinogenic effects [6–9]. Antioxidative effects of tea catechins are characterized by the ability to inhibit free radical generation and to scavenge free radicals, among other effects [6]. They also influence activation of transcription factors such as nuclear factor kappa B (NF-κB), a multipotential promoter of inducible nitric oxide synthase and adhesion molecules [10]. The major tea catechins are epigallocatechin-3 gallate (EGCG), epigallocatechin (EGC), epicatechin-3 gallate (ECG), and epicatechin (EC). Among them, EGCG is the most active polyphenol [11] and primarily responsible for the green tea effect [12]. In addition, EGCG demonstrated potent antioxidant properties [13]. EGCG possesses two triphenolic groups in its structure, which are reported to be important for its strong activity [14]. Luczay et al. arranged their antioxidative properties as follows: EGCG = EGC >> ECG = EC [15–17]. Antioxidative properties of catechins are manifested by their abilities to inhibit free radical generation, to scavenge free radicals, and to chelate transition metal ions, which are catalysts of free radical reactions [16]. Based on the standard one-electron reduction potential values, catechins can scavenge free radicals generated in an organism [18–21]. It is also noteworthy that EGCG is thought to act as an antioxidant in biological systems. Yin et al. revealed that EGCG increased cell viability, decreased reactive oxygen species (ROS) formation, and improved mitochondrial membrane potential in hippocampal neurons that had been exposed to lead. They concluded that EGCG is a potential agent in the treatment of chronic lead intoxication through its antioxidative character [22].

Oxidative stress is an important factor in organ and tissue injury [23]. Oxidative stress is defined as damage to cells, tissues, and organs caused by ROS. ROS are generated exogenously and intracellularly and include superoxide anion, hydrogen peroxide, hydroxyl radicals, and peroxynitrite. The principal intracellular sources of ROS include the mitochondrial electron transport system, peroxisomes, cytochrome P-450, and NADPH oxidase enzymes [24]. It is also known that exogenous factors involved in the generation of ROS are inflammatory cytokines, chemotherapeutic drugs, and toxins. Antioxidants constitute the defense mechanism against oxidative stress injury and include both enzymes and nonenzymatic factors. Copper–zinc and manganese superoxide dismutase (CuZn-SOD and Mn-SOD), catalase, and glutathione peroxidase (GPX) are key antioxidant enzymes. In contrast, glutathione and vitamins A, C, and E are also known to be the major nonenzymatic antioxidant molecules. The balance between ROS production and antioxidant defenses defines the degree of oxidative stress [25]. Whereas ROS play an important role in cell proliferation, differentiation, and apoptosis [26–28], oxidant signals can alter and denature nucleic acids, carbohydrates, lipids, and proteins, resulting in cell toxicity. The deleterious effects of oxidative stress have been reported in the pathophysiology of aging [24, 25] and neoplastic [29], hypertensive [30, 31], cardiovascular [32–34], and chronic kidney diseases [35–38].

Although the characteristics of tea catechins have been well documented [39], their effects on oxidative stress in cardiovascular diseases have not been well investigated. Recently, we have reported the effects of tea catechins on oxidative

stress–related cardiovascular diseases, such as myocardial ischemia [40], acute myocarditis [41], and rejection after heart transplantation [42]. In this chapter, we review these reports and other investigations.

2 Catechins Suppress Oxidative Stress in Myocardial Ischemia

Myocardial ischemia and ventricular remodeling causes significant damage leading to cardiac death. It is well known that NF-κB–related inflammation is enhanced by ischemia and reperfusion of myocardium. Oxidative stress is known to cause adverse effects by increasing the infarct size or altering ventricular remodeling [43]. Results from Askari et al. [44] revealed the relationship between inflammation and modulation of left ventricular remodeling. Several leukocyte-derived enzymes are responsible for the increase of oxidative stress in the myocardium after acute myocardial infarction [45]. In ischemic conditions, superoxide is generated by nicotinamide adenine dinucleotide phosphate oxidase [46], hypochlorous acid by myeloperoxidase (MPO) [47], and peroxynitrite from inducible nitric oxide synthase [48]. The systems lead to the oxidation of proteins and lipids in the infarct zone. By identifying the oxidation products present after inflammation, the responsible oxidant systems should be elucidated. It is noteworthy that statins can block inflammation in several of these systems by limiting the generation of superoxide [49, 50]. Results from previous studies suggest that altered protease activation may contribute to remodeling [51]. Oxidative stress directly affects protease activation, and the phenotype observed in MPO-knockout mice was similar to that observed in urokinase and plasminogen-knockout mice [52]. Protease activation follows a well-described cascade of events. Urokinase cleaves plasminogen to plasmin, whereas plasmin can cleave pro–matrix metalloproteinase-9 to its active form, matrix metalloproteinase-9 (MMP-9). Urokinase is inhibited by binding to the plasminogen activator inhibitor-1 (PAI-1), leading to the generation of an irreversibly inactivated molecule. Each of these proteases has been shown to significantly affect the inflammatory response after acute myocardial infarction [53].

Activation of NF-κB induces adhesion molecules, cytokines, and MMPs involved in myocardial ischemia. Thus, decoy against NF-κB reduces myocardial inflammation induced by ischemia/reperfusion injury [54]. As proinflammatory factors are key components in the positive feedback loop of inflammation, the inhibition is an effective therapy for myocardial reperfusion injury by preventing inflammation [55–58]. The effects of catechins are induced by the suppression of several inflammatory factors including ROS induced by NF-κB [10]. To clarify the role of catechins in the ischemic heart, we produced a rat myocardial ischemia model by ligation of the left anterior descending coronary artery, and this was continued for 28 days. After ischemic injury, the nontreated ischemia group showed significant decline of blood pressure compared with the nontreated sham-operated group. However, catechin administration suppressed the decline of the blood pressure compared with that of the nontreated ischemia group. Echocardiography revealed that the nontreated ischemia group showed significantly impaired left ventricular

contraction compared with that of the nontreated sham-operated group. However, the catechin treatment significantly improved left ventricular wall motion compared with that of the nontreated ischemia group. Pathologically, the anterior wall of each heart of the nontreated ischemia group was completely fibrotic, and the remaining area showed interstitial fibrosis and cell infiltration. However, catechin-treated hearts showed significantly less infarct size, infarct length, left ventricular circumference, and left ventricular inner diameter than those of the nontreated ischemia group. Immunohistochemically, increased numbers of CD4, CD8, CD11b, intercellular adhesion molecule-1 (ICAM-1), and ED-1 positive infiltrating cells were observed in the nontreated ischemia group, whereas catechin administration suppressed the numbers significantly. To prove the effect of catechins on NF-κB, we performed a sensitive multiwell colorimetric assay. It revealed that increased NF-κB activity was observed in hearts in the nontreated ischemia group. However, this enhanced NF-κB activity was abolished by the catechin treatment. Finally, to reveal the role of MMPs, the infarct region and myocardium were separated under a dissecting microscope and used with zymography as previously reported. This showed that increased gelatinase (MMP-2 and MMP-9) activity was observed in hearts in the nontreated ischemia group. However, this enhanced gelatinase activity was decreased by catechin administration [40]. We clearly revealed that catechins prevented chronic ventricular remodeling after ischemic injury because of the suppression of proinflammatory factors. Oxidation and inflammation have a significant role in left ventricular remodeling after acute myocardial infarction. Administration of catechin, a compound that downregulates the oxidative stress–induced modulation of the biological function of components of the MMP cascade, could inhibit the negative remodeling that occurs after myocardial infarction and improve patient outcomes.

3 Catechins Suppress Oxidative Stress in Myocarditis

Myocarditis is a serious disease in humans. Patients with myocarditis may present with rapidly progressive heart failure, shock, or arrhythmia in its severe form. Although acute myocardial inflammation is an essential etiology for the progression, no effective treatment has been elucidated [59–63]. Experimental autoimmune myocarditis (EAM) is a rat model that is characterized by severe myocardial damage and multinucleated giant cell infiltration. This has been used as a disease model of human acute myocarditis [64–68]. Liu et al. investigated the therapeutic role of thioredoxin-1 (TRX-1), a redox-regulatory protein with anti-oxidant and anti-inflammatory effects, in a murine myocarditis model [69]. They revealed that TRX-1 attenuates myocarditis by suppressing chemokine expressions and leukocyte chemotaxis in the murine model. TRX-1 is well known as a scavenger of ROS. The study using 8-OHdG, a marker for tissue oxidative damage, showed that 8-OHdG was strongly expressed in the heart with myocarditis, whereas the area immunopositive for 8-OHdG was much smaller in the heart with TRX-1 treatment. It also has been reported that, in the initial stage of EAM, cardiac dendritic cells and infiltrating

macrophages and neutrophils attack the cardiomyocytes, forming rosette figures as a sign of active cardiomyocytolysis. Subsequently, the infiltration by macrophages and T lymphocytes plays a crucial role in the generation of myocarditis [70]. A recent study showed that ROS are involved in the antigen-presenting function of dendritic cells and that antioxidants suppress the activation of dendritic cells [71]. TRX-1 has radical scavenging functions; thus, administration of TRX-1 may also attenuate the activation of dendritic cells in this model. Shioji et al. [72] also reported that the suppression of dendritic cell functions by immunoglobulin therapy in the early phase attenuated giant cell autoimmune myocarditis. The evidence strongly suggests that the reducing activity of TRX-1 plays an important therapeutic role in EAM. In addition, chemokines, such as macrophage inflammatory protein-1 (MIP-1) and MIP-2, have recently been reported to be involved in the recruitment of inflammatory cells in EAM [73–76]. In the study, the cardiac expression of chemokines was markedly suppressed by TRX-1. Thus, the authors concluded that the attenuation of EAM by TRX-1 is due to its suppression of chemokine-induced chemotaxis of inflammatory cells in the initial phase of EAM.

To clarify the role of catechins in myocarditis, we produced a rat EAM model. The rats were supplemented with diet containing catechins or diet with saline without catechins for controls. After the induction of EAM, the catechins significantly reduced the heart weight/body weight ratio compared with that of nontreated EAM controls. Echocardiogram revealed the catechins improved cardiac function compared with that of the controls. Pathologically, nontreated control EAM animals showed severe myocardial cell infiltration and fibrotic lesions. However, the catechin treatment showed significantly less myocardial cell infiltration and fibrosis areas compared with those in controls. Immunohistochemistry revealed that enhanced expression of CD4, CD8, CD11b, ICAM-1, and NF-κB in infiltrating and arterial endothelial cells was observed in nontreated EAM hearts, whereas the catechins suppressed the expression. To examine expression of cytokine mRNA in EAM hearts, RNase protection assay was used. Tumor necrosis factor (TNF)-α mRNA level was markedly decreased in the catechin-treated group compared with that of the control group. On the other hand, mRNA levels of Th2 cytokines such as interleukin (IL)-4 and IL-10 in the catechin-treated group were markedly enhanced compared with those of the control group. We revealed that myocardial cell infiltration, fibrosis, and proinflammatory cytokines were enhanced in the EAM progression, and the catechins suppressed the development of these changes with suppressed cytokine expression and oxidative stress [41].

4 Catechins Suppress Oxidative Stress in Transplant Rejection

Transplantation has been established in humans; however, acute rejection and graft arterial diseases (GADs) are still problems [77–80]. Systemic biomarkers of oxidative stress are increased in kidney transplant recipients [81–86]. Fuentes et al. demonstrated the effects of *N*-acetylcysteine (NAC) on oxidative stress,

lipids, and renal function in 25 patients with renal transplantation. They revealed that NAC treatment increased high-density lipoprotein cholesterol and antioxidant molecules in relation to glutathione peroxidase, with a positive relationship on renal function [87]. Moratalla et al. showed a possible causal influence of subclinical glucose metabolism impairment on the presentation of left ventricular diastolic dysfunction via the impaired oxidative stress status in kidney transplantation [88]. In cardiac transplantation, Nilakantan et al. examined whether continuous treatment of recipients with the superoxide dismutase (SOD) mimetic manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTmPyP) provided protection using rat acute cardiac transplant models. They revealed that the beneficial effect of MnTmPyP on graft function was related to oxidative stress and by decreasing apoptotic signaling, rather than by an effect on inflammatory cytokine gene expression [89]. Murata et al. also showed that a single injection of the macrocyclic SOD mimetic M40401 before revascularization decreased the extent of development of coronary artery disease in rat cardiac allografts [90]. The study suggests that ROS play a critical role in reperfusion injury in cardiac allografts. To confirm the antioxidative effects of catechins on rejection, we have used a class II allo-mismatch combination of mice to perform cardiac transplantation [91]. Some recipient mice were orally supplemented with tea catechins; the other transplanted mice were supplemented with normal water without the catechins for control. Although severe myocardial cell infiltration and fibrosis was observed in nontreated allografts at day 60, tea catechins markedly attenuated myocardial cell infiltration and fibrosis. Immunohistochemically, enhancement of CD4, CD8, CD11b, ICAM-1, and vascular cell adhesion molecule (VCAM)-1 expression was observed in nontreated allograft myocardium and coronary arteries. However, catechin markedly attenuated expression of all these factors. RNase protection assay was used to examine expression of cytokine mRNA in hearts. Levels of Th2 cytokine IL-10 was significantly elevated in the catechin-treated group compared with that of the nontreated group [42]. In the study, we have demonstrated that tea catechins reduced both myocardial remodeling and GAD formation with suppression of cell adhesion molecules. Because blockade of cell adhesion molecule resulted in suppression of rejection and induction of immunologic tolerance [92–95], adhesion molecule–related oxidative stress is critical in transplantation. Therefore, catechins may be clinically effective for suppression of transplant rejection because they effectively suppress oxidative stress.

5 Summary and Future Direction

In this review, we summarize the beneficial effects of catechins in cardiovascular diseases focusing on oxidative stress. Catechins exert cardiovascular protective effects through multiple mechanisms, including not only antioxidative but also anti-inflammatory, antihypertensive, antiproliferative, antithrombogenic, and lipid-lowering effects [96–100]. Although epidemiologic studies have shown a positive

correlation between green tea consumption and cardiovascular health, detailed pathophysiology of catechin effects against oxidative stress has not yet been clarified. Therefore, further investigation is needed to develop a new antioxidative strategy to suppress clinical diseases using catechins or their related compounds.

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Vascular Aging and Oxidative Stress: Hormesis and Adaptive Cellular Pathways

Nathalie Thorin-Trescases and Eric Thorin

Abstract The biochemical basis of aging in general and of vascular aging in particular is the progressive failure of maintenance and repair systems due to cumulative molecular damage. One of the major sources of damage is reactive oxygen species (ROS). Indeed, all organisms living in an aerobic environment are exposed to ROS on a continual basis, generated as by-products of normal cellular metabolism and taken up from the external environment. In fact, oxidative damage is widely used as a biomarker of aging and diseases. At the cellular level, molecular damage and oxidative stress lead to cellular senescence. Endothelial senescent cells are proinflammatory, proatherosclerotic, and prothrombotic and could participate in the process of aging and atherosclerosis. ROS, however, also play a crucial role in physiologic cell function when present at a low physiologic concentration. Therefore, we believe that the exposure of the endothelium to physiologic oxidative stress during its maturation phase determines vascular longevity. This process is known as hormesis: mild stress activates different endogenous mechanisms of repair and maintenance to protect cells against subsequent stresses. In the case of endothelial cells, exposure to mild oxidative stress during the maturation phase of the endothelium will activate protective pathways involved in stress resistance. It is the cumulative and chronic stress that induce progressive failure of protective mechanisms, leading to higher susceptibility to diseases, cellular senescence, aging, and ultimately death.

Keywords Vascular aging · Oxidative stress · Hormesis · Endothelium

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1 Introduction

The free radical theory of aging first described by D. Harman half a century ago [1] led to intensive research. It is clear from the literature that levels of oxidatively damaged lipids, proteins, and DNA increase with age in various tissues of different species [2]. To counteract the potential damage, more than a dozen mechanisms of repair and maintenance have been classified (for review, see [3]). Clear relationships between longevity and efficiency of mechanisms of maintenance and repair have been established. These mechanisms are activated by mild stress, including oxidative stress, providing a protective response against subsequent stress: this is the hormesis response. In the context of aging, as hormesis increases stress resistance and as higher stress resistance increases life span, hormesis could slow down or control the aging process [4]. Aging, senescence, and death are the final manifestations of unsuccessful repair and maintenance systems. Endothelial cells are very sensitive to oxidative stress, and endothelial dysfunction is a hallmark of vascular aging [5]. We believe that hormetic exposure of the endothelium to physiologic oxidative stress early in life determines its resistance to risk factors for cardiovascular diseases and thus determines vascular longevity.

1.1 Aging: Different Theories of Aging

Aging is characterized by a progressive decline in biological functions with time. This results in a decreased resistance to various forms of stress, leading to an increased susceptibility to numerous diseases. Aging can be defined as the progressive failure of maintenance and repair systems, with a consequent accumulation of molecular heterogeneity and damage in nucleic acids, proteins, and lipids. As the homeostatic ability of a living system is primarily due to its maintenance and repair systems, it is the progressive failure of these systems that is the biochemical basis of aging [3, 6, 7–9]. This also applies for vascular aging and endothelial aging [5, 6, 10].

Many theories (more than 300 [11]) of aging have attempted to explain the causes and mechanisms of aging, but because aging is multicausal and a very complex process, there should not be one “single” theory of aging [3, 9, 10]. Although there is no universal cause or phenotype of aging [3, 9], the multiple causes of aging include failure of major organ systems (heart, major blood vessels, brain, etc.) due to inflammation of tissues and/or loss of immune function [12], greatly increased incidence of tumors [6, 13], accumulation of genetic damage or mutations [8, 13, 14, 15], abnormal modifications of proteins [9, 10], and damage by ROS at different molecular levels [1, 16].

There are three major sources of damage within a cell [9]: (1) endogenous and exogenous ROS and other free radicals, (2) glucose and its metabolites, (3) spontaneous errors in DNA transcription or in protein synthesis. To counteract the potential damage, more than a dozen mechanisms of repair and maintenance have been classified (for review, see [3]). They include DNA repair to prevent spontaneous lesions,

protein repair and proteases to remove defective proteins, immune response, defense against neoplastic transformation, apoptosis to clear damaged cells, and detoxification of exogenous and endogenous toxic substances (antioxidant enzymes, for example). Clear relationships between longevity and efficiency of mechanisms of maintenance and repair have been established. This led to the disposable soma theory of aging [7], where the duration of life is primarily controlled by maintenance mechanisms and stress responses. Similarly, the catastrophe theory of aging [9] incorporates the central role of macromolecular damage: protein synthesis errors and their accumulation could play a crucial role in aging.

One of the major mechanistic theories of aging, still prevalent today, is the free radical theory [1, 16], better known as the oxidative stress theory [10]: all organisms living in an aerobic environment are exposed to ROS on a continual basis, as ROS are continually generated as by-products of normal cellular metabolism and taken up from the external environment. In fact, oxidative damage is widely used as a biomarker of aging and disease: cumulative cellular oxidative damage to macromolecules, including lipid peroxidation and oxidation of DNA and proteins, induce irreversible functional changes leading to aging, senescence, and ultimately death. One of the primary molecular mechanism contributing to aging could be the progressive shift in the cellular redox environment. However, the main criticisms raised against the oxidative stress theory of aging involve its lack of incorporation of the physiologic and beneficial roles of free radicals in cell function [17]. In addition, the lack of expected benefit from antioxidant therapies to extend longevity confirms that ROS and redox environment are not the unique pathway involved in the complex mechanism of aging. Indeed, secondary antioxidant treatment does not prevent atherosclerosis in humans [18] and does not clearly slow the rate of aging (for review, see [2, 10]). This could be due to the fact that secondary antioxidant treatment is added on top of an already (irreversible?) damaged cellular background. In addition, secondary antioxidant prevention does not specifically target the defective defense and repair mechanisms. The controversial and even contradictory results from antioxidant clinical trials [19] or experiments manipulating antioxidants by either a pharmacological or genetic approach support further that aging is a complex and multifaceted phenomenon that cannot be explained by a single theory.

1.2 Cellular Aging or Cellular Senescence

Cellular senescence is an irreversible condition in which damaged cells remain metabolically active [20] but are unable to proliferate [21, 22]. Cellular senescence is used as a cellular model for understanding mechanisms underlying the aging process. Aging is closely linked with damage, and DNA damage response is a central mediator in triggering cellular senescence [13, 14].

Senescence can be triggered by cell divisions, leading to cumulative telomere attrition down to a threshold length at which cells enter replicative senescence [23].

Telomeres, the end of linear chromosomes composed of repetitive 5′-TTAGGG-3′ DNA sequences, shorten at each cell division due to the end-replication problem and to the high susceptibility to oxidative stress of GGG [24]. The exact critical threshold value for telomere length is not known, and the question of how many short telomeres are required to trigger senescence is unsolved [13], but it has been postulated that the restriction on cell turnover imposed by replicative senescence could contribute to part of the decline in tissue function observed in aging [25]. In healthy humans, telomere shortening is age-dependent [26], and telomere shortening was reported in vivo [23, 27], although it has been proposed that replicative senescence could be too tardive to occur in vivo [7, 28, 29]. In individuals with shorter telomere length in blood cells, a higher risk of mortality rate from cardiovascular diseases was observed; although just an association, this suggests that telomeres contribute and are a predictor of mortality in age-related diseases [30, 31].

Cellular senescence can also occur prematurely, independently of replicative age, after exposure to multiple types of stress (stress-induced premature senescence; SIPS) such as oxidative stress or oncogenes [21]. SIPS can be viewed as an acute form of senescence, induced by stressful stimuli. Oncogene-induced cellular senescence is a tumor suppressive mechanism that stops proliferation of cells expressing high levels of oncogenes. This type of senescence is established independently of telomere attrition [13]. Concerning oxidative stress–induced senescence, we recently reported that in vascular endothelial cells isolated from active smokers, in a highly pro-oxidative and proinflammatory environment, SIPS predominates and that telomere shortening does not account for premature endothelial dysfunction in smokers [32]. In addition, we observed that in vascular endothelial cells from atherosclerotic patients with various risk factors for cardiovascular diseases, there was an interplay between replicative senescence and SIPS [33, 34].

Cellular senescence could be seen as a simple reductionist view of aging, but recent evidence shows that senescence occurs in vivo [13, 27, 35, 36]. Cellular senescence has been linked with aging and cardiovascular diseases: endothelial senescent cells are proinflammatory, proatherosclerotic, and prothrombotic [32, 33, 34, 37, 38] and could participate in the process of aging and atherosclerosis. The estimates of abundance of senescent cells in vivo varies from <1% to >15% [35]. The fraction of senescence-associated β-galactosidase–positive cells, a marker of senescence, increases with age in the skin of healthy human beings [39]. Similarly, senescent endothelial cells (β-galactosidase–positive cells) were found to accumulate after endothelial denudation in the rabbit carotid artery [40], and enlarged flattened senescent endothelial cells were observed in atherosclerotic plaque of human aorta and coronary arteries [38, 41]. The presence of accumulated DNA-damage response foci at telomeres and the increased detection of heterochromatin markers in the skin of aged baboons also suggest that replicative senescent cells play a role in vivo during aging [42, 43]. There is a large amount of evidence showing that telomere shortening occurs in vivo; for example, endothelial telomeres shorten with age, and this attrition is accelerated in areas prone to atherosclerosis [26]. It has been reported that p16 expression, a cyclin-dependent kinase inhibitor that is

involved in growth arrest, is increased in aged mice stem cells [44]. In humans, increased levels of p16 were found in myocardial cells of hearts from aged patients with heart failure [45].

Taken together, although there is no real biomarker exclusive to all types of senescent cells, these data suggest that senescent cells accumulate in vivo and have a physiologic relevance. Cellular senescence is beneficial in the context of cancer, as it is an efficient tumor-suppressive pathway [20, 35, 36], but is also detrimental because it causes growth arrest and thus contributes to decrement in tissue renewal and function [20]. It is not known to what extent the secreted factors by senescent cells contribute to the tissue or generalized inflammatory environment observed in aging.

1.3 Vascular Endothelial Aging

> It has been said that one is as old as one's arteries. In view of the supreme importance of endothelium in arterial function, I should like to modify. . .. this statement by saying that one is as old as one's endothelium [46].

This visionary statement implies that endothelial cells (ECs) evolve with time and that aging could modulate their functions. Endothelial dysfunction is a hallmark of normal aging in humans [5]. It has been postulated that vascular aging may be a primary factor in the overall aging process and that alterations at cellular, tissue, and organ levels may be secondary to age-related vascular dysfunction (for review, see [10]). Healthy EC aging can be divided in two phases: maturation and maintenance. During maturation, we believe that reactive species are essential in triggering adaptation to the growth and the hormonal changes.

Free radical generation is often considered a deleterious by-product of oxygen metabolism, but despite their potential to cause damage, ROS play a crucial role in physiologic cell function [17]. ROS are critical components of a variety of cellular processes such as development, growth, and proliferation [47] and of the immune system [17, 48]. Hydrogen peroxide (H_2O_2) and nitric oxide, chemically more stable than other ROS, are signaling molecules in multiple biological processes [49, 50, 51]. Among others, H_2O_2 has the potential to alter expression of different genes thought to play a role in vascular biology [52]. H_2O_2 diffuses throughout the cell, is nontoxic at physiologic concentrations, and is an established mitogen [53]. In summary, at moderate concentrations, ROS-mediated responses protect the cells against oxidative stress and inflammation and allow cellular homeostasis [17].

At high concentrations, free radicals are deleterious and play a pathophysiologic role: ROS have been implicated in the pathogenesis of atherosclerosis [54] and essential hypertension [55]. In addition, ROS influence vascular function and structure [17, 37, 56]. Thus, during the endothelial maintenance phase, it is likely that cumulative oxidative stress–induced damage progressively leads to endothelial aging [57, 58, 59].

Based on these data, we hypothesize that the modeling of the endothelium in response to physiologic oxidative stress during the maturation phase determines the subsequent endothelial response to risk factors for cardiovascular diseases and thus determines vascular longevity. In other words, vascular functional longevity is largely set during maturation.

To support this hypothesis, we tested the impact of an antioxidant treatment (polyphenol catechin) initiated before or after endothelial maturation on endothelial dysfunction of aged mice (Gendron and Thorin, unpublished data). Renal artery dilations to acetylcholine were recorded, vascular gene expression measured, and splenocyte adhesion to the endothelium was quantified in mice treated with catechin for the last 3 (postmaturation) or 9 months prior to study at 12 months of age. The antioxidant initiated before adulthood was not as beneficial for the endothelium as the treatment initiated in mature (9 month old) mice: only the late treatment with catechin prevented endothelium dysfunction associated with aging, normalized adhesion of splenocytes, and prevented the age-dependent rise in inflammatory COX-2 and decline in sirtuin-1, known to induce stress resistance. Mice treated with the antioxidant after adulthood seem to have benefited from the maturation of the endothelium initially exposed to low physiologic oxidative stress. In contrast, in mice treated with the antioxidant before complete endothelial maturation, excessive preventive protection against oxidative stress was deleterious to endothelial function: age-dependent endothelial dysfunction (decrease in acetylcholine-induced relaxation) was not prevented, inflammatory COX-2 expression was increased, and antioxidant Mn-SOD expression was decreased. Thus, these results suggest that low ROS levels contribute to the expression of antioxidant defenses. These data also demonstrate that postmaturation antioxidant treatment preserves endothelial function and avoids inflammation, possibly by contributing against ROS-mediated damage and preserving the defense system essential for the proper maintenance of the endothelium.

Similarly, it is likely that the lack of defensive mechanisms explains the overreaction to the “Western way of life” of the population of the island of Nauru in the Central Pacific Ocean [60]: Nauru is a small coral atoll in the Pacific, where phosphate mining began in 1906. With it, consequent industrial development and wealth changed the lifestyle of the population at the independence of the island in 1968: 10 years after becoming wealthy, the population became largely invalid through diabetes and cardiovascular diseases (CVD) due to a considerable intake of imported calories [61], with the highest incidence of obesity (90%) and of type 2 diabetes (40%) in adults worldwide [62]. This high prevalence of obesity and CVD is responsible for premature mortality and very high mortality rate [60]. Their traditional diet of fruits, vegetables, and fish and their protected lifestyle was favorable to the cardiovascular system in a strictly protected environment; a rapid switch to a highly proatherogenic diet and to a stressful lifestyle has been disastrous. One hypothesis is that the endothelium could not develop its defense mechanisms because of the significantly limited ROS stimulation during its maturation. The phenomenon in which mild stress exposure can produce a protective response against subsequent stresses is called *hormesis*.

1.4 Hormesis in Aging

Hormesis is a term used by toxicologists to describe the biphasic dose-response to a drug or an environmental agent, characterized by a beneficial effect at low dose and an inhibitory effect at high dose [63]. Hormesis is the scientific basis for homeopathy and acupuncture [64] and is a fundamental concept in evolutionary theory: To avoid extinction and to survive changes in variation in temperature, pH, or redox environment, cells had to develop mechanisms of protection [28, 63, 65]. In the context of aging, as hormesis increases stress resistance by induction of protective mechanisms and as increment in stress resistance increases life span, one possibility is that hormesis could slow down or control the aging process [4]. Aging, senescence, and death are the final manifestations of unsuccessful repair and maintenance systems. Hormesis, in contrast, contributes to the biological amplification of adaptive responses leading to beneficial cell performance [66].

The mechanistic basis underlying the phenomenon of hormesis could be schematized as follow [8, 9, 66, 67]: A mild stress activates a molecular sensor, which activates a specific transcription factor. This upregulates anti-stress genes that encode for proteins or enzymes to counteract the original stress. As an example, DNA damage by mild ROS exposure activates the telomere machinery and upregulates the expression of enzymes involved in DNA repair [14, 15, 36]. The telomeric location of various proteins involved in DNA repair supports this process [68]. Another example is oxidative stress that induces upregulation of antioxidant enzymes and molecules to maintain cellular homeostasis [67, 69]. In this context, it has been reported that the mRNA level of different genes coding for DNA repair and antioxidant proteins is highly increased in aged mice [70].

Hormesis is a real biological phenomenon, and hormetic responses have been reported in various biological systems for various stresses [67]. Caloric restriction, mild physical exercise, and mild exposure to ROS are considered as hormetic stressors, leading to adaptive beneficial processes that could retard the aging process.

Caloric restriction (i.e., a long-term moderate reduction in food intake and thus a low-intensity stressor) is the most commonly used intervention that has been reported to extend life in yeast, insects, mice, rats, and monkeys (for review, see [66]). Much evidence from the literature suggests that caloric restriction reduces the level of oxidative damage in lipids, proteins, and DNA (for review, see [2]). The proposed mechanism involved is a decreased endogenous production of ROS and an increased stress resistance via sirtuin-1 [71] and/or increased activity of the endogenous antioxidant enzymes (for review, see [2]). Some studies also suggest that caloric restriction may reduce oxidative damage by enhancing the repair and turnover of lipids, proteins, and DNA (for review, see [2]). Whether caloric restriction has a similar effect in human is unknown. Some recent reports suggest that caloric restriction promotes some beneficial effects in human beings (for review, see [66]), but one should consider that the unintentional exposure to caloric restriction of humans makes the “experimental” conditions uncontrolled. Thus, hormesis for longevity under caloric restriction is likely to be limited to laboratory animals

exposed to a controlled environment. In addition, restricted food intake mimics the period of caloric restriction that is considered normal in the wild [72].

Similarly, the long-term beneficial effects of repeated moderate exercise can be explained by hormesis. Indeed, increase in ROS and other free radicals induced by physical exercise activates adaptive responses inducing antioxidant enzymes and oxidative damage repair systems [69, 73, 74]. Because the induced adaptive mechanisms are systemic, the generalized resistance to stress and the improved physiologic functions associated with exercise could explain its preventive and beneficial effects [69]. Furthermore, exercise-induced adaptive responses seem effective even when training is initiated late in life, thus regular exercise could retard age-related physiologic decline in the elderly [75].

It has been reported that exposure of isolated human skin keratinocytes to subtoxic concentrations of hydrogen peroxide extended cellular life span by slowing down replicative senescence, reducing telomere shortening, and lowering intracellular ROS [76]. The beneficial effects of trace amount of H_2O_2 on life span of keratinocytes were more advantageous than antioxidant treatment such as ascorbic acid. Thus, low concentrations of H_2O_2 extended the life span of cultured keratinocytes by a hormesis effect.

Some criticisms to the beneficial role played by hormesis in aging have been put forward. First, if we consider that aging is a stochastic process with random molecular disorder, it cannot be affected by hormesis: hormesis may affect longevity, but not aging per se [77]. Second, it has been proposed that the mild hormetic stress can only be repeated a limited number of times, otherwise chronic mild stress could accelerate the aging process [78]. Third, all cell types do not respond in a similar way to identical stressful conditions; a given stress favoring hormesis in a young tissue could be deleterious in an old tissue [79]. Finally, N. Minois [80] asked this very pertinent question: “Can human beings have a use of hormetic factors, or are they already benefiting from their effects?” Indeed, because humans are exposed in everyday life to various endogenous and environmental stresses, it is plausible that hormesis responses are already established. It is possible that antioxidant protections, for example, are already optimized in mammals, as the correlation between aging and antioxidant levels is very weak [81]. In contrast, in a very protected environment, such as an artificially controlled laboratory environment, or in protected conditions (island vs. mainland), beneficial effects of mild stress are more obvious [72].

2 Summary and Conclusions

In conclusion, cellular aging, hormesis, SIPS, apoptosis, and necrosis are different net results of the interaction of the cells with their environment. These net results depend not only on the intensity and duration of the stress but also on the efficiency and kinetics of the defense/repair/elimination mechanisms [28, 67]. Our hypothesis is that hormesis is crucial to cell maturation, with activation by mild stress of

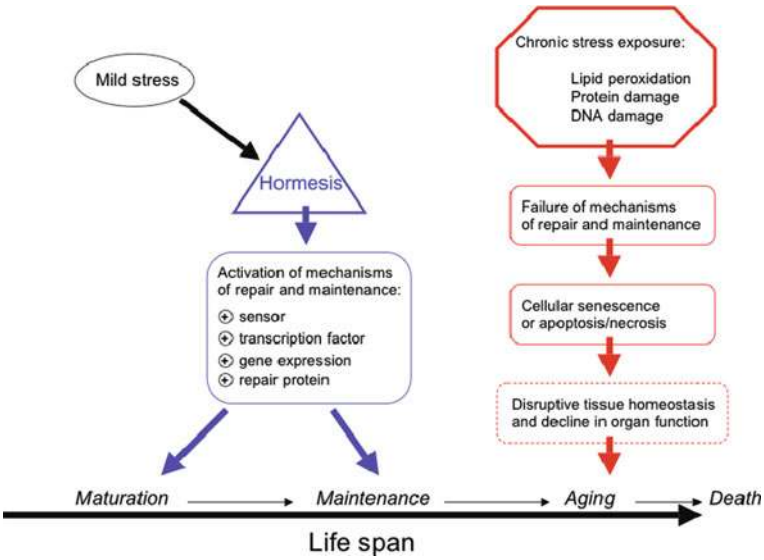

Fig. 1 Importance of hormesis in cell maturation, with activation by mild stress of mechanisms of repair and maintenance to protect cells against subsequent stresses. Cumulative and chronic stress induce progressive failure of these mechanisms, leading to cellular senescence, aging, and ultimately death

mechanisms of repair and maintenance to protect cells against subsequent stresses. In the case of endothelial cells, exposure to mild oxidative stress during the maturation phase of the endothelium will activate protective pathways. It is the cumulative and chronic stress that induce progressive failure of these mechanisms, leading to higher susceptibility to diseases, cellular senescence, aging, and ultimately death. This hypothesis is summarized in Fig. 1.

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Role of Oxidative Stress in Mediating Elevated Blood Pressure with Aging

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Abstract Blood pressure increases with age in men and women. Whether and how oxidative stress plays a role in mediating hypertension has been under intense study for the past 10 years. Although most animal studies have shown that oxidative stress will increase blood pressure, the clinical trials using antioxidants to treat hypertension in humans have not been successful. One theory for how oxidative stress increases blood pressure is that oxidants cause an increase in renal vasoconstriction that increases sodium and water reabsorption by the kidney. However, whether the blood pressure in males and females responds the same to increases in oxidative stress is not clear. There are several problems with regard to the study of oxidative stress: the lack of consistent measurements of oxidative stress, lack of specific inhibitors of oxidants or scavengers of reactive oxygen species, and the lack of specific, selective ways to increase oxidative stress in animal models. Problems in human studies contribute additional roadblocks to answering the questions of whether and how oxidative stress contributes to hypertension, such as genetic differences in the population, lack of power to take into consideration that men and women may respond differently to antioxidants, and lack of separation of genders in analyses of the data. This review will address these issues.

Keywords Hypertension · F2-isoprostanes · Menopause · Oxidative stress · Sexual dimorphism

1 Introduction

Blood pressure increases with aging in men and women. Using ambulatory blood pressure monitoring, studies in humans have definitively shown that men have higher blood pressures than do age-matched premenopausal women. However, after

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menopause, blood pressures increase significantly in women to levels that are similar to or higher than those in men until very old ages when blood pressure becomes similar in men and women [1, 2].

The role that oxidative stress plays in mediating the increase in blood pressure with aging is controversial. Animal studies consistently show that oxidative stress impacts blood pressure and cardiovascular–renal disease, yet few clinical trials using antioxidant supplementation have had positive results to reduce blood pressure or protect against negative cardiovascular outcomes in humans. Thus whether and how oxidative stress plays a role in mediating hypertension and cardiovascular disease with aging is not clear.

2 The Role of Oxidative Stress in Control of Blood Pressure

Rajagopalan and colleagues reported several years ago that infusion of high doses of angiotensin II (Ang II) increases blood pressure via an oxidative stress–mediated mechanism. They found that giving superoxide dismutase prevents the increase in blood pressure and vascular dysfunction that occur with Ang II [3]. Studies performed by other investigators verified that giving superoxide dismutase mimetics, such as tempol, protected against the so-called "slow pressor" response to Ang II in which the increase in blood pressure occurs over several days but was prevented by treatment of animals with tempol [4]. Studies in recent years have shown that infusion of endothelin into animals also causes increases in oxidative stress and leads to hypertension [5].

In addition to giving vasoconstrictors to increase blood pressure, infusing oxidants into animals also increases blood pressure. Cowley and colleagues reported that infusing hydrogen peroxide directly into the renal medulla elevated blood pressure in rats [6]. In addition, we have found that infusion of molsidomine, a superoxide anion donor, further increases blood pressure in already hypertensive animals but not in normotensive controls [7]. Thus, oxidative stress plays a role in mediating increases in blood pressure in many different hypertensive models.

2.1 Blood Pressure, Oxidative Stress, and the Kidney

The key organ for long-term control of blood pressure and body fluid volume is the kidney [8, 9]. According to the renal–body fluid feedback concept, chronic increases in arterial pressure occur as the result of abnormalities in the relationship between renal perfusion pressure and sodium excretion. That is, in order for long-term increases in arterial pressure to occur, a reduction in the kidneys' capacity to excrete sodium and water must be present. A common defect that has been found in all forms of hypertension examined to date, including genetic and experimental models and human essential hypertension, is a hypertensive shift in the "pressure natriuresis relationship."

Therefore, there have been many studies that have evaluated how renal function is affected by oxidative stress. One way oxidative stress can impact blood pressure is by causing renal vasoconstriction that could reduce glomerular filtration rate leading to increased sodium reabsorption. An increase in reactive oxygen species (ROS) leads to an increase in F2-isoprostanes, nonenzymatic products of arachidonic acid [10]. F2-isoprostanes have been shown to have a vasoconstrictor effect in the kidney [11].

Figure 1 provides a tentative hypothesis regarding how oxidative stress may increase blood pressure. Ang II and endothelin, both potent vasoconstrictors, increase blood pressure and oxidative stress. Both of these vasoconstrictors increase expression of NADPH oxidase, the most common source of ROS, in kidneys of animals [3, 12]. Inhibition of NADPH oxidase expression and subunit movement to the plasma membrane, and thus activation, with apocynin results in reductions in blood pressure in hypertensive rats [13, 14]. Furthermore, oxidative stress can increase endothelin synthesis [15], thus setting up a vicious cycle. In fact, Skalska and colleagues recently reported that in aging humans with hypertension, plasma endothelin levels were positively correlated with lower plasma vitamin C concentration and lower ferric reducing ability of plasma, an indicator of the antioxidant capacity of the plasma [16].

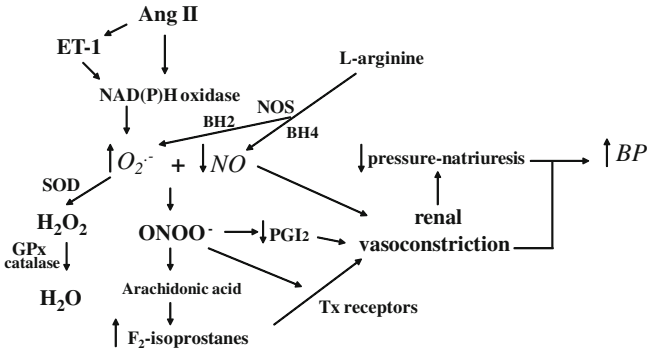

Fig. 1 Schematic of the possible ways oxidative stress could increase blood pressure. Oxidative stress in the form of superoxide is removed from the cell by superoxide dismutase (SOD) and converted to hydrogen peroxide (H_2O_2). H_2O_2 is then converted to water by glutathione peroxidase (GPx) and catalase. Angiotensin II (Ang II) activates NADPH oxidase, the most common producer of superoxide. Ang II can also increase expression of endothelin (ET-1) also leading NADPH oxidase. Nitric oxide (NO) is produced by NO synthase (NOS). However, if tetrahydrobiopterin (BH4) is oxidized to dihydrobiopterin (BH2), NOS produces superoxide rather than NO. Superoxide and NO combine to form peroxynitrite ($ONOO^-$), which causes NO to be unavailable for vasodilation and thus leads to renal vasoconstriction. Peroxynitrite decreases expression of prostacylin (PGI2), a vasodilator, and increases the production of F2-isoprostanes, vasoconstrictors from arachidonic acid. Peroxynitrite also increases expression of thromboxane (Tx) receptors, which are thought to be the receptors for F2-isoprostanes. The increase in vasoconstrictors increases renal vasoconstriction leading to a rightward shift and reduction in the pressure–natriuresis relationship and hypertension

Another source of vasoconstriction in the kidney is the interaction between superoxide and nitric oxide (NO). The combination of superoxide and NO produces peroxynitrite, a potent oxidant [17] (see Fig. 1). The interaction between superoxide and NO leads to loss of bioavailability of NO to cause vasodilation and thus results in vasoconstriction. Furthermore, though peroxynitrite is a vasodilator, tachyphylaxis to peroxynitrite results not only in loss of vasodilator response to peroxynitrite itself but also to loss of response to other vasodilators as well [18]. Thus, peroxynitrite could mitigate the vasodilator effect of NO as well. In addition, peroxynitrite inhibits prostacyclin synthase, thereby decreasing the vasodilator prostacyclin (PGI_2), and stimulates production of thromboxane A_2 receptors [19, 20]. Thromboxane A_2 is another vasoconstrictor. In addition, F2-isoprostanes, nonenzymatic products of oxidation of arachidonic acid, are thought to work through thromboxane receptors to cause vasoconstriction. Thus, increases in oxidation can cause vasoconstriction. Under normal conditions, NO is produced by NO synthase using the cofactor tetrahydrobiopterin. In the presence of an oxidative environment, such as the presence of peroxynitrite, tetrahydrobiopterin is oxidized to dihydrobiopterin. Dihydrobiopterin then interacts with NO synthase to produce superoxide rather than NO, the so-called "uncoupling of NO synthase" [21]. Thus, the increase in oxidative stress sets up a positive feedback to cause further increase in ROS via NO synthase.

The fact that oxidative stress causes renal vasoconstriction is well accepted at this point. Therefore, an increase in oxidative stress with aging should contribute to the increase in blood pressure that occurs with aging. It is not clear then why the clinical studies using antioxidants have not been successful in reducing blood pressure and lowering cardiovascular disease risk.

2.2 Sex Differences in Oxidative Stress

One possibility for the lack of a depressor response to antioxidants in aging individuals is that there may be sex differences in oxidative stress that may not be elucidated in clinical studies or factored for in design of clinical studies. In many clinical trials, they have not been powered to separate data in men and women. Thus, if men responded to antioxidants to reduce blood pressure, but women did not, the study may show negative results.

Estradiol downregulates angiotensin converting enzyme, which converts Ang I to Ang II, thus decreasing the amount of Ang II that is present to cause oxidative stress. In addition, estradiol causes a downregulation of angiotensin AT1 receptors [22], thus decreasing Ang II effectiveness to increase oxidative stress. Estradiol also increases NO production both by increasing intracellular calcium to increase NOS activity and by increasing synthesis of endothelial NOS [23]. Estradiol should offset the increase in superoxide by increasing NO production. In addition, estradiol increases expression of superoxide dismutase to remove superoxide from the oxidative milieu. There is also some evidence that estradiol downregulates the expression

of endothelin [24]. Thus, women should have less oxidative stress than do men. Furthermore, postmenopausal women should have higher levels of oxidative stress than do premenopausal women and men.

As mentioned above, F2-isoprostanes represent the non-cyclooxygenase-mediated conversion of arachidonic acid that reflects levels of lipid peroxidation [25, 26]. In many studies, men have been shown to exhibit higher levels of markers of oxidative stress than women, as measured by higher levels of 15-F2t-isoprostanes (F2-isoprostanes) and thiobarbituric acid–reactive substances (such as malondialdehyde) in the plasma [27–29]. However, whereas postmenopausal women have been shown to have higher levels of another isoprostane, 8-iso-prostaglandin F(2α), than their premenopausal counterparts [29], another study of 121 men and 177 women, aged 19–78 years, showed that plasma malondialdehyde and F2-isoprostanes were significantly higher in women than in men of all ages. Similar observations were made in the Insulin Resistance Atherosclerosis Study [30].

One reason for the discrepancies in whether there are sex differences in oxidative stress in aging individuals may be a consequence of the indicator of oxidative stress used to date. Though the measurement of F2-isoprostanes has been heralded frequently as the most sensitive measure of oxidative stress, Helmersson and Basu found that there were significant (42%) day-to-day variations in plasma and urinary F2-isoprostane levels in 13 healthy men and women on 10 consecutive days [31]. These data suggest that in order to measure differences in oxidative stress in humans, the cohorts must be sufficiently large, and the differences in levels of oxidative stress between groups must be substantial. However, these precautions may not eliminate the discrepancies between relative changes in F2-isoprostanes compared with changes in other measures of oxidative stress, as shown in the Block study, in which measurements of F2-isoprostanes were at odds with malondialdehyde levels [32].

Though the measurements of oxidative stress are not consistent among groups and thus may give additional reasons why many of the clinical trials with antioxidant therapies have not been successful in protecting against cardiovascular outcomes, including lowering blood pressure, another aspect of the human studies should be taken into consideration. It is very likely that there may a genetic component to the relative contribution that oxidative stress may make in mediating cardiovascular disease, and thus the response to antioxidants may reflect those genetic differences. For example, in the Women's Angiographic Vitamin and Estrogen (WAVE) trial, premenopausal and postmenopausal women underwent baseline and follow-up coronary angiography after vitamins E and C therapy for up to 36 months [33]. There was a significant improvement with antioxidant vitamins in coronary minimum luminal diameter as measured with angiography in women who exhibited the Hp 1-1 allele of haptoglobin. However, this was not found in women with Hp 2-2 allele. In contrast, neither flow-mediated, endothelium-dependent dilation nor nitroglycerin-induced vasodilation was improved in any of the women taking vitamins E and C in this trial [34]. These data suggest that there may be a genetic component involved in the mechanisms by which oxidative stress mediates cardiovascular disease. Other investigators suggest that differences in improvement

of coronary outcomes with antioxidant vitamins could be explained by differences in the relative risk levels in the cohorts, with individuals in low-risk populations not showing improvements with antioxidant treatment and thus skewing the results [34]. Therefore, in order for the differences in the response to antioxidants to be measurable/detectable, the cohorts should be carefully chosen and be as homogeneous as possible. However, not only is this requirement difficult to satisfy in a clinical setting, but also the selection of a homogeneous population would undermine the scientific validity of the clinical study, making the findings difficult to be extrapolated to the general population.

2.3 Postmenopausal Hypertension

Menopause occurs on average at 51 years of age and, as mentioned earlier, is associated with an increase in blood pressure in many women and with frank hypertension in others. Numerous mechanisms have been proposed to be implicated in the elevation of blood pressure in postmenopausal women including the generation of ROS. Also as mentioned above, in postmenopausal women, oxidative stress increases, as determined by increases in plasma malondialdehyde or plasma F_2-isoprostane [29, 35]. Furthermore, the longer the time since menopause occurs, the more oxidative stress is present in women [36]. Despite the evidence that oxidative stress increases in postmenopausal women, there is no evidence that the increase in oxidative stress contributes to their elevated blood pressure with age.

3 Studies in Aging Animals Linking Oxidative Stress and Blood Pressure

3.1 Studies in Spontaneously Hypertensive Rats

Blood pressure in male spontaneously hypertensive rat (SHR) is higher than that in female SHR, and this occurs shortly after puberty [37]. However, once the females cease estrous cycling at 10–12 months of age, their blood pressures increase to levels similar to or higher than those in males [38]. In trying to determine the mechanisms responsible for the higher blood pressure in young SHRs, we evaluated the role of oxidative stress. We found that blood pressure in males, aged 3–4 months, decreased with all antioxidants that were tried – tempol, apocynin, and vitamins E and C [13, 39, 40]. In contrast, blood pressure in young females was unresponsive to all antioxidants [40]. We also determined whether increasing oxidative stress, using molsidomine, would further increase blood pressure in SHR. We found that molsidomine increased blood pressure further in young male SHRs, but not in young male WKY rats, a normotensive control strain [7]. We also found that molsidomine failed to increase blood pressure further in young female SHRs.

3.2 Measurement of Oxidative Stress

With these data in mind, we wondered whether the reason why blood pressure was not affected by antioxidants in female SHRs was that they had little if any oxidative stress as measured by typical markers. We found in fact that measurement of F2-isoprostanes (15-F2t-isoprostanes), measured by gas chromatography–mass spectroscopy give equivocal results not only between groups but also between tissues and fluids from the same rats [40]. For example, in plasma there were no differences in F2-isoprostanes between male and female SHRs. We also found that there was no difference in plasma total antioxidant status between the groups, which supports the lack of sex differences in oxidative stress in the plasma. In contrast, in whole kidney tissue, F2-isoprostane levels were slightly higher in males than in females, suggesting that renal tissue oxidative stress was higher in males than in females. In contrast, in urine samples, female SHRs had significantly higher levels of F2-isoprostanes than did males. The F2-isoprostanes were measured at the same time from tissues and fluids from the same rats, removing the possibility of error from using different rats.

In order to evaluate further whether sex differences in oxidative stress are present in tissues of SHR, we used the well-accepted method of lucigenin chemiluminescence in tissue homogenates, which is an index of superoxide production. We measured both the basal production, the capacity of the tissue to produce superoxide per se, and the NADPH-stimulated production, which is an indication of the NADPH oxidase activity as the substrate was added in excess [41, 42]. Basal renal superoxide anion levels were similar in the medulla of males and females but higher in the cortex of females. NADPH-stimulated superoxide anion production, an indicator of NADPH oxidase activity, was similar in the cortex of males and females but significantly higher in the medulla of females. These data, just as the F2-isoprostane data, show sex differences in oxidative stress but not uniformly in one sex or the other. One would anticipate that many of the problems of measuring oxidative stress in humans would be alleviated in genetically homogeneous rats, as the genetic component and the variations in cardiovascular health status should be eliminated. However, we have not found this to be the case in our studies of SHR.

3.3 Measurement of Antioxidant Enzyme Expression

To characterize fully the oxidative stress pathways in SHR, we measured protein expression of antioxidant enzymes in kidney tissue. The antioxidant systems are shown in Fig. 1. The protein expression of Mn-SOD (present in renal mitochondria) was not different between males and females, but CuZn-SOD was higher in the cortex of males yet similar in the medulla of males and females [40]. These data are consistent with those of Sullivan and colleagues who also found no difference in expression of the SOD isoforms in the medulla of male and female SHRs [43]. Furthermore, we found that expression of glutathione peroxidase was higher

in cortex of male SHRs than that in females but was not different between groups in the medulla. Consistent with our data, Sullivan and colleagues were unable to find a sex difference in renal medullary hydrogen peroxide production [43]. In contrast, whereas Sullivan and colleagues found a modest increase in medullary catalase activity with no change in catalase expression in SHRs, we found that whole kidney catalase expression was significantly higher in males than in females, which could suggest that cortical catalase expression may also be higher in males than in females. Thus, the levels of CuZn-SOD, glutathione peroxidase, and catalase were all higher in the kidneys of males than in kidneys of females. We hypothesize that an increase in the expression in the levels of these antioxidant enzymes in males suggests that males have more oxidative stress in their kidneys, as we found in other studies that the response to an increase in oxidative stress in kidneys of normotensive animals is to increase expression of the antioxidant enzymes [7]. Whether there are similar sex differences in expression of antioxidant enzymes in kidneys of humans is not clear, nor is it known whether intrarenal antioxidant levels change with aging in humans.

3.4 Functional Studies to Evaluate the Role of Oxidative Stress on Blood Pressure in SHR

The fact that urinary F2-isoprostane excretion is higher in female SHR is reminiscent of the higher levels of thromboxane, a vasoconstrictor that can impact renal function and blood pressure, that are excreted into the urine of female SHRs compared with that in males [44]. These data were interesting to us because F2-isoprostanes are thought to exhibit biological activity via thromboxane receptors [45]. In order to determine if thromboxane receptors played a role in mediating the blood pressure in male and female SHRs, rats were infused chronically with a thromboxane receptor antagonist [44]. The antagonist had no effect on blood pressure in female SHRs but reduced blood pressure in males. As a thromboxane synthase inhibitor had no effect on blood pressure in either males or females, we reasoned that it was not likely that thromboxane was the ligand for the thromboxane receptor but perhaps F2-isoprostane, and that the increased isoprostane may play a role in the hypertension of male SHRs but not in females.

From our studies in young SHRs, we determined that there were sex differences in measures of oxidative stress and in response to antioxidants. We then began to evaluate the role that oxidative stress may play in controlling blood pressure in aging SHR. We found that male SHRs exhibited increased renal vasoconstriction with reductions in glomerular filtration rate and renal plasma flow compared with that in age-matched females (16–18 months of age) [46]. To address the role that oxidative stress played in mediating the renal vasoconstriction that occurred with aging in male SHR, we gave male and female SHRs either tempol or vitamins E and C, beginning at 8 months of age, and studied them at 16 months of age [40]. As found in young female SHRs, blood pressure in aging females was unresponsive to tempol

antioxidant treatment. Despite the lack of a depressor response, F2-isoprostane levels were reduced slightly with tempol treatment in females, suggesting that oxidative stress was reduced. Also as found in young male SHRs, tempol reduced the blood pressure significantly in aging males. F2-isoprostanes were reduced slightly with tempol in aging males. However, in aging males, vitamins E and C failed to reduce their blood pressure, unlike what we found in young males, whereas in females, vitamins E and C reduced their blood pressure by a small but significant amount (approximately 10%). F2-isoprostanes were not reduced in females with vitamins E and C, suggesting that the reduction in blood pressure with vitamins E and C in aging females was independent of oxidative stress. In males, vitamins E and C slightly reduced F2-isoprostanes, despite no depressor response.

These data were surprising and difficult to interpret until years later when we found a study by Barella and colleagues in which they reported that vitamin E in rats reduces cholesterol biosynthesis [47]. Cholesterol is the substrate for sex steroid synthesis. We had shown previously that androgens are an important mediator of hypertension in male SHR, as castration attenuates the increase in blood pressure in male SHRs to similar levels as found in females [37]. Furthermore, the depressor response to the NADPH oxidase is not present in males that are castrated [13]. We had shown previously that plasma testosterone levels decrease with age by about 70% in male SHR. We therefore postulate that the reduction in androgens with aging in male SHR is the reason why vitamins E and C were unable to reduce blood pressure in aging males. In contrast, in aging females, we found that plasma testosterone is increased fourfold compared with that in young females [48]. In addition, we have unpublished data that blockade of the androgen receptor with flutamide reduces blood pressure in aging female SHRs, suggesting that androgens contribute to their hypertension (Yanes and Reckelhoff, unpublished data). Thus we postulate that if vitamin E reduced testosterone biosynthesis in aging females, this was the mechanism by which blood pressure was reduced in aging females with vitamins E and C.

Future studies will be necessary to confirm these hypotheses. However, they point to other considerations in human studies of antioxidants and their impact on hypertension. The mechanisms we believe are in play with antioxidant treatment may not be the whole picture. Furthermore, it is not clear that the mechanisms that have been identified as being important in oxidative stress–mediated increases in blood pressure hold true in humans.

It is still not clear from animal studies how or why oxidative stress affects blood pressure. As mentioned previously, when tetrahydrobiopterin is oxidized to dihydrobiopterin, NOS synthesizes superoxide rather than NO. This is the NOS uncoupling phenomenon. As proof of this concept, we gave young intact and castrated male SHRs tetrahydrobiopterin for 2 weeks with the hypothesis in mind that androgens are a causative factor in NOS uncoupling leading to higher blood pressure in males than in castrated males. We found that indeed tetrahydrobiopterin reduced blood pressure in intact males but had no effect on castrated males [49]. However, when we evaluate the levels of testosterone in the rats, we found that tetrahydrobiopterin reduces plasma testosterone levels in male SHRs to levels not too dissimilar from

that in castrated males. When we then fixed the plasma testosterone levels in castrated males to levels found in intact males, tetrahydrobiopterin failed to reduce the blood pressure in the intact males. Again, these data point to the need for caution in prescribing effects to the oxidative stress cascade when they may be due to interactions in other less complex systems.

3.5 Studies to Mimic Endothelial Dysfunction in Aging

In another series of studies, we sought to mimic the endothelial dysfunction that occurs with long-term hypertension as found in aging individuals. In these studies, we employed tempol as an antioxidant and also blocked NOS with nitro-L-arginine methyl ester (L-NAME) for 2 weeks in young male SHRs [50]. In rats given only tempol, blood pressure and oxidative stress, as measured by lucigenin chemiluminescence, were decreased. With L-NAME alone, oxidative stress was not changed, but blood pressure increased, as has been previously shown by ourselves and others [51]. However, when tempol as given with L-NAME, although oxidative stress was reduced, as found with tempol alone, blood pressure did not decrease. These data are extremely important for the determination of the contribution to oxidative stress in mediating hypertension in aging individuals. Perhaps another reason why blood pressure has not been lowered in aging hypertensive humans in clinical trials is that these individuals have likely been hypertensive for several years, and with aging and hypertension there is likely endothelial dysfunction and low levels of NO. In this case, when there is little NO, antioxidants are incapable of reducing blood pressure as antioxidants work by scavenging superoxide and freeing up NO to become bioavailable to cause vasodilation and loss of sodium and water by the kidney leading to reductions in blood pressure. Thus, we should not expect antioxidants to reduce blood pressure in aging humans with endothelial dysfunction.

3.6 Lack of Tools to Study ROS and Blood Pressure Regulation

One additional problem with the study of oxidative stress in humans or experimental animals is that antioxidants or scavengers of ROS are nonselective. For example, though tempol is thought to be a superoxide dismutase mimetic, it also affects the sympathetic nervous system, independent of oxidative stress, and may decrease renal sympathetic nerve activity [52]. Sympathetic activity is important in modulating renal hemodynamics and renin release and can thus affect blood pressure regulation. Tempol also causes a reduction in NO bioavailability [53]. If tempol acted only as a superoxide dismutase scavenger, it would decrease the amount of superoxide leaving a greater concentration of NO to cause vasodilation. Apocynin, the NADPH oxidase inhibitor, not only prevents the subunits of NADPH oxidase from migrating and assembling at the plasma membrane but also decreases the synthesis of the subunits [13]. In addition, apocynin can cause oxidative stress on its

own. As for factors that could increase oxidative stress in an experimental setting, Ang II and endothelin are thought to increase oxidative stress, but they affect many other systems and have direct effects of sodium and water handling by the kidney, independent of oxidative stress. Finally, molsidomine, also known as SIN-1, increases superoxide production, but it also releases NO. Thus, the background level of oxidative stress plays an important role in whether the net effect of molsidomine is to produce NO, superoxide, or peroxynitrite [7]. Until there are better tools with which to study oxidative stress, it will be difficult to determine the contribution of oxidative stress to blood pressure control in aging individuals.

4 Summary and Conclusions

Oxidative stress increases with aging. How it effects blood pressure is still not completely understood. It is likely that there are sex differences in age-related oxidative stress. It is possible that there are different reactive oxygen and nitrogen species produced in men and women that impact blood pressure differently. Aging women may be able to upregulate the synthesis of antioxidant enzymes better than do men to compensate for increases in ROS and thus decrease their impact on blood pressure. It is also possible that blood pressure in women is independent of oxidative stress as we have found in SHRs. Finally, the tools to study oxidative stress, the scavengers, the methods to assess ROS, and the methods to increase oxidative stress must be improved to be more specific and selective in order to evaluate fully the contribution of oxidative stress to blood pressure regulation. All these possibilities would explain why clinical studies using antioxidants fail to show a reduction in blood pressure in aging individuals. However, in all likelihood, the fact that long-term hypertension, as occurs in most aging individuals, is associated with endothelial damage and subsequent dysfunction is the major reason why clinical trials using antioxidants have been unsuccessful. Without the ability to produce NO, antioxidants will not be able to reduce blood pressure.

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Part III
The Nervous System

Melatonin, Oxidative Stress, and the Aging Brain

Stephen Bondy and Edward H. Sharman

Abstract The changes associated with brain aging are discussed with emphasis on altered oxidative and inflammatory events and on mitochondrial dysfunction. Many of these changes are exacerbated in a variety of age-related neurologic diseases. This commonality has led to the idea that similar therapeutic approaches may be used in the treatment of several apparently unrelated neurodegenerative disorders. When aspects of these diseases are modeled in experimental animals and cell lines, the application of melatonin has been reported to be advantageous. The means by which melatonin can be protective probably is mediated by way of activation of melatonin receptors, of which several subtypes can be identified. This can initiate a sequence of intracellular signaling events and by activation of transcription factors lead to altered gene expression. The culmination of this cascade can lead to increased levels of antioxidant enzymes and depressed levels of inflammation. Melatonin may be useful both in the retardation of nonpathologic brain aging and in the amelioration of chronic brain disease associated with aging. The inexpensive nature and ready availability of melatonin together with its very low toxicity reinforce the need to place the beneficial properties of this agent on a firmer basis.

Keywords Melatonin · Aging · Neurologic disease · Neurodegeneration · Oxidative stress · Inflammation

1 Introduction

Brain senescence involves excess free radical generation, elevated levels of basal immune and inflammatory activity, together with impaired mitochondrial function.

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A large number of genetic phenotypic changes occur with senescence. Some of the general changes taking place include deletions of specific portions of mitochondrial DNA [1, 2], altered immune function, and an increasingly pro-oxidant milieu concomitant with reduced antioxidant activity. Such changes have often been described as associated with brain aging [3–6]. Common neuropathologic changes include the deposition of lipofuscin and other insoluble materials such as amyloid plaques and neurofibrillary tangles. These inclusions are resistant to normal intracellular proteolytic processes and thus accumulate within and around the cell. Though such changes may characterize specific neurodegenerative disorders, they are also found with normal aging. For example, as well as being characteristic of Alzheimer's disease, amyloid plaques are present in half of the brains from an apparently normal elderly population [7]. Such indigestible inclusions consist of protein and carbohydrate components. Their accumulation is likely to disrupt cell function, in a parallel manner to the more spectacular accretion and consequent distortion of neuronal geometry seen in genetic gangliosidoses. Peptides within these complexes are cross-linked in a β-configuration, and the formation of β-sheets forms the basis for their resistance to normal ubiquitinylation and cleavage by proteases.

1.1 Oxidative Stress and Brain Aging

There are several reports suggesting that some age-related damage to the brain may be caused by excessive activity of reactive oxygen species [3, 6]. Many key intracellular constituents are vulnerable to oxidative or nitrosylative damage, and this may increase with age (reviewed in [8]). Macromolecules are especially liable to injurious oxidative modification. These include proteins [8–11], nucleic acids [12], and lipids [13]. Although an increased level of pro-oxidant activity with age has frequently been reported, the establishment of a causal relation whereby oxidant events result in impaired neurologic or behavioral status is more difficult to unequivocally demonstrate. Evidence of this nature includes the finding that treatment of aged gerbils with a free radical spin trapping agent, α-phenyl-*N*-*tert*-butyl nitrone (PBN), can reverse some age-associated loss of recall ability as tested by the use of a radial arm maze [14]. In a mouse line developed for an unusually rapid onset of senescence, PBN can also diminish the degree of free radical–induced protein oxidation, and this is concomitant with improved behavioral performance [15]. Together with PBN-induced improvement of ischemic damage in stroke models using rats and marmosets, this has led to a phase 3 clinical trial for treatment of acute ischemic stroke with a derivative of PBN, disodium 4-[(*tert*-butylimino) methyl] benzene-1, 3-disulfonate *N*-oxide (NXY-059) [16].

1.2 Inflammation and the Aged Brain

The dysregulation of immune response mechanisms within the aging brain has been widely reported in recent years. Several indices of immune activity in the nervous system are persistently elevated with age despite the absence of provocative stimuli [17–20]. The microglia of the brain can act as the brain's macrophages, and

their level of activation is markedly increased in the white matter of the brains of aged rats [21] and primates [22]. The significant role of microglia in promoting adverse inflammatory processes is exemplified by the report that inhibition of microglial activation reduces the degree of damage to dopaminergic neurons caused by the selective neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [23]. However, elevated basal inflammatory activity in aged animals is not accompanied by keener immune surveillance and an improved ability to respond to an inflammatory stimulus of exogenous origin. On the contrary, the ability of the intrinsic immune system within the CNS to react to a material such as lipopolysaccharide is considerably diminished in the elderly mouse [19]. Such a dampening of the ability of immune defenses to detect relevant extraneous stimuli implies an impaired ability to mount an effective response to pathogens. Persistent levels of basal low-level inflammatory activity in the absence of exogenous stimuli is likely to be undesirable and to have deleterious consequences. The lung illustrates the injurious nature of a prolonged and futile immune response where the chronic presence of mineral particles leads to an ineffectual phagocytic attack by alveolar macrophages. This ultimately leads to severe pathologic changes involving inflammatory cytokines [24]. Analogous futile inflammatory responses in the brain may be toward aggregated amyloid peptide and other insoluble proteinaceous inclusions [25]. Thus, the elevated levels of microglial activity found in aged animals [22] may reflect a nonproductive aberrant response to endogenous factors, which cannot be resolved by raising an immune response.

1.3 Mitochondrial Dysfunction and Brain Aging

The mitochondrion is the site of the respiratory chain, and a side effect of this activity is the generation and diffusion into the cytoplasm of reactive oxidizing species; hence it also constitutes a major target of cumulative oxidative events. In fact, most of the reactive oxygen species produced within the cell have their origins from such leakage out of the respiratory chain. The electron transport is a very efficient process, but it has been estimated that around 2% of oxygen utilized escapes complete reduction to water and can form transient reactive intermediates [26]. Moreover, the efficiency of mitochondrial respiration may be further reduced with aging leading to a greater production of superoxide and other oxidant species [27–29]. After a chemical challenge, mitochondria from aged mice produce more reactive oxygen species than do mitochondria from the corresponding young mice [30]. The reduced efficiency of aged mitochondria can thus elevate levels of oxidative damage. The closest targets are most likely to be at risk, and these are structural elements of the mitochondria themselves.

It has been proposed that the increased levels of free radical generation within the aged brain are attributable to reduced efficiency of mitochondrial energy production [31, 32]. Impaired respiratory function may lead to oxidative stress and also to impairment of antioxidant enzyme systems [33]. Decline of mitochondrial functioning has also been associated with various age-related neurodegenerative

diseases including Alzheimer's, Parkinson's, and Huntington's diseases and amyotrophic lateral sclerosis [34]. Potential targets of oxidative damage to mitochondria include both their inner and outer membranes, and mitochondrial DNA (mtDNA). mtDNA is relatively exposed in comparison with nuclear DNA as it is located near the mitochondrial inner membrane and not enclosed within basic histones as is nuclear DNA. Furthermore, the mitochondrial DNA repair mechanism lacks the effectiveness and sophistication of its nuclear counterpart. Consequently, the degree of DNA damage in aging humans is 10 times greater in the mitochondria than in the nucleus [35]. In addition, the extent of mitochondrial damage is 15-fold greater in people over 70 years of age than in the younger population. This is evidenced by greater levels of gene deletions and by a much higher level of 8-hydroxy-2-deoxyguanosine in mtDNA [36]. As judged by deletion frequencies, the mutation rate of mtDNA is many fold greater in old mice relative to their young counterparts [37]. A high level of mtDNA deletions has been found to lead to reduced efficacy of mitochondrial respiration [38]. The substantia nigra is especially vulnerable to oxidative damage due to the potential of dopamine for catalytic oxidation. This may account for the selective vulnerability of dopaminergic midbrain neurons to DNA deletions in both Alzheimer's disease and Parkinson's disease [39]. Mitochondrial deletions are a driving force behind premature aging and may also have a causal role in many age-related pathologies [40]. It may be that mitochondria containing characteristic deletions, although less efficient in performing oxidative phosphorylation than normal mitochondria, are able to divide faster [41]. This raises the issue of whether such "malignant mitochondria" can gradually supplant healthy mitochondria during aging [42].

Mitochondrial dysfunction can also play an important role in the regulation of processes of cellular self-destruction, apoptosis, autophagy, and necrosis [8, 9, 43]. Control of these processes that relate to cell death is very responsive to imbalances in cellular homeostasis as gauged by such measures as Ca^{2+} levels [44] and cellular redox status [45].

2 The Treatment of Chronic Neurodegenerative Disorders

Therapeutic approaches to the most prevalent disease associated with human brain aging, Alzheimer's disease (AD), have often included anti-inflammatory regimens. These treatments have been validated by epidemiologic studies suggesting that the extended use of anti-inflammatory agents (e.g., the chronic use of aspirin by patients with arthritis) reduces the risk of AD [46]. Other epidemiologic data have suggested that antioxidants may be of value in lowering the incidence of AD [47]. Such reductions in incidence of AD attributed to anti-inflammatory or antioxidant agents could also be the result of deceleration of changes occurring during normal brain aging, rather than from effecting a direct mitigation of AD-specific changes. The search for agents with properties that target the nervous system and restore a biochemical and behavioral profile trending toward that found in younger animals may ultimately be of value in treatment of a range of distinct neurologic diseases.

The selection of appropriate materials whose prolonged usage may lead to beneficial changes within the aging central nervous system poses some distinctive problems. For example, broad, nonspecific reduction of pro-oxidant activity within the brain may disrupt those physiologic signaling events that rely on reactive oxygen species. This includes the involvement of superoxide anion in the inflammatory response. Nitric oxide (NO) is a relatively stable free radical that can combine with superoxide to form a very potent oxidant, peroxynitrite, but NO also plays an important role in the modulation of both intracellular and intercellular signals. NO has a series of key functions relating to blood supply and neurotransmission. Although protein nitrosylation is elevated with aging [22, 48], global inhibition of nitric oxide synthase aimed at protecting against neurotoxic damage [49] could have unanticipated adverse consequences, and thus such a broad-based strategy could be unsuitable. NO may be important in the prevention of cardiovascular aging by modulation of vascular tone [50].

2.1 The Potential Retardation of Brain Aging by Melatonin

The basis for selecting melatonin as an agent having the potential for slowing the onset of age-related oxidant events is based on reports from several laboratories [51–54]. A proportion of ingested melatonin can access the brain in an unmodified form [55]. On the other hand, the ability of several other potentially beneficial compounds to reach the brain is often limited due to their excessively lipophilic or water-soluble characteristics. For example, treatment of humans in one arm of the DATATOP study investigating the potential mitigation of Parkinson's disease (PD) involved α-tocopherol administration. Even after many months of such treatment, levels of this vitamin were not equilibrated within the cerebrospinal fluid, and were still rising [56]. Melatonin may be of *specific* value to the CNS where it is able to induce antioxidant genes [57]. This may account for its ability to attenuate lipopolysaccharide (LPS)-induced inflammatory responses [19, 58, 59] and to ameliorate the indices of anxiety induced by LPS [60]. β-Amyloid–induced interleukin secretion and production of reactive oxygen species are also suppressed by melatonin [61, 62]. Supercoiled mtDNA in a low entropy state in brain can be converted into more random circular and linearized forms by ethanol, and this can be attenuated by concurrent treatment with melatonin [63]. There is a considerable amount of evidence for the utility of melatonin in partially reversing changes in both the biochemical and behavioral profiles of older animals [48, 64]. Pinealectomy, which removes the major source of melatonin synthesis within the brain, may speed up changes characterizing aging [65, 66].

The use of melatonin to slow down the progression of undesirable age-related events is also supported by reports that either melatonin treatment or pineal grafting can retard the thymic involution that characterizes aging [33, 67]. It may be that aging is fundamentally an event programmed by the pineal gland [68].

The potential utility of melatonin in the deceleration of brain aging can be roughly classified into three major areas of study: (1) The application of melatonin as mitigation of overall systemic aging, including beneficial effects not confined

to the CNS such as maintenance of an effective immune function and extension of the life span. (2) The use of melatonin in the mitigation of undesirable changes associated with nonpathologic brain aging not associated with a specific neurologic disease. (3) The potential benefit of melatonin use in treatment of distinct neurologic diseases. This latter benefit has been investigated using genetic mouse lines modeling specific neurodegenerative diseases of humans.

2.1.1 Melatonin and Overall Phenotypic Aging

Extension of life span of both vertebrate and invertebrate species treated with melatonin has been described on several occasions [69–71]. This has been attributed to its ability to induce antioxidant enzymes [72] and to reduce age-related elevation of lipid peroxidation [73]. Such findings imply that melatonin has effects on the general systemic well-being rather than acting on a single organ. Such extension of longevity by melatonin has also been reported for several single-celled organisms [74]. In parallel, pinealectomy can reduce longevity [75]. This apparently beneficial effect of melatonin is further evidenced by the ability of melatonin to restore the reproductive cycle of aged mice [76] and the effective functioning of the systemic immune system of aged animals [77]. The age-related increase in susceptibility to the onset of cancer with age can be partially relieved by melatonin [75]. An inverse relation exists in several tissues, between melatonin levels and age-related oxidative damage to DNA, suggesting that this agent may exert antioxidant effects in a wide range of tissues [78].

2.1.2 Specific Effects of Melatonin on Events Relating to Brain Aging

Some of the biochemical, physiologic, and behavioral changes associated with brain aging in the absence of clinical disease include memory deficits, cerebral arterial thinning, and deposition of lipofuscin and amyloid plaques. All of these may be attenuated by chronic treatment with dietary melatonin [79–83]. Melatonin can restore the ability of the aging brain to respond to an inflammatory stimulus [19], and this is in concordance with reports of favorable effects of melatonin on the circulating immune system (described earlier). However, melatonin can also reduce excess immune responsivity (discussed in a later section). Rather than being simply pro- or anti-inflammatory, melatonin has a subtle immunoregulatory capacity.

2.1.3 The Potential for Melatonin Treatment of Specific Neurologic Disease

There are many descriptions of the potentially beneficial effect of melatonin on various neurologic and psychiatric disorders and their relevant animal models. These disorders typically become increasingly prevalent with age.

A review must judge the preponderance of current evidence and summarize the most established findings to serve as a platform for design of new studies. Considering age-related diseases with a slow rate of progression such as AD and

PD as well as aging itself, an obvious generalization is that any beneficial effects of melatonin are likely to require prolonged administration, most likely preceding the appearance of overt signs of disease. For instance, in the case of AD, a disease characterized by depressed levels of melatonin in the cerebrospinal fluid [84], preventing plaque deposition may require that exposure to exogenous melatonin be initiated in the young animal, preceding the appearance of any amyloid deposits [85].

Several reports suggest the potential effectiveness of melatonin for treating PD. Inhibition of lipid peroxidation, hydroxyl formation, and protection of nigral dopaminergic neurons in MPTP-treated rodents can be protected against by melatonin (reviewed in [86]), and exogenous melatonin has been shown to protect against L-DOPA auto-oxidation in the rat thereby increasing the availability of this drug to the striatum [87]. However, there may be a hazard in using melatonin to treat relatively acute animal models of PD involving MPTP or 6-hydroxydopamine, as the PD-like symptoms in this case may actually be worsened by melatonin application (reviewed in [88]), perhaps by inhibition of dopamine release [89].

Melatonin is also protective in preventing the oxidative damage that follows ischemia/reperfusion brain injury. This effect, which is evidenced by reduced infarct volume, necrotic neuronal death, together with diminished neurologic deficits and increased number of surviving neurons, can shield brain function [90]. The beneficial properties of melatonin in countering stroke injury can be manifested even if administered 24 h after an ischemic lesion [91].

Studies using animal models have often focused on the effect of melatonin on three major processes associated with many neurologic diseases as well as non-pathologic brain aging. This classical triad consists of (i) oxidative damage to macromolecules, (ii) persistent inflammation, and (iii) low level but prolonged hyperexcitation (excitotoxicity). All of these have been described as being mitigated by melatonin. These three classes of event, though differing, are closely interrelated and can often occur together. In consequence, it can be difficult to pinpoint the primary event that leads to a cascade of secondary changes. However, the antiexcitatory properties of melatonin are likely to be indirect changes derived from the initial antioxidant properties of melatonin [92, 93].

The problem of determining the primary initial locus of melatonin's actions and elucidating the likely chain of events that ensues is discussed next.

3 Processes That May Underlie the Ability of Melatonin to Modulate the Aging Process

3.1 Melatonin as an Antioxidant

Melatonin is present in bacteria, plants, eukaryotes, and fungi as well as all phyla of multicellular animals, and it has been suggested that its initial evolutionary role was as an antioxidant [74]. Melatonin has been reported to possess antioxidant properties

in both tissue culture and in intact animals. An unresolved issue is whether melatonin acts as an antioxidant *directly* or acts by promotion of key pathways involved in the disposition of free radicals [94]. The evidence for a direct effect rests on the fact that melatonin can act as a powerful free radical scavenger in isolated cell-free systems [95, 96]. However, it has also been reported that melatonin can act as a pro-oxidant in such systems [52, 97]. A key factor in determining this issue may be consideration of levels of free melatonin within the brain. Melatonin is present there at a concentration (around 4 pM) that is only 5% of that found in serum [55]. Therefore – unless it were to be highly concentrated in a localized area – melatonin can make little direct free-radical scavenging contribution in comparison with predominant antioxidant species such as glutathione (present in millimolar amounts) and α-tocopherol (in cells at ca. 1 μM [98]).

Melatonin is present in cerebrospinal fluid (CSF) at concentrations actually higher than serum, suggesting that the pineal may be a source of blood-borne melatonin [99]. This could account for the diurnal flux of melatonin content in both blood and cerebrospinal fluid [100]. The evidence for the involvement of activation of melatonin receptors in accounting for melatonin's antioxidant potential is described in Section 3.3.

3.2 The Role of Melatonin in the Regulation of Immune Function

Basal levels of inflammatory cytokines, their transcription factors, and glial fibrillary acidic protein (GFAP), a marker of astroglial and intrinsic immune activation, are elevated within the aging brain [46, 101, 102]. These evidences of immune activation are even more pronounced in AD [102]. Melatonin treatment reduces these age-related increases in proinflammatory activity [48, 103, 104]. The causal relation between inflammatory and oxidative events is unclear as inflammation involves oxidative activity, and free radicals can reciprocally recruit an immune response.

3.3 Melatonin Receptors and Enzyme Induction

Within the brain, there are three major plasma membrane receptors for melatonin (Table 1). Two of these, MT1 and MT2, are coupled to G-proteins whose activation leads to depression of levels of cyclic AMP. The third, MT3 or NQO2, is a quinone reductase with poorly understood in vivo function. However, the existence of additional melatonin binding sites in the nucleus of many cell types suggests mechanisms of action other than through plasma membrane receptors [105]. The specificity of melatonin may reside in its properties as a neurohormone, which affects transcriptional events in the CNS [57], rather than as a nonspecific antioxidant.

Table 1 Melatonin receptor subtypes and characteristics

Name	GenBank designation	UniProt designation	Other designations	Properties
Melatonin receptor type 1a	Mtnr1a	MTR1A (Q61184)	Mel1a, ML1a, ML1, MT1	353aa plasma membrane GPCR
Melatonin receptor type 1b	Mtnr1b	MTR1B (Q8CIQ6)	Mel1b, ML1b, MT2	364aa plasma membrane GPCR
NAD(P)H dehydrogenase, quinone 2; NRH:quinone oxidoreductase 2	NQO2	NQO2 (Q9JI75)	ML2, MT3	230aa zinc-binding cytoplasmic enzyme

Note: aa = amino acid

A wide range of antioxidant enzymes is induced by melatonin including glutathione peroxidase, catalase, and superoxide dismutases [106]. These protein changes are paralleled by altered levels of gene expression of oxidative enzymes [107]. In addition, levels of some pro-oxidant enzymes such as lipoxygenase and nitric oxide synthetase are depressed after melatonin treatment [48, 108]. Several kinds of cytoplasmic melatonin receptor involve G-protein transduction and modulate transcription. MT1 activation depresses cAMP responsive-element binding protein (CREB) and stimulates extracellular-signal-regulated kinase (ERK) [109]. MT2 levels are depressed in AD [110]. Changes in these signaling pathways may form the basis of the alteration in gene expression effected by melatonin.

The interaction of melatonin with its MT3 receptor may contribute to understanding melatonin's action at pharmacological concentrations, for, despite tight binding to MT3 (K_i = 280 nM), melatonin inhibits MT3 only modestly (IC_{50} = 43 μM). MT3 has been characterized as a "toxicity enzyme" [111], based on the reduced sensitivity of MT3-knockout mice to the toxic effects of the MT3 substrate menadione [112]. Thus, melatonin inhibits the (possibly toxic) activity of MT3 activity at just the concentrations where it is found to be protective pharmacologically [111].

Knockout strains of mouse lacking either MT1 or MT2 have been developed [113]. These mutants indicate that there is a limited functional redundancy between the receptor subtypes in the suprachiasmatic nucleus.

3.4 The Link Between Aging and Circadian Events

While the endogenous circadian clock seems to decline more slowly with age, the body's ability to synchronize properly with external light cycles is diminished more rapidly [114]. There is a connection between the length of the photoperiod and aging. The mean life span of the prosimian primate *Microcebus murinus* is

decreased 28% by an accelerated photoperiodic regimen [115]. Moreover, a shortened diurnal cycle is associated with diminished nocturnal melatonin secretion [116] and can hasten the appearance of several behavioral deficits associated with aging [117]. It is interesting that the extreme longevity of healthy centenarians is associated with a pronounced diurnal flux of melatonin [118]. Light suppresses melatonin production in humans. In consideration of other evidence presented, this linkage between reduced melatonin levels and accelerated aging may be more than merely correlative. Levels of both MT1 and MT2 receptors are very high within the suprachiasmatic nucleus (SCN), the site of circadian rhythm regulation. However, levels of expression of both MT1 and MT2 mRNAs in the SCN are identical in senescence-accelerated and senescence-resistant mouse strains [119].

3.5 Melatonin and Mitochondria

There are many references documenting the ability of melatonin to maintain indices of mitochondrial health. These include downregulation of Bax, caspases, and inhibition of mitochondrial DNA fragmentation, apoptosis, cytochrome c release, and closure of the permeability transition pore. Depression of these adverse events is accompanied by induction of Bcl-2 and improved function of the respiratory chain and ATP synthesis [120–122]. Maintenance of mitochondrial glutathione levels has been also attributed to melatonin [94, 123].

Concepts concerning the possible mechanisms of melatonin's protection of mitochondrial function fall broadly into two classes; namely, direct effects of melatonin upon mitochondria and indirect effects after induction of repression of key proteins. There is little evidence of a direct effect of melatonin upon mitochondrial constituents. Direct inhibition of the mitochondrial permeability transition pore has been shown in isolated systems, but this requires micromolar concentrations of melatonin [124]. The weight of evidence suggests that mitochondrial protection is largely consequent to the induction of antioxidant enzymes and repression of apoptotic pathways. The low levels of melatonin present in tissues cannot afford much direct antioxidant power, but much amplification is provided by any action by way of gene regulation.

3.6 Summary of Mechanisms of Melatonin Action and Suggestions for Future Work

The simplest way to account for the multiplicity of effects of melatonin is to posit an early alteration of gene expression consisting of depression of mRNAs for immune-related cytokines and elevation of those for antioxidant proteins. This would lead to many of the reported enzymic changes resulting from melatonin treatment. Upstream of this may be activation of transcription factors after binding to cytoplasmic melatonin receptors or changes due to melatonin acting directly on

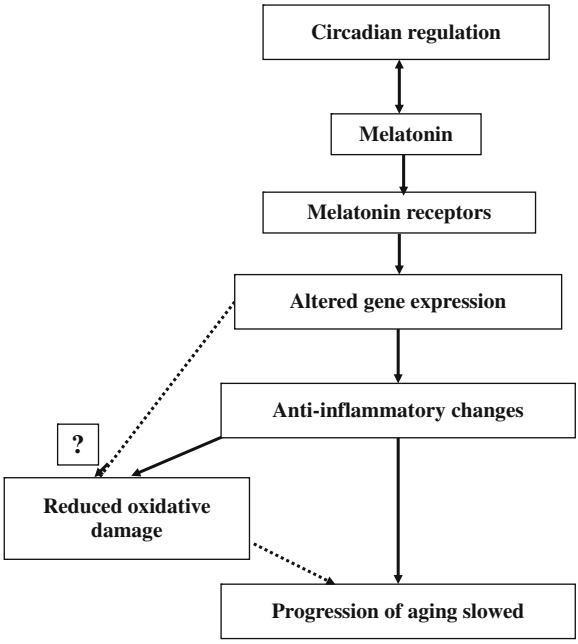

Fig. 1 Potential relationships between regulated melatonin levels and their consequent effect on brain aging

nuclear receptors. Melatonin receptors are not well characterized, and no specific antagonists are available. Thus, circulating melatonin levels, which fluctuate under circadian influence but which are depressed with age, could dynamically influence levels of oxidative and inflammatory activity (Fig. 1). Further research in characterizing melatonin receptors could lead to better delineation of the sequence of events by which melatonin exerts its effects.

4 Conclusions

The median age of the U.S. population is rapidly increasing, and this will lead to a corresponding increase in the incidence of many age-related syndromes. Aging is often associated with both memory and motor deficits including impaired locomotor, postural, and balancing skills. The potential for major increases in incidence of neurodegenerative disorders will be especially pronounced in view of the declining cardiovascular death rate. Retardation of the appearance of changes found with nonpathologic aging could postpone the clinical onset of diseases such as Parkinsonism and Alzheimer's disease. It may be that one of the most rewarding approaches to mitigation of the societal effects of these diseases lies in the deceleration of changes associated with normal cerebral senescence that are not specifically associated with any neurologic disease. Although the onset of neurologic disease generally does not represent merely an acceleration of normal aging, it is obviously based on a platform

of aging. Both normal aging and pathologic processes in part involve changes in the same loci. The identification and protection of such common targets can be valuable in the development of strategies designed to delay the manifestation of common neurodegenerative disorders. The slow progression over a substantial portion of an individual's adult life span with these conditions suggests that, to be effective, treatments such as dietary supplementation need to be followed over many years. This lengthy treatment of essentially "well" patients requires that both the effectiveness and safety of such regimens be rigorously investigated – initially in animal models – before their widespread use can be confidently advocated.

As only 9% of Americans eat the recommended five servings of fruits and vegetables daily [4], the opportunity for retarding neural aging by modifying the intake of exogenous nutrients is high. Dietary supplementation as a means of delaying age-related neural disorders is more likely to be adhered to than any regimen based on caloric restriction, which is known to retard aging processes. However, no dietary supplement has yet been found as effective as caloric restriction in extending life span [125]. The consumption of melatonin on a regular basis may help to mitigate some aspects of brain aging and appears to pose very little downside risk. Any potential benefits will occur over a long time and will thus be hard to definitively document in humans. Data derived from animal studies suggest the value of melatonin. Aging is a multifactorial process, and the advocacy of a single remedial agent is clearly incomplete. Unlike the focused pharmacological remediation for a specific disorder, amelioration of the wider range of changes associated with normal aging is obviously best addressed with a multiplicity of nutritional and physiologic modifications. However, melatonin has utility as a means of retarding some aspects of brain aging. In addition to the experimental evidence described above, melatonin has several positive features that enhance its candidacy as a therapeutic agent. Melatonin is an evolutionarily ancient neurohormone that has very low toxicity and no carcinogenic properties, and this makes it a very safe compound. It is also readily available and has a low cost. Melatonin is likely to constitute an inexpensive and nonhazardous means of maintaining the functionality of the aging brain.

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The SAM Strain of Mice, a Higher Oxidative Stress, Age-Dependent Degenerative Disease, and Senescence Acceleration Model

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Abstract The Senescence-Accelerated Mouse (SAM) strain of mice is a group of related inbred strains consisting of a series of SAMP (accelerated senescence-prone) and SAMR (accelerated senescence-resistant) strains. Compared with SAMR strains, SAMP strains show accelerated senescence processes, shorter life spans, and earlier onset and more rapid progression of age-associated pathologic phenotypes similar to human geriatric disorders. Based on these observations, the SAM strain was developed as a model for senescence acceleration and age-associated disorders. Numerous studies using this strain of mice have been conducted for more than 30 years to clarify mechanisms of senescence acceleration and pathogenesis of age-associated disorders. Many of the aforementioned mechanisms highlight (a) the oxidative stress status that results from (b) mitochondrial dysfunction as critical factors in possible mechanisms that accelerate the senescence process and cause and/or aggravate age-dependent degeneration of various body tissues. Because the SAM strain was developed as an animal model of geriatric disorders, many experiments to intervene with senescent phenotypes were done from the very beginning. Recently, many substrates with protective effects on mitochondria were tested to determine their effectiveness for treating senescent phenotypes and age-associated disorders seen in SAMP mice. The aforementioned SAM strains can serve as useful tools to understand cellular mechanisms of age-dependent degeneration and to develop clinical interventions.

Keywords Senescence-Accelerated Mouse · Higher oxidative stress status · Mitochondrial dysfunction · Interventions of senescent phenotypes

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1 The SAM Strain of Mice Is a Unique Model for Senescence Acceleration

The Senescence-Accelerated Mouse (SAM), originally established by Dr. Toshio Takeda and his colleagues, is a unique mouse model for senescence acceleration and age-associated disorders. In this section, we briefly review the history of the establishment of the SAM strain. SAM strains of mice were generated through selective inbreeding processes. The recombination of genes from the gene pools, which were produced by the unexpected outbreeding between AKR/J and unidentified strain(s), occurred successively during this inbreeding process. Therefore, SAM can be considered to represent a group of related inbred strains.

Life history findings from SAM mice suggested that senescence processes in SAMP mice are more characteristic of "accelerated senescence" rather than "premature senescence." The SAM strain of mice provided a concrete idea of accelerated senescence as a biological phenomenon.

1.1 A Brief History of the Development of SAM Strains of Mice – A Mechanism to Generate Mice

The SAM strain of mice is actually a group of related inbred strains consisting of a series of SAMP (accelerated senescence-prone) and SAMR (accelerated senescence-resistant) strains. Around 1973, while maintaining the line of AKR/J mice that had been donated in 1963 by the Jackson Laboratory (Bar Harbor, ME, USA) to the Chest Disease Research Institute, Kyoto University (Kyoto, Japan), Dr. Takeda and his colleagues became aware of certain litters in which most mice showed senescent phenotypes in behavior and gross appearance at an early age, along with shorter life spans. They confirmed that these phenotypes were inherited by subsequent generations and in 1975 selected five litters with early senescence as progenitors for the senescence-prone series (P series). Three litters in which the aging process seemed normal were also selected as progenitors for the senescence-resistant series (R series). Thereafter, in addition to routine sister–brother inbreeding, retrospective pedigree selection [1] was used based on the degree of senescence [2–5] at 8 months of age, the life span, and the incidence and degree of age-associated pathologic phenotypes. These mice were categorized as Senescence-Accelerated Mouse (SAM) in 1981 [2].

When Dr. Takeda and his colleagues selected SAM strain progenitors, a low incidence of thymic lymphoma suggested that the genetic background of SAM strains deviated from the authentic AKR/J strain [6]. Routine genetic monitoring techniques revealed that there was at least one genotype in each SAM strain that differed from that of the AKR/J strain [3, 7]. In some loci, three kinds of genotypes were determined. The history of the development of the SAM strain and these results strongly suggest that unexpected outbreeding had occurred prior to 1973 as the most plausible mechanism to explain the generation of SAM strains. The recombination of

genes from gene pools is assumed to have occurred successively during the selective inbreeding process. Therefore, SAM strains are considered to represent a group of related inbred strains, which was confirmed by genetic typing using microsatellite markers [8].

1.2 Accelerated Senescence and SAM Mice

The SAM strain of mice provided a concrete idea of accelerated senescence as a biological phenomenon. In the development of this strain, numerous data relating to life history of mice were accumulated and analyzed.

In both SAMP1 and SAMR1 mice, body weight increased rapidly up to 20 weeks of age, and there were no differences between these two strains for both males and females [2]. The time at which the reproductive capability was reached in SAMP3, SAMP11, and SAMR1 mice was between 39 and 45 days after birth, and there were no differences among strains. These data indicate no significant differences in development and maturation between SAMP and SAMR mice.

A Gompertzian function indicated that the increase in the age-specific death rate of SAMP strains was greater than in SAMR strains [2].

The degree of senescence (evaluated based on their gross appearance and behavior using a grading score [4, 5]) of SAMP mice increased steeply. The difference between SAMP and SAMR mice, which are maintained under conventional conditions in Kyoto colonies (maintained until October 2002 at Kyoto University), became obvious after 6 months of age. Scores for SAMP and SAMR mice leveled out at 16 and 24 months of age, respectively, and there were no differences between maximum scores [4].

The primary identifiable cause of death in SAM strains includes contracted kidneys, abscesses, pneumonia, and lymphoid cell neoplasms. Although there were some differences in the frequency of the cause of death in each strain, there was no strain-specific cause of death [2, 9].

In our studies, the words “aging” and “senescence” have been used in different contexts. Aging consists of biological phenomena observed in an individual organism from the time of fertilization to the time of death. Strehler [10] proposed four criteria for delineating age-associated changes: universality, intrinsicality, time dependence, and deleteriousness. Based on this concept, we believe that the first three criteria delineate “aging,” whereas all four criteria together delineate “senescence.” In this context, “senescence” can be considered an aging process, which occurs after maturation. Atrophy can be a phenotype of “senescence,” and hypoplasia or maldevelopment should be discriminated from atrophy.

There can be three kinds of senescence processes: “normal senescence,” “accelerated senescence,” and “premature senescence.” Conceptually, two kinds of aberrant senescence processes, “premature aging” and “accelerated senescence,” can be differentiated. “Premature aging” is an early-onset senescence process. In “accelerated senescence,” the rate of the aging process is accelerated but the onset is unaffected. “Accelerated senescence” and “premature aging” cannot be observed

in isolation. They become apparent only when they are compared with the senescence process that we assess as "natural" or "normal." In SAMP mice, normal development and maturity of reproductive function are observed, and values for many (but not all) physiologic and pathologic parameters are the same as those in SAMR mice from juveniles to adults. Due to the rapid progression of the senescence process, the median survival time of each SAMP strain is about 37–73% of the corresponding SAMR1 strain, and the degree of senescence at 8 months of age in SAMP strains is about 1.5–3.5 times higher than in the age-matched SAMR1 strain under conventional rearing conditions in Kyoto colonies [3]. These findings suggest that senescence processes in SAMP mice are more characteristic of "accelerated senescence" rather than "premature senescence." Following this concept, we can speculate that the short life span observed in SAMP mice results from senescence acceleration. However, we can also postulate conceptually that the short life span is not necessarily associated with senescence acceleration, and conversely senescence acceleration does not always result in a shorter life span.

Having established SAM strains of mice, several questions regarding the manifestation of senescence acceleration in SAMP mice have been addressed. One question is whether the acceleration of the senescence process is evident only at the level of the individual mouse, or also at the level of cultured cells. To answer this question, we devised a tissue culture method and investigated the aging process in vitro in cells isolated from SAM mice [11–16]. Fibroblast-like cells were isolated from the dorsal dermis of newborn mice from SAMP11 and SAMR1 strains. All cultures from both strains showed a crisis in growth, and then immortalized. At senescence/crisis, all cultures were composed of morphologically characteristic senescent cells. These cultures showed the least saturation density and the longest population doubling times [11]. Flow cytometric analyses of the DNA content of confluent cells and chromosome analyses in mitoses revealed that diploid cells were being replaced with polyploid cells until the senescence/crisis [13]. However, in cultured cells from SAMP11 mice, these changes occurred more rapidly and at earlier population doublings (PDs). The senescence/crisis of SAMP11 cells (9–11 PDs) occurred significantly earlier ($p < 0.05$) than that of SAMR1 cells (13–19 PDs). An accelerated in vitro senescence has also been reported in primary cultures of dermal fibroblasts from SAMP6 mice [17]. This evidence tends to support various in vivo observations for age-associated changes of cells in accelerated senescence-prone SAMP mice as described in the following sections.

Genetic analyses revealed that four loci on chromosomes 4, 14, 16, and 17 contained genes responsible for the senescence acceleration observed in SAMP strains [8].

2 The SAM Strain of Mice Is a Unique Model for Several Age-Dependent Disorders

Systematic studies on various age-associated pathologies of SAM mice have revealed that these pathologies are suitable models for several geriatric disorders

observed in humans. They include senile osteoporosis, degenerative joint disease, age-related deficits in learning and memory, olfactory bulb and forebrain atrophies, presbycusis, retinal atrophies, senile amyloidosis, immunosenescence, senile lungs, and so on (for a review, see [3, 6, 7, 16, 18–21]). Many of these age-associated pathologies form from age-dependent degenerative changes of cells that might have a higher susceptibility to factors that cause senescence acceleration, including oxidative stress.

2.1 The SAM Strain of Mice Is a Model for Age-Associated Disorders

Pathology analyses were vigorously performed on SAMR and SAMP strains of mice throughout their life spans. Each SAMP strain showed relatively strain-specific age-associated pathologies. Many of these characteristic pathologies were similar to those often observed in elderly humans and assessed as animal models for geriatric disorders. Initial reports of these pathologies revealed the following:

Neuronal and sensory system: deficits in the learning and memory of SAMP8 mice [22] and SAMP10 mice [23], olfactory bulb and forebrain atrophy of SAMP10 mice [24], abnormal circadian rhythm in SAMP8 and SAMP10 mice [22, 25], presbycusis of SAMP1 mice [26], atrophy of the retina in SAMP1 mice [27], senile cataracts in SAMP1 and SAMP2A mice [28].
Immune system: immunosenescence in SAMP1 mice [29, 30] and in SAMP8 mice [31].
Vascular system: medial thickening of the aorta [32], damage to the blood–brain barrier [33].
Respiratory system: hyperinflation of the lung [34], induced emphysema [35, 36].
Digestive system: Crohn's disease [37].
Musculoskeletal system: senile osteoporosis [38], degenerative joint disease [39].
Metabolic system: senile amyloidosis [40].

Over 30 years, using SAM strains of mice, voluminous studies were performed to clarify the cause, mechanism, and course of geriatric disorders [3, 6, 21]. Studies that developed intervention methods for geriatric disorders were also performed extensively using SAM mice [3, 6, 20, 21].

Age-associated pathologies are polygenic/multigenic traits. Therefore, it is hard to detect causative genes. Among these pathologies, the most successful study to detect causative genes consisted of a series of studies for senile osteoporosis using SAMP6, SAMP2, and their congenic strains. Studies were done to detect genes that determine the low-peak bone mass of SAMP6 mice. This work did not relate to the senescence process but rather related to the development of bone. However, the peak bone mass at maturation was the most important factor for the onset of

senile osteoporosis [41]. A classic cross-mating experiment of SAMP6 mice with a low-peak bone mass and SAMP2 mice with a high-peak bone mass suggested that strain-specific bone mass in these strains was inherited as a polygenic characteristic and controlled by a small number of genes [42]. We did quantitative trait linkage analyses for low-peak bone mass and identified two significant quantitative trait loci on chromosomes (Chr) 11 and 13 and one suggestive locus on Chr X that linked to the peak bone mass [43]. A congenic strain (P6.P2-*Pbd2*^{b}), which carried a single genomic interval from Chr 13 of SAMP2 on a SAMP6-derived osteoporotic background, confirmed that the Chr 13 locus Pbd2 regulated bone density in mice [44]. Osteoblast cultures from SAMP6, P6.P2-*Pbd2*^{b} mice and molecular genetic studies identified Sfrp4 (secreted frizzled-related protein 4) within the Pbd2 locus was responsible for lower-peak bone mass in the SAMP6 strain [45].

2.2 Age-Dependent Degenerative Change of Tissues in SAMP Mice

Cotran [46] classified geriatric (age-associated) disorders in humans into two categories: age-related disorders and age-dependent disorders. The former category was thought to be "simply prevalent in advanced years of life and offered opportunities in prevention, control, and treatment," whereas the latter category was thought to be "an inevitable part of senescence as a direct consequence of physiological senescence." It is noteworthy that age-associated pathologic phenotypes observed in SAMP mice contain several "age-dependent" disorders. This means that the age-dependent functional decline of parenchymal cells (and also mesenchymal cells) such as cell proliferation, cell survival, and differentiated cell functions occurs in an accelerated and specific manner in SAMP mice and that these functional changes result in senile degenerative diseases.

We can show two typical examples of age-dependent degenerative changes of tissues in SAMP mice. We observed an increase in aortic wall thickness as well as branching, breakage, and disorganization of the elastic lamellae and an increase in thin collagen fibrils between medial smooth muscle cells and hypertrophy, but a significant decrease in the number of medial smooth muscle cells occurred in both SAMR1 and SAMP11 mice with advancing age. These alterations observed in SAMP11 mice occurred earlier and in a more pronounced fashion than in SAMR1 mice. The lumen of the aorta dilated in SAMR1 mice but narrowed in SAMP11 mice. Based on our findings, we suggested that the aorta of SAMR1 mice might reflect the physiologic process of aging, whereas the SAMP11 mice showed earlier changes due to the senescence acceleration of vascular cells, which was exaggerated by elevated blood pressure [32].

Detrimental effects from aging are best observed in postmitotic tissues such as the brain [47]. Among SAMP strains of mice, SAMP8 and SAMP10 strains have been used as models for age-associated neurodegeneration. The cellular and molecular basis of some of these age-associated pathologic phenotypes of the brain,

including age-associated deficits in learning and memory in SAMP8 and SAMP10 mice, has been reviewed [16, 18, 19]. Recent studies revealed that a higher oxidative stress status is an important factor, which generates neurodegeneration, and is discussed in the next section.

3 SAMP Strains of Mice Show a Higher Oxidative Stress Status as a Unique Biological Characteristic

The higher oxidative stress status observed in SAMP mice is partly caused by mitochondrial dysfunction and may be a cause of the senescence acceleration and age-dependent alterations in cell structure and function. Based on our recent observations, we discuss a possible mechanism for mitochondrial dysfunction resulting in the excessive production of reactive oxygen species and a role for the hyperoxidative stress status in age-dependent disorders in SAMP mice.

3.1 A Higher Oxidative Stress Status is a Primary Characteristic of SAMP Strains of Mice

From the initial observation of higher lipid peroxide (LPO) levels in the skin of 3- to 4-month-old SAMP1 mice by Komura et al. [48], a higher oxidative status was detected in various organs including the brain, liver, heart, eye, and so forth [49–61].

In the brain, Liu et al. [49] observed that the amount of LPO increased at 11 months of age in both SAMP8 and ddY mice compared with that in those 3 months of age. In their study, the superoxide dismutase (SOD) activity of SAMP8 mouse brains was higher than that of ddY mouse brains at both 3 and 11 months of age. Nomura et al. [50] observed slight but significantly higher LPO levels in 11- to 12-month-old SAMP8 males than in SAMR1 males.

Sato et al. [51], however, observed transiently higher LPO and protein carbonyl levels at 4–8 weeks of age in the cerebral cortex but not in other regions of the brain. Over this period, the net generation of reactive oxygen species (ROS) increased in cerebral cells, whereas glutamine synthetase, an enzyme highly sensitive to ROS, decreased in activity. Catalase activity also decreased by 75%, and the acyl-CoA oxidase, a microperoxisomal H_2O_2-producing enzyme, increased 1.6-fold [52]. Using a method involving chemical derivatization and high-performance liquid chromatography on SAMP8 and SAMR1 mice at 3–9 months of age, Matsugo et al. [53] could detect an age-dependent increase in levels of LPO in the heart, liver, lung, and kidney, but not in the brain. However, LPO levels in brains from SAMP8 mice were higher than that in brains of SAMR1 mice at all ages. Recent studies have revealed that LPO levels were increased in younger (2- to 5-month-old) SAMP8 mice [54, 55]. Taken altogether, these findings indicate a higher oxidative status in the SAMP8 mouse brain, and slight discrepancies in results from these reports may be due to different methods used to detect LPO.

Cortical synaptosomal membranes from 10-month-old SAMP8 mice showed structural characteristics of free radical oxidative stress in terms of electron paramagnetic resonance spin labeling (EPR), protein carbonyl content, and the activity of glutamine synthetase [56]. Furthermore, they treated SAMP8 mice at this age for 2 weeks with a free radical scavenger, *N-tert*-butyl-a-phenylnitrone, and observed a return toward normal values for relevant EPR parameters. Proteomic analyses revealed that specific protein carbonyl levels of lactate dehydrogenase 2, dihydropyrimidinase-like protein 2, α-spectrin, and creatine kinase were significantly increased in 12-month-old SAMP8 brains compared with 4-month-old SAMP8 brains [57], and the carbonyl modification of hippocampal cholinergic neurostimulating peptide precursor protein was significantly increased in 9-month-old SAMP8 brains compared with age-matched SAMR1 brains [58].

We demonstrated that MDF cells from SAMP11 mice showed an increase in the production of ROS from mitochondria and impairments of mitochondrial function compared with murine dermal fibroblast-like (MDF) cells from SAMR1 mice [15]. In this study, we also demonstrated that the activity and expression of antioxidant enzymes and the glutathione concentration were not impaired in MDF cells from SAMP11 mice. We previously showed that MDF cells from SAMP11 mice exhibited accelerated senescence/crisis in vitro and an elevated LPO content compared with cells from SAMR1 mice [11, 12, 14]. The mean concentration of LPO in fibroblast-like cells from primary cultures of SAMP11 mice was 55% higher than that in cultures from SAMR1 mice. Four millimolar aminoguanidine supplementation reduced the lipid peroxidation of cell layers from SAMP11 cultures to levels in SAMR1 cultures and delayed the stage of the senescence/crisis from 9 to 13 PDs. This treatment did not affect SAMR1 cultures [14]. Previous results indicated that the senescence/crisis of MDF cells was determined, at least in part, by the intracellular oxidative stress level.

3.2 Mitochondrial Dysfunction Is Observed in SAMP Mice

Park et al. [61] reported that mitochondrial CuZn-SOD activities decreased in livers from SAMP1 mice from a very young age to old age compared with those in SAMR1 mice. They suggested that the impaired transport of CuZn-SOD into mitochondria after cytosolic synthesis was the underlying mechanism and proposed mitochondrial dysfunction as a factor contributing to the senescence acceleration. After their work was published, several studies supported the mitochondrial dysfunction observed in SAMP mice. Nakahara et al. [62] studied oxidative phosphorylation in livers of SAMP8 and SAMR1 mice. They found that the respiratory control ratio decreased during aging in SAMP8 mice and estimated that the mitochondrial capacity to produce ATP might fall below that needed to maintain the normal cell metabolism by 18 months of age. They also observed that the amount of Bcl-x in the liver mitochondria was slightly decreased in SAMP8 mice. Milder effects of aging on mitochondrial functional parameters were observed in SAMR1

mice. We also observed a higher redox state and higher activity for mitochondrial respiration with a lower respiration control ratio in mitochondrial fractions from brains of 2-month-old SAMP8 males compared with SAMR1 mice [63]. However, unlike mitochondrial diseases observed in humans, there were no significant age-associated increases in serum lactate/pyruvate levels in either SAMP8 or SAMR1 mice, and there were no further significant differences between SAMP8 and SAMR1 mice [64]. Based on our observations, we proposed that a mild but inefficient hyperactive state can exist as a change in the mitochondrial electron transport system before age-associated mitochondrial dysfunction develops. In the electron transport system, decreased activities for complex I and complex III were observed [65], but the activity of succinate dehydrogenase was not decreased [63]. Kurokawa et al. [66] observed that the Mn-SOD activity in the cerebral cortex of 10-week-old SAMP8 mice was decreased by about 50% compared with that in age-matched SAMR1 mice. However, interestingly, there was no difference in the expression of this protein between the two strains, and they suggested that posttranslational modification reduced enzymatic activity. Fujibayashi et al. [65] observed a small but significantly greater amount of multiple mitochondrial DNA deletions in SAMP8 mice brains at 4 and 8 weeks of age. Xu et al. [67] analyzed mitochondrial membrane potentials ($\Delta\psi_m$) of platelets from SAMP8 and SAMR1 mice using fluorescent probe JC-1 and found that platelets from 2-month-old SAMP8 mice showed lower $\Delta\psi_m$ compared with that in platelets from age-matched SAMR1 mice.

We analyzed mitochondrial alterations in cultured MDF cells from SAMP11 mice [15]. We found an increase in mitochondria with low $\Delta\psi_m$ and degenerative changes in ultrastructures with advancing days in culture. Double labeling of superoxide with hydroethidine and mitochondria with MitoTracker Green revealed that most superoxide-producing sites were mitochondria. These results suggest that an increased production of ROS from dysfunctional mitochondria may be the cause of in vitro accelerated senescence. As respiratory chain complex dysfunctions can result in an increased production of ROS [68, 69], the mutation(s) in the gene(s) encoding mitochondrial respiratory chain complex subunits warrant investigation. To determine whether gene(s) associated with accelerated senescence/crisis in SAMP11 cells are located on nuclear DNA or mitochondrial DNA, we performed reciprocal cross-breeding using SAMP11 and SAMR1 mice. MDF cells from SAMP11, SAMR1, P11·R1F_1, and R1·P11F_1 neonates were cultured, and the senescence/crisis was evaluated as previously described [11]. MDF cells both from P11·R1F_1 (14.9 ± 2.4 PDs) and from R1·P11F_1 (15.7 ± 0.6 PDs) neonates reached senescence/crisis at PDs comparable with those from SAMR1 (16.0 ± 0.9 PDs) neonates, and interestingly, no maternal effect was observed [16]. These results imply that determinant gene(s) for senescence/crisis are not located on mitochondrial DNA but on nuclear DNA, and that SAMR1 traits are dominant over SAMP11 traits. Based on the microsatellite map of SAM mice [70], we listed respiratory chain complex–related genes in chromosomal regions where microsatellite polymorphisms between SAMP11 mice and SAMR1 mice exist [16]. Whether SAMP11 mice actually possess mutations in these genes remains to be elucidated.

3.3 A Higher Oxidative Stress Status Is a Possible Cause of Senescence Acceleration and Degeneration of Cells in SAMP Mice

Harman [71] initially proposed that free radicals were important factors in aging. The hyperoxidative status mentioned earlier may be associated with damage to the DNA, proteins, and lipids of cells and may impair cellular proliferation, cell survival, and various cell functions. The age-associated increase in hepatic DNA single-strand breaks was accelerated in SAMP1 mice compared with that in SAMR1 mice [72]. We also observed that age-associated DNA damage was accelerated in the brain, heart, muscle, kidney, liver, and intestines of SAMP1 mice compared with that in SAMR1 mice [73]. DNA repair decreased drastically with advancing age in the same organs of SAMP1 mice [74]. An age-associated increase in DNA–protein cross-links was observed in the brain, heart, kidney, lung, and liver of SAMP1 mice but was evident only in brain and heart from NMRI mice [75]. Choi et al. [76] observed that 8-hydroxyguanine levels in the liver and brain were higher in SAMP1 and SAMP8 mice from 2 to 13 months of age than in SAMR1 mice, and a low activity in 8-hydroxyguanine DNA glycosylase (OGG1) (10–40% of the SAMR1 level) was observed in these SAMP mice. The authors also found a mutation in the Ogg1 gene from Arg (SAMR1) to Trp at codon 304. However, Mori et al. [77] could not support the notion that this mutation was responsible for the accelerated senescence and short life span of SAMP1 mice.

The previous observations may explain the acceleration in age-associated increases in chromosomal aberrations in bone marrow cells [78] and in the mutation frequency of splenic lymphocytes at the hypoxanthine phosphoribosyl transferase (Hprt) locus in SAMP1 mice [79]. Although the exact mechanisms are still unknown for the senescence acceleration in SAMP strains, one mechanism that accelerates, exacerbates, or can cause age-associated cell degeneration in SAMP mice at an early age is possibly elevated oxidative stress.

The SAMP8 strain of mice is characterized by deficits in learning and memory from a relatively young age [80]. SAMP8 mice have drawn attention in gerontologic research on dementia, as amyloid beta protein is implicated in the pathogenesis of age-associated brain dysfunctions [81–86]. As mentioned in the previous sections, one of the conspicuous features of SAMP8 mice is the presence of a higher oxidative stress status and mitochondrial dysfunction [49–58, 62, 63, 65–67]. Successful interventions with various antioxidants, as described in Section 4.2, further support the idea that oxidative stress and mitochondrial dysfunction are directly linked with the age-associated neurodegeneration phenotype of SAMP8 mice. A recent in vitro study suggests a mechanism for oxidative stress–induced neurodegeneration in SAMP8 brains [87]. Cultured astrocytes from SAMP8 mice showed reduced viability compared with astrocytes from SAMR1 mice when they were exposed to 400 μM hydrogen peroxide. The results suggested that changes in the peroxide-detoxifying potential of astrocytes from SAMP8 mice might lead to the inadequate protection of neurons from oxidative stress.

The SAMP10 strain of mice is a model of brain aging in which senescence is characterized by cerebral atrophy and several neurodegenerative phenotypes [23, 24, 88–92]. Recently, some molecular abnormalities have been identified in SAMP10 mice, including the absence of normal fibroblast growth factor 1 protein [93] and a reduced expression of MAb6B4 epitopes on aggrecan in perineuronal nets from cerebral cortices [94]. However, whether these molecular alterations have a causal relationship with neurodegeneration remains to be elucidated.

There is evidence that a higher oxidative stress status is present in SAMP10 brains and may have a causal relationship with the pathogenesis of neurodegeneration observed in this animal model. Levels of 8-oxodeoxyguanosine (8-oxodG) in brains from 15-month-old SAMP10 mice were significantly higher than in 2-month-old mice [95], and the activity of glutathione peroxidase in the cerebral cortex was significantly lower in 12-month-old SAMP10 mice than in 2-month-old mice [96]. The administration of green tea catechins, which are potent antioxidants, reduced oxidative DNA damage, restored the activity of glutathione peroxidase, and prevented memory disturbances [95–97]. We recently reported that ubiquitinated inclusions were formed in neurons from limbic-related forebrain structures, and their formation was associated with decreased proteasome activity [92]. Inclusions had ultrastructural features of fused lipofuscin granules. A higher oxidative stress status may underlie these changes, as oxidative damage is implicated in lipofuscinogenesis [98] and an impairment of proteasomal activity [99]. Sasaki et al. [100] have directly demonstrated an increase in superoxide production in the SAMP10 brain using "real-time bioradiography" to detect superoxide-dependent chemiluminescence in freshly prepared brain slices. Superoxide-dependent chemiluminescence at both the baseline state and under reoxygenation after hypoxic treatment was higher in SAMP10 mice than in SAMR1 mice, even at 2 months of age. Further, an age-dependent increase in chemiluminescence occurred more prominently in SAMP10 mice than in SAMR1 mice.

To elucidate whether ROS produced in neurons play a direct role in degenerative changes, we developed a primary culture of neurons from SAMP10 and SAMR1 embryos and analyzed their in vitro phenotypes. Unfortunately, however, under culture conditions, neurons isolated from SAMP10 mice did not show any remarkable phenotypes in terms of survival rates and oxidative stress levels when they were maintained without exogenous toxic stimuli [16]. These in vitro results are not consistent with the above-mentioned ex vivo observations by Sasaki and colleagues [100]. This discrepancy may be attributed to culture conditions, in which cellular maintenance and the measurement of oxidative stress levels were performed in "sandwich" cultures, where cells are considered to be in an environment of low oxygen concentration [101]. Another possible explanation for the discrepancy is that the main source of ROS in the brain might not be neurons but rather glial cells. Microglia-derived oxygen radicals have been implicated in neurodegeneration and neuronal death [102, 103]. Thus, activated microglia and/or astrocytes may be the origin of ROS in the SAMP10 brain. The fact that the superoxide-dependent chemiluminescence in the brain was greater in the white matter than in the gray matter [100] may have some implication for the origin of these ROS. The potential for

glial cells from SAMP10 mice to produce ROS needs to be investigated, and coculture experiments of neurons and glia together may clarify the role of glia-derived ROS in neurodegeneration.

Recently, we proposed a working hypothesis that links the neuroinflammatory theory of neurodegeneration [104] to a higher oxidative stress status using SAMP10 mice [105]. We demonstrated that there was a higher proinflammatory status in atrophy-prone brain regions of SAMP10 mice [105]. This proinflammatory status is characterized by a higher expression of interleukin-1β and interferon-γ mRNA throughout the entire life span and an age-associated increase in the expression of tumor necrosis factor-α, interleukin-6, and monocyte chemotactic protein-1 mRNA. An age-associated increase in activated microglia was also observed in atrophic brain regions. Proinflammatory cytokines may exert a toxic effect on neurons, mainly via an excitotoxic mechanism [106, 107]. Proinflammatory conditions can be induced in the central nervous system by several noxious stimuli, including viral, bacterial, and parasitic infections, trauma, ischemia, tumor, and a higher oxidative stress status (see the discussion in [105]). However, there is no evidence supporting the presence of these stimuli, except for oxidative stress, in brains from SAMP10 mice. The observation that superoxide production in the brain appears to be higher in SAMP10 mice than in SAMR1 mice even at 2 months of age [100] further supports our hypothesis that a higher oxidative stress status from a younger age may induce the higher proinflammatory status observed in atrophy-prone regions of SAMP10 brains.

4 Interventions of Mitochondrial Dysfunction, Senescence Acceleration, and Age-Dependent Disorders

Because the SAM strain of mice was developed as an animal model of geriatric disorders, many experiments to intervene with senescence phenotypes were done from the very beginning [3, 20, 21], including caloric restriction and administration of nutrients, chemicals, medicines, and traditional herbal medicines. Some manipulations successfully improved age-associated pathologies on the point of their degree and/or their onset. However, mechanisms of effects still remain in speculation. Recent studies highlighted that the improvement in mitochondrial dysfunction may relate to interventions of age-associated pathologies in SAMP mice.

4.1 Interventions of Senescence Acceleration and Age-Dependent Disorders

Since the initial study by McCay et al. [108], chronic caloric restriction has been established as the most reliable and reproducible method to extend the life span of organisms. Chronic caloric restriction was also applied to SAM mice shortly after the establishment of strains. A 40% restriction in caloric intake from 6 weeks of

age decelerated the senescence process and extended mean and maximal life span (24.3 and 65.9%, respectively) in SAMP1 mice [109] and also improved immunosenescence [110] and senile amyloidosis [109]. Many kinds of nutrients, foods, and traditional herbal medicines have been tested for effects to extend life span, to decelerate the senescence process, and to improve age-associated pathologies [3, 20, 21].

Recently, two unique methods have been applied to intervene with age-associated pathologies of SAM mice. One is acupuncture, the Chinese traditional medicine, and the other is intra-bone marrow–bone marrow transplantation (IBM-BMT), a contemporary regenerative medicine.

Ding et al. [111] examined molecular events in SAMP10 mice brain hippocampi associated with aging and acupuncture effects. They found that acupuncture could completely or partially reverse some gene expression profiles in the hippocampus associated with aging. Acupuncture modified the expression of several genes including Hsp84, Hsp86, and YB-1, which are closely related to oxidative damage. They concluded that acupuncture could be useful in retarding molecular events associated with aging in mammals.

Iwai et al. [112] reported that age-related dysfunctions of the systemic immune system and accelerated presbycusis in SAMP1 mice were corrected by allogeneic IBM-BMT. They proposed that the relationship between age-related systemic immune dysfunctions and neurodegeneration mechanisms open up new avenues for treatment of presbycusis, for which there is no effective therapy. IBM-BMT was also effective for treating other degenerative disease models, such as osteoporosis in SAMP6 mice [113] and emphysema in Tsk mice [114].

4.2 Improvements in Mitochondrial Dysfunction

Like our observations, mitochondria are an attractive target for interventions in accelerated senescence and age-associated pathologies because they play a pivotal role in energy production, oxidative damage, and senescence [68, 69]. Strategies to protect mitochondria from age-related damage include the prevention and repair of oxidative damage in mitochondria and an enhancement in mitochondrial energy production. In studies using SAM mice, many substrates that have protective effects on mitochondria were tested for their effectiveness in treating the senescent phenotype and age-associated disorders seen in SAMP mice. Among these, the efficacy of melatonin has been extensively investigated using SAMP8 mice. Melatonin, secreted by the pineal gland, is a multifunctional agent with antioxidant activity [115]. When administered in drinking water, melatonin reduced oxidative damage in brain tissues [116, 117], plasma, and erythrocytes [118], in mitochondria in the liver [119], diaphragm, and heart [120, 121], and improved mitochondrial respiratory chain complex function [119, 120]. Tea catechins, which also have antioxidative effects, were effective in reducing oxidative damage in the brain and preventing forebrain atrophies and cognitive dysfunctions in SAMP10 mice [95, 97]. When

combined with habitual exercise, catechin administration increased mRNA levels of mitochondria-related proteins in skeletal muscles of SAMP1 mice [122]. Extracts from plants with antioxidative effects [123–127], antioxidant-enriched diets [128], and mitochondrial nutrients [129] such as α-lipoic acid [130], coenzyme Q10 [131], and acetyl-L-carnitine [132], were also effective to various degrees for treating age-associated phenotypes in SAMP mice.

5 Conclusions

In this chapter, we described the history of the development of SAM mice and their characteristic senescent phenotypes, accelerated senescence, and age-associated pathologies. We discussed relationships between age-dependent degenerative changes and pathologic phenotypes observed in SAMP strains and the higher oxidative stress status and mitochondrial dysfunction as primary characteristics that cause degenerative changes. We also described attempts to intervene with the senescence phenotype in SAMP mice. Butterfield and Poon [133] demonstrated that alterations in gene expression and protein abnormalities were relevant to age-associated learning and memory deficits in the SAMP8 strain. These genes and proteins were functionally categorized into neuroprotection, signal transduction, protein folding/degradation, cytoskeleton/transport, immune response, and ROS production. In his review, Liu [129] described that "mitochondrial nutrients" can directly or indirectly protect mitochondria from oxidative damage and improve age-associated mitochondrial and cognitive dysfunctions. Taken altogether, mitochondrial dysfunctions cause slight but continuous oxidative stress, alter gene expression, and cause protein abnormalities that can play an important role in age-dependent degenerative changes of various cells and tissues. SAMP strains of mice are useful tools to investigate mechanisms of age-dependent disorders and in the development of clinical interventions.

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Antioxidants Combined with Behavioral Enrichment Can Slow Brain Aging

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Abstract In the brain, oxidative damage, loss of synapses, and loss of growth factors such as brain-derived neurotrophic factor (BDNF) are linked to age-associated cognitive decline and Alzheimer's disease (AD). Antioxidant treatments to reduce oxidative damage have provided only modest benefits in human clinical trials. Cognitive and physical training, which may promote synapse growth and maintenance and increase BDNF, has proved to be more promising in small clinical trials in normal aged individuals and AD patients. Using a canine model of human brain aging, which naturally accumulates oxidative damage and cognitive dysfunction, we tested the hypothesis that combining treatments that can reduce several age-associated neuropathologies will prove to be more efficacious than targeting a single pathologic cascade. Aged beagles were either fed an antioxidant/mitochondrial cofactor diet (vitamins E and C, fruits and vegetables, lipoic acid and carnitine) or provided with behavioral enrichment that can increase BDNF and synapse and neuron growth (environmental, social, and cognitive enrichment with physical exercise) or treated with a combination of both the diet and behavioral enrichment. Cognitive improvements were observed for each treatment alone, but the combination approach, in particular, led to larger improvements in learning scores, to maintenance of cognitive ability, and to recovery of impaired memory function. Notably, each treatment selectively reduced different types of neuropathology in the brain. The use of a combination of antioxidant supplement or diet and other lifestyle modifications (increased social activity, physical activity, and cognitive engagement) may work additively and be beneficial for healthy human brain aging.

Keywords Acetyl-L-carnitine · Alzheimer's disease · Beagle · Beta-amyloid · Dog · Canine · Lipoic acid · Oxidative damage

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1 Introduction

An important goal of aging research, the preservation of cognitive function throughout life, is becoming more critical as our population ages. In parallel with the shift of our population to older individuals is an increased risk of developing age-associated neurodegenerative diseases such as Alzheimer's disease (AD). Brain aging is accompanied by the accumulation of several types of neuropathology that may all have different etiologies and/or stem from similar molecular cascades and interact. Thus, interventions that promote healthy brain aging may prove to be more beneficial if a combinatorial approach is taken. In this chapter, two types of age-associated neuropathology are briefly described (oxidative damage, synapse/neurotrophic molecule loss) along with possible treatments (antioxidants, behavioral enrichment) that may target each and lead to greater cognitive and brain benefits if combined. A longitudinal study of aging in beagles where animals were provided with either an antioxidant diet or with behavioral enrichment or with a combination of both treatments is described. The results of the study in beagles strongly support the hypothesis that combination treatment approaches may be more beneficial for supporting healthy aging than single targeted interventions.

2 Antioxidants and Healthy Brain Aging

Aging in most species studied to date is accompanied by the progressive accumulation of oxidative damage in many tissues [1–4]. The brain, in particular, is vulnerable to oxidative damage as it consumes approximately 20% of the body's total oxygen, has a high content of polyunsaturated fatty acids, and has lower levels of endogenous antioxidant activity relative to other tissue [5–7]. Normal metabolic processes result in the release of reactive oxygen species (ROS), which in turn can lead to oxidative damage to proteins, lipids, DNA, and RNA [1]. ROS are produced primarily from mitochondria [8], intracellular organelles that are themselves vulnerable to oxidative damage [9]. Thus, mitochondrial dysfunction and production of ROS may be a key contributor to the deleterious effects of aging on the brain [10–18].

Studies in both normal and pathologic human brain aging provide correlative evidence in support of a role of oxidative damage in age-associated cognitive losses. Studies of human autopsy tissue show higher levels of oxidative damage to DNA/RNA [19–21], proteins [20, 22–25], and lipids [21, 25, 26] in aged brain compared with that in young brain. Oxidative damage may also play a role in age-associated neurodegenerative diseases such as AD [27–29]. AD is associated with further increases in protein [20, 23, 24, 30–37], lipid [21, 26, 38–41], DNA [42–44], and RNA/DNA oxidative damage [12, 45, 46]. In addition, endogenous antioxidant activity in the AD brain is reduced relative to that in age-matched controls [20, 25, 47]. Proteins particularly vulnerable to oxidative damage have been identified by proteomics, and a subset of these proteins may be directly or indirectly involved in

the production and accumulation of AD neuropathology [48]. Mitochondrial function also appears compromised with age and disease in the human brain [3, 4, 14, 49]. In normal aging, mitochondrial respiratory chain activity declines [50], and mitochondrial metabolism-associated enzymes such as aconitase decrease [51] and the rate of somatic mitochondrial DNA mutations increase [17, 52]. In AD, similar types of losses in respiratory chain activity [15, 53, 54] and increases in mitochondrial DNA mutations [55] are observed but are higher when compared with that in age-matched controls. Further, in AD, decreased cytochrome oxidase activity in the posterior cingulate cortex is correlated with hypometabolism observed via positron emission studies [56]. A gene array study in the cingulate cortex shows that energy metabolism–related genes expression levels are decreased, and specifically a 65% reduction in expression of mitochondrial electron transport chain genes, occurs in AD [57]. Thus, mitochondrial dysfunction and the production of ROS combined with lower endogenous antioxidant activity may lead to increasing oxidative damage to molecules critically important to neuronal function.

Based on correlative human neuropathology studies, antioxidants would be predicted to be associated with healthy aging, might serve to reduce the risk of developing AD, and may improve cognitive function in AD patients. However, studies in humans have shown either a positive effect of antioxidant use on cognition and risk reduction for developing AD [58–60] or no significant effects [61–64]. There have been few systematic and controlled clinical trials evaluating the effects of antioxidants on cognition in aged individuals or patients with AD. Vitamin E delays institutionalization in AD patients, suggesting some beneficial effects [65]. However, vitamin E alone did not improve cognition in patients with mild cognitive impairment, which is thought to be a precursor to AD [66]. Further, in nondemented elderly women, vitamin E treatment was associated with little improvements in cognition [63].

In addition to studies regarding the effects of cellular antioxidants on cognition and risk of AD, there exist several studies on the effects of targeted cofactors that improve mitochondrial function, including acetylcarnitine (ALCAR) and lipoic acid (LA). ALCAR and LA may improve mitochondrial function and reduce the production of ROS, thus also reducing oxidative damage to proteins, lipids, and DNA/RNA [67]. In studies where ALCAR was administered to patients with moderate to severe AD, either improved cognition and/or slower deterioration has been observed [68–71]. In early-onset AD patients (less than 65 years of age), only small cognitive improvements were noted [72], but in other studies of younger patients with AD (less than 61 years), there was evidence for slowed disease progression [73, 74]. When the results of all these studies were combined in a meta-analysis, there were clear benefits of ALCAR administration in patients with AD, particularly with respect to slowing cognitive decline [75]. Further, combining ALCAR with acetylcholinesterase therapy in AD may provide additional benefits [76]. Similar evidence of maintenance of function was observed in a study of nine patients with AD or related dementias receiving 600 mg/d of LA for an average of 337 days [77]. In a follow-up study of 48 patients for a longer 48-month treatment period, maintenance of function similar to that in the smaller study was observed [78].

When taken together, however, studies of dietary or supplemental antioxidant intake in humans reveal variable results and appear far less robustly associated with positive functional outcomes than those reported in the rodent-aging literature [9, 18, 79–86]. There may be many possible reasons for this. Variability in the outcomes of human antioxidant clinical trials may reflect inconsistencies in the amount of supplements provided, their form and source, their duration and regularity of use, and also the challenges of determining the exact background of dietary intake of antioxidants. Notably, combinations of antioxidants may be superior to single-compound supplementation [87], and dietary intake of antioxidants has been shown to be superior to supplements in human studies on cognition and risk of developing AD [88, 89]. Further, in elderly women, supplementation with a combination of vitamins E and C can lead to improved memory [90]. Thus, single-antioxidant administration (e.g., vitamin E alone) may prove to be more efficacious if administered in combination with other antioxidants (e.g., vitamin C, which helps to recycle vitamin E) and administered through diet rather than as a tablet supplement. As will be described in later sections, the combination of antioxidants administered through an enriched diet proved to be a potent approach to improving cognition and reducing brain pathology when tested in a canine model of human brain aging.

3 Behavioral Enrichment and Healthy Brain Aging

Despite the progressive accumulation of oxidative damage, the brain retains significant neuroplasticity with age, and several environmental stimuli can promote neuron growth, synapse growth, and the release of growth factors. All of these structural and molecular events may lead to maintenance of intact cognition. In humans, environmental stimuli associated with lifestyle factors such as physical, social, and intellectual activity are associated with healthy brain and cognitive aging and reduced risk of developing AD. For example, aerobically fit individuals show less age-related brain atrophy [91]. Further, a 6-month physical exercise intervention (45 min three times per week) improves attention and executive function in 55- to 78-year-old individuals [92]. Several studies have shown that increased mental activity can reduce the risk of dementia [93–95]. In a study of 469 older adults (>75 years), those with a high frequency of participation in cognitive and physical activities had a reduced risk of dementia [94]. Systematic and controlled clinical trials using cognitive training interventions reveal that significant cognitive improvements can be elicited in AD patients [96, 97] and in healthy elderly controls [98].

The use of aged rodent models has been instrumental in elucidating how environmental factors promote brain neuroplasticity and the neurobiological mechanisms underlying these effects. Synapse loss [99], reduced neurogenesis [100–102], and decreases in brain growth factors [103] are all observed in the aged rodent brain. An enriched environment that includes play toys, social interaction with other rats, and a larger cage size can increase synaptic growth and drive neurogenesis [104, 105] and increase the growth factor, brain-derived neurotrophic factor (BDNF) [106], in

aged animals. Further, physical exercise, usually a component of behavioral enrichment, increases BDNF, cerebral blood flow, induces molecules mediating synaptic plasticity, generates new neurons, and improves learning [107] (also see review by [108]). Interestingly, BDNF alone when injected into aged brain can lead to neuroprotection by reversing synapse loss and restoring learning and memory deficits [109]. Given that in the aged human brain, and in AD in particular, a significant loss of synapses and synapse-associated proteins [110–124] and BDNF is observed in brain regions required for intact memory function [125–127], behavioral enrichment may help to improve or maintain cognitive function. Small clinical trials using interventions that modify environmental factors, primarily through cognitive training protocols, have shown positive clinical outcomes, and several larger studies are ongoing [128].

4 Antioxidants and Behavioral Enrichment in the Canine Model of Human Aging

To explore whether or how oxidative damage or environmental factors contribute to brain aging, we have been working with aged beagles. The canine model of aging provides advantages for the study of oxidative damage and development of AD pathology that complements currently used model systems such as transgenic mice and non-human primates. Unique features of the canine model include long life spans, which permits longitudinal studies; the natural accumulation of oxidative damage and AD-like neuropathology; and absorption of dietary nutrients similar to that in humans [129].

4.1 Neurobiological and Cognitive Features of the Aged Dog

Neurobiologically, dogs naturally accumulate beta-amyloid (Aβ) [130–134], which is a toxic protein implicated in AD [135]. Neuron loss also occurs in the hilus of the hippocampus [136, 137] in aged dogs along with a reduced ability to generate new neurons [138]. Less neurogenesis is correlated with poorer cognitive performance [138]. By magnetic resonance imaging, cortical atrophy and ventricular widening can be observed with age [139, 140] and is linked to both Aβ and cognition [141]. As with many other species studied to date, the brains of aged dogs progressively accumulate oxidative damage to lipids, proteins, DNA, and RNA [142–148], making them a useful model system in which to evaluate the effects of antioxidants.

In parallel with these neuropathologic events in the aged canine brain, there is a progressive loss of a subset of cognitive functions [149–155]. However, not all aged individuals are equally affected; some aged dogs can learn and remember information as well as younger dogs do. Other aged dogs can show severe cognitive

impairments [150, 156]. Some types of learning and memory are more vulnerable to aging than others. For example, simple visual discrimination learning and procedural learning remains relatively intact with age in dogs [150]. In contrast, visuospatial function [155] and complex learning ability [157] are compromised with age. In a sensitive measure of spatial learning and memory, subtle age impairments can be detected as young as 6 years of age [155]. Oxidative damage may also be associated with behavioral decline. Rofina and collaborators found that increased oxidative end products in aged canine brain [147, 148, 158] correlates with severity of behavior changes due to cognitive dysfunction. For example a significant correlation was found between behavior changes and several measures of oxidative damage [148]. Thus, we tested the hypothesis that a diet rich in a broad spectrum of antioxidants in a longitudinal study of aging in old beagles would lead to improved cognition and reduced oxidative damage in the brain. In addition, we proposed that combining an antioxidant diet with behavioral enrichment to stimulate neurogenesis, synapse growth and/or maintenance, and neuronal growth factor release would provide added cognitive benefits.

4.2 Cognitive Benefits of Antioxidants and Behavioral Enrichment in Aged Dogs

We completed a longitudinal study of the effects of dietary antioxidant intervention and behavioral enrichment on cognitive function of aged beagle dogs. The experimental subjects were a group of 48 aged beagles (10–13 years of age). Each animal was assigned into one of two diet groups using a counterbalanced design based on extensive baseline cognitive testing. No differences existed between cognitive ability of groups prior to dietary intervention [159]. The two diets were formulated to meet the nutrient profile for the American Association of Feed Control Officials recommendations for adult dogs [160]. Control and test diets were identical in composition, other than inclusion of a broad-based antioxidant and mitochondrial cofactor supplementation to the test diet. The control and enriched foods had the following differences in formulation, respectively, on an as-fed basis: *dl*-α-tocopherol acetate (120 ppm vs. 1,050 ppm), L-carnitine (<20 ppm vs. 260 ppm), *dl*-α-lipoic acid (<20 ppm vs. 128 ppm), ascorbic acid as Stay-C (<30 ppm vs. 80 ppm), and 1% inclusions of each of the following (1 to 1 exchange for corn): spinach flakes, tomato pomace, grape pomace, carrot granules, and citrus pulp.

The behavioral enrichment protocol consisted of housing animals in pairs (social enrichment), providing two 20-min outdoor walks per week (physical exercise), and continuous cognitive testing (cognitive enrichment). The cognitive enrichment consisted of a landmark discrimination task [161], an oddity discrimination task [157], and a size discrimination learning and reversal task [159, 162]. Each of these tests measured distinct cognitive domains to minimize practice effects and served as a behavioral enrichment test battery (i.e., control animals did not receive this training).

On an annual basis, a second battery of tests was used, and all animals were given cognitive testing on measures of discrimination learning, reversal learning, and spatial memory, which were different tasks than those used for the behavioral enrichment treatment. To minimize practice effects, we used different stimulus objects for each successive discrimination and reversal problems, and in addition, we selected objects that were more difficult for animals to distinguish. This prevented possible floor effects. For the spatial memory task, identical testing procedures were used each year, and we predicted that all animals may improve with repeated exposure but that treated animals would benefit more.

After 1 month of treatment, we tested animals for spatial attention ability using a landmark discrimination task [149]. We observed rapid improvements in function on this measure of learning [161]. Approximately 6 months after starting the dietary intervention, the dogs were tested on a series of oddity discrimination learning tasks [157]. Old dogs fed the antioxidant diet made significantly fewer errors on the more difficult tasks than old controls. Additional improvements in cognition in response to the antioxidant-enriched diet were observed on repeated measures of visual discrimination and reversal learning [159]. Spatial memory was also improved in aged dogs provided with the antioxidant enriched diet after long-term treatment (>2.0 years) [163].

Behavioral enrichment also led to significant improvements in visual discrimination learning and reversal learning throughout the study [164] that was in some tasks greater than that observed in antioxidant-treated dogs. However, the most exciting outcome from the study was observed in the combination treatment group. First, the combined treatment was associated with the greatest benefits in learning throughout the study relative to each treatment alone [164]. Second, the combination treatment was associated with a significant maintenance of cognitive function over the duration of the study [159]. Third, there was recovery of function on a measure of spatial memory [163]. These results suggest that combination treatment approaches that include both an antioxidant diet and behavioral enrichment can improve, maintain, and potentially restore cognition.

4.3 Neurobiological Changes in Response to Antioxidants and Behavioral Enrichment

Our next experiments were intended to identify the neurobiological mechanisms underlying improved cognition in response to each treatment alone (antioxidant diet vs. behavioral enrichment) or to the added cognitive improvements when combined. In the brain, we observed decreased protein oxidation and increased endogenous antioxidant enzyme activity in response to the antioxidant diet [145]. These effects were more robust in animals receiving both the antioxidant diet and behavioral enrichment. The extent of diffuse Aβ in the brains of treated animals was also reduced but only in the dogs provided with the antioxidant diet [165]. However, in analyzing which brain regions were affected by antioxidant treatment, we found that

the prefrontal cortex remained unaffected, whereas more posterior and ventral cortical regions (parietal cortex and entorhinal cortex) showed less Aβ in response to the diet. Based on previous work [131, 132], we have learned that Aβ deposition occurs at different ages in distinct cortical regions in the canine brain. The prefrontal cortex shows Aβ accumulation between 8 and 9 years of age, whereas parietal and entorhinal cortex accumulates Aβ at later ages (>10 years). Thus, given that dogs were started on the treatment between 10 and 13 years of age, the antioxidant treatment was not able to reduce preexisting Aβ (prefrontal cortex) but prevented further Aβ accumulation in the parietal and entorhinal cortex. In contrast, we have found that behavioral enrichment does not modify Aβ levels but may improve or enhance the effects of the antioxidant diet by maintaining neuron number in the hippocampus, a region critically involved with learning and memory [136]. Surprisingly, given previous studies in rodent models showing increased neurogenesis in response to behavioral enrichment, a similar outcome was not observed in the aged dog brain [138]. The results of these studies strongly suggest that antioxidants and behavioral enrichment improve brain function through independent molecular pathways but in combination can target multiple age-associated neuropathologies.

5 Summary and Conclusions

Age-associated cognitive decline is linked to a progressive accumulation of oxidative damage and losses in synapses and growth factors in the brain. Cognitive dysfunction, oxidative damage, neuron loss, and synapse loss are particularly pronounced in AD. Human clinical trials evaluating single antioxidants, such as vitamin E, have provided only modest benefits to aged individuals and AD patients. Further, retrospective studies of antioxidant use (supplements or dietary) variably report positive or no consistent association with intact cognitive function or in a reduced risk of developing AD. Using the canine model of aging, we demonstrate that one possible approach to improving cognitive function and reducing neuropathology in both normal aging and in patients with AD is to combine therapies and antioxidants that target independent molecular pathways. Specifically, the use of antioxidant supplements in addition to other lifestyle modifications (increased social activity, physical activity, and cognitive engagement) may work additively and be beneficial for healthy human brain aging and may improve cognition in AD patients.

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Role of Nitric Oxide in Neurodegeneration and Vulnerability of Neuronal Cells to Nitric Oxide Metabolites and Reactive Oxygen Species

Debomoy K. Lahiri, Chandramallika Ghosh, Jack T. Rogers, Stephen Bondy, and Nigel H. Greig

Abstract Nitric oxide (NO) is produced during the oxidative deamination catalyzed by nitric oxide synthase (NOS) that converts L-arginine to L-citrulline. NO can also be released chemically from a group of compounds called NO donors, such as sodium nitroprusside (SNP). NO directly or through its metabolites is believed to be involved in several disorders, including Alzheimer's disease (AD). This chapter summarizes the role of NO and oxidative stress in neurodegeneration and describes the experimental evidence of increased vulnerability of neuronal cells to NO metabolites and other reactive oxygen species (ROS). As NO is a highly labile, unstable free gas, levels of the stable end products, such as nitrite and nitrate (NOx), were measured. When different cell types were treated with SNP, a significant level of NOx was detected in a time- and dose-dependent manner, which was more than the spontaneous release by SNP. Astrocytic, glial, and epithelial cell lines released significantly higher levels of NOx compared with neuronal cell lines after SNP treatment. Neuronal cells were more sensitive to SNP-induced cytotoxicity, as determined by lactate dehydrogenase assay. SNP-mediated toxicity is known to be due, in large part, to the accumulation of cyanide ions, and the ability of cells to protect themselves against this toxicity depends upon their levels of NO metabolites. Cell lines that generate more NOx, such as astrocytic and epithelial, are better protected against the SNP-induced toxicity than are less NOx-protecting neuronal cell lines. Our results suggest that various cell types metabolize SNP differently and that neuronal cell lines are more vulnerable than other cell types to SNP treatment. As neuronal cell lines lack an NO-generated protective mechanism, these cells are potentially primary targets for neurodegeneration by toxic agents including the free radicals and peroxynitrites.

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1 Introduction

Alzheimer's disease (AD) currently afflicts more than 5 million people within the United States of America, and this number will likely triple in the forthcoming 40 years given the increasing numbers of aged citizens. At present, the few agents that are FDA-approved for the treatment of AD have demonstrated only modest effects in modifying clinical symptoms for relatively short periods, and none has shown a clear effect on disease progression [1, 2]. The process of discovering and developing new drugs is lengthy and expensive, and more mechanistic-based studies are currently needed to expand available therapeutic options [1]. Neurodegeneration in AD is believed to be mediated by multiple events, including neurotransmitter imbalance, free-radical formation, and abnormal protein depositions, mostly induced by amyloid beta peptide (Aβ) and hyperphosphorylated tau protein [3–5]. Moreover, recent evidence suggests that oxidative stress and nitric oxide–mediated events play important roles in the pathogenesis of neurodegenerative disorders, such as AD and Parkinson's disease (PD) [6–8]. Nitric oxide (NO) is a small, diffusible, lipophilic, and transient free radical gas that mediates significant and diverse signaling functions in nearly every organ system within the body [9]. This chapter is presented in two parts: The first reviews the role of NO and free radicals in neurodegeneration, and the second summarizes experimental evidence for the increased vulnerability of neuronal cells to metabolites of NO and reactive oxygen species.

1.1 Neuronal Death and Survival Under Oxidative Stress in AD and PD

Current research indicates that several known gene mutations causing familial AD (Aβ precursor protein [APP], presenilin-1, or presenilin-2 gene) and familial PD (Parkin, PINK-1, or DJ-1) are associated with increased oxidative stress [6]. Further, several known genetic (e.g., apolipoprotein E-epsilon 4 variant [APOEε4]) and environmental (e.g., metals or pesticides exposure) risk factors of sporadic AD and/or PD are associated with increased oxidative stress. At the clinical level, patients at the early stages of AD and PD together with cellular and animal models of these diseases provide consistent evidence that oxidative insult is a major early event in the pathologic cascade leading to AD and PD. Research on the molecular mechanisms of longevity studies suggests that prolongevity gene products, such as forkhead transcription factors and sirtuins, may participate in the insulin-like signaling pathway and oxidative stress resistance against aging. Therefore, an enhancement of prolongevity signaling (e.g., caloric restriction) has been suggested to be a

promising approach as antioxidative strategy against age-associated neurodegenerative diseases [6].

1.2 Superoxide and NO in Senescence and Aging

It has been known for some time that although they themselves are mostly harmless species, superoxide and NO are precursors of the reactive species hydroxyl radical and peroxynitrite and thereby are initiators of aging and various pathologies. In addition, dietary modulation of age-related changes in cerebral pro-oxidant status has been previously suggested to be both feasible and potentially valuable [10]. Furthermore, the role of the physiologic free radicals, superoxide and NO, in senescence and aging development and the mechanisms of processes mediated by these radicals has recently been reviewed [11].

1.3 Role of Reactive Oxygen Species and Reactive Nitrogen Species in Oxidative and Nitrosative Stress and in Aging

The free radical theory of aging attributes cellular pathology to the cumulative accumulation of reactive oxygen species (ROS), although it remains controversial. Molecular interactions between ROS and reactive nitrogen species (RNS), such as NO, suggest that in biological systems one effect of increased ROS is the disruption of protein S-nitrosylation, a ubiquitous posttranslational modification system [12]. In this manner, ROS may not only damage cells but also disrupt widespread signaling pathways. Hence, the interrelationship between oxidative and nitrosative stress in the context of aging and the cardiovascular system are currently being investigated [12].

1.4 Role of NO in Aging, AD, Obesity, and Heart Disease

NO may play a role in the progression of AD and PD [13]. Lipopolysaccharides (LPSs), as well as other bacterial and viral products, cause inducible nitric oxide synthase (iNOS) synthesis that, in turn, produces copious amounts of NO. LPSs similarly activate cytokine and iNOS production in the cardiovascular system and thereby lead to coronary heart disease. Fat is a major supply of NO stimulated by leptin. As fat stores enlarge, the release of leptin and NO rises in parallel in a circadian rhythm with a peak at night. Hence, NO could be to blame for increased coronary heart disease in the presence of obesity. Aging of the anterior pituitary and pineal, with resultant lowered secretion of pituitary hormones and the pineal hormone melatonin, respectively, may be caused by NO. Thus, antioxidants, such as melatonin, vitamin C, and vitamin E, may likely play important roles in reducing or eliminating the oxidant damage produced by NO [13].

1.5 Role of NO and Cellular Stress Response in Brain Aging and Neurodegenerative Disorders

NO and other ROS and RNS exert important roles in the brain, such as neuromodulation, neurotransmission, and synaptic plasticity, but are also involved in pathologic processes such as neurodegeneration and neuroinflammation [14]. Acute and chronic inflammation induces an increased nitrogen monoxide formation and nitrosative stress. NO and its toxic metabolite, peroxynitrite, inhibit components of the mitochondrial respiratory chain leading to cellular energy deficiency, dysfunction, and, ultimately, to cell death. Within the brain, the vulnerability of different brain cell types to NO and peroxynitrite exposure are influenced by factors such as the intracellular reduced glutathione and cellular stress resistance signal pathways. Hence, neurons, contrary to astrocytes, appear exceptionally susceptible to the effect of nitrosative stress. Recent studies support this position for neurologic disorders such as AD, PD, amyotrophic lateral sclerosis (ALS), multiple sclerosis, and Huntington's disease. To endure different types of injuries, brain cells have developed integrated adaptive responses, termed *longevity assurance processes*, encompassing a number of genes dubbed *vitagenes* and components of the heat shock protein (HSP) system, including HSP70 and HSP32, to identify and control assorted forms of stress. Calabrese and colleagues [14] suggest that maintenance or recovery of the activity of vitagenes may delay the aging process and lower the occurrence of age-related diseases thereby providing prolongation of a healthy life span.

1.6 Interplay Between Superoxide and NO in Aging and Diseases via Many Physiologic Functions

Free radicals, superoxide and NO, are critical signaling molecules that mediate a wide variety of physiologic functions, including phagocytosis and vasorelaxation [15]. Nonetheless, regulation errors may result in free radical–mediated damaging processes in cells and tissues. Afanas'ev [15] has suggested that an interplay between superoxide and NO may impact and be responsible for the development of aging and diseases. In particular, the superoxide-mediated proton leak may lead to the inhibition of oxidative phosphorylation, and the competition between NO and superoxide ($O_2^{-\bullet}$) in their reactions with cytochrome oxidase may be a cause of mitochondrial aging [15].

2 NO and Its Physiologic Role

NO is a labile and highly reactive species that is enzymatically generated during the oxidation of L-arginine to L-citrulline by nitric oxide synthase (NOS) [9, 16, 17] (Fig. 1). NO can also be generated by a group of compounds that are collectively known as NO donors, such as sodium nitroprusside (SNP). SNP, a prototypical nitrovasodilator, is considered to produce its vasorelaxant action by releasing NO.

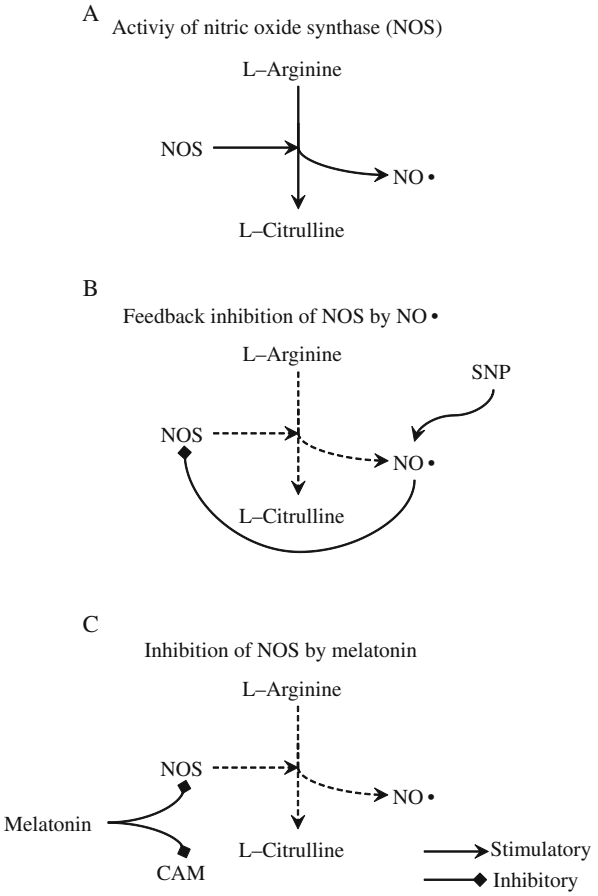

Fig. 1 Regulation of NOS activity: Schematic diagram showing the activity of NOS and its regulation by NO and melatonin. (**a**) Activity of NOS, (**b**) feedback inhibition of NOS by NO, and (**c**) inhibition of NOS by melatonin

The compound is hence used to examine pathways induced by NO in cell lines of different origins and to study the susceptibility of cells to SNP-mediated toxicity. NO plays a fundamental role as a neuronal messenger involved in neurotransmitter release, long-term potentiation, and gene transcription. Under specific conditions, however, NO and its metabolites become neurotoxic [18, 19]. In addition, a recent study suggests that NO mediates toxicity in paraquat-exposed SH-SY5Y cells and that 7-nitroindazole exerts a protective role [20].

2.1 Aβ, NO, and Synaptic Plasticity

The peptide Aβ plays a major role in AD. Consequently, much research is under way to understand the mechanisms by which Aβ is involved in AD pathogenesis. Aβ has

been shown to markedly impair hippocampal long-term potentiation (LTP), a commonly studied cellular model of synaptic plasticity that is considered to underlie learning and memory. Notably, the NO pathway is involved in synaptic dysfunction after Aβ elevation in AD [21] brain. Hence, researchers are currently investigating the exact role of the NO/cGMP/cAMP-regulatory element binding (CREB) pathway in Aβ-induced changes of basal neurotransmission and synaptic plasticity in the hippocampus, a structure within the temporal lobe of the brain fundamental for memory storage [21].

2.2 Aβ Fragment Impairs Memory and Increases NO in the Temporal Cortex of Rats

Aβ(25–35) has been demonstrated to impair memory and increase NO in the temporal cortex of rats [22]. In particular, injection of the fraction Aβ(25–35) caused an increase of neuronal nitric oxide synthase (nNOS) and iNOS immunoreactivity in the temporal cortex and hippocampus. In addition, there was a significant increase of reactive astrocytosis, which was accompanied by neuronal damage in the temporal cortex and hippocampus of rats injected with Aβ(25–35).

3 The Vulnerability of Different Cell Types to ROS and RNS Insults

The vulnerability of different cell types to ROS and RNS is incompletely understood. This issue has been addressed by studying the susceptibility of different cell types to SNP-mediated damage and by quantitatively analyzing the level of secreted derivatives of APP, specific forms of which have previously been shown to be neuroprotective [23–25]. SNP has been used as an exogenous source of NO to define the role of free radicals, superoxides, and ROS in general metabolism and viability of astrocytic and neuroblastoma cell cultures. Consequent to the instability of NO, its stable end products such as nitrite and nitrate (NOx) are generally measured, rather than the parent compound. An additional reason for selecting SNP is that, in addition to NO, SNP also chemically generates KCN, which is toxic to cells. Unlike cyanide, NO is labile and converts to its stable end products, such as nitrite and nitrate, through a series of chemical steps involving the participation of superoxides. As each cell type generates various levels of superoxides, the production of NOx therefore varies among different cell lines. The vulnerability of a specific cell type depends, in an inverse manner, upon the accumulation of nitrite and nitrate. For example, astrocytic and epithelial cell lines generate a considerable amount of NOx, and these cell lines are more resistant against the SNP-mediated damage than neuronal cell lines. These results suggest that cell lines of different origins responded differentially to NO-induced cellular insults, that APP processing was altered as a

consequence of SNP treatment, and that neuronal cell lines lack an effective protective mechanism against free radicals–mediated insult compared with astrocytic cell lines.

4 Use of SNP to Study the Susceptibility of Different Cell Types Toward Free Radicals

In the absence of SNP, nitrite concentrations proved to be below the detection limit of all the cell lines tested at every time point. However, SNP was found to release NOx when incubated with the cell culture medium alone. When the SNP-containing medium was added to the cells, there was an increased release of NOx in the conditioned medium in a time- and dose-dependent manner. This observed increase was significantly higher than the spontaneous NOx released from SNP in the medium. This finding indicates that cellular processes participate in the generation of NOx under these conditions.

5 Different Cell Types Generate NOx Differently When Treated with SNP

Measurement of NOx levels in conditioned medium from different cell lines demonstrated that SNP has different NOx generating capacity depending on the cell type. In all cell types, NOx continued to accumulate in a time-dependent fashion. Two groups of cell types were observed: one with a high level of NOx release, epitomized by astrocytic, glial, and epithelial cells, and one with a low NOx release, as found in neuronal cells. As an example, the SNP-induced NOx release from C6 and U-138 cells was significantly higher at all measurement points (6, 12, 24, 32, and 48 h) than that determined in PC12, IMR-32, and N1E-115 cells under the same conditions. Compared with the conditioned medium, the level of NOx could not be detected in intracellular extracts of different cell types after SNP exposure.

5.1 Increased Levels of NOx Release in SNP-Treated Astrocytic and Epithelial Cell Lines

The SNP-mediated release of NOx at 100 μM SNP is described below. In the conditioned medium of astrocytic U-138 cells, the NOx release was 10 μM at 12 h and 25 μM at 24 h. In C6 cells, NOx release was 24 μM at 12 h and 38 μM at 24 h. In the conditioned medium of HeLa cells, NOx release was 34 and 45 μM at 12 and 24 h, respectively (Table 1). The SNP-mediated release of NOx at 300 μM SNP is the following: In U-138 cells, the NOx release was 13 μM at 12 h and 36 μM at 24 h. In C6 cells, NOx release was 32 μM at 12 h and 60 μM at 24 h. In the conditioned medium of HeLa cells, NOx release after 300 μM treatment was 46 and

Table 1 Effect of 100 μM SNP on the release of nitrite and nitrate (NOx) in different cell lines[a]

	SNP (100 μM) effect on release			
Cell lines	NOx (M) 12 h	LDH (%) 12 h	NOx (M) 24 h	LDH (%) 24 h
C6	24.12 ± 0.20	3.60 ± 1.00	37.51 ± 0.21	4.50 ± 1.25
HeLa	34.06 ± 0.31	5.80 ± 1.60	45.16 ± 1.64	15.50 ± 1.60
N1E-115	16.80 ± 1.20	52.60 ± 4.72	24.80 ± 0.96	49.66 ± 1.96
U-138	10.00 ± 0.95	4.00 ± 0.66	24.69 ± 1.16	4.60 ± 0.66
IMR-32	4.10 ± 0.75	0.00 ± 0.00	15.25 ± 0.73	0.00 ± 0.00

[a]Cell lines of different origins were cultured and treated with 100 μM SNP for various time periods, and levels of NOx and LDH were measured as described previously [23]. Cytotoxicity was expressed as the percentage of LDH activity from the plain conditioned medium (0%) to total LDH activity in the medium when cells were completely lysed (100%).

Table 2 Effect of 300 μM SNP on the release of nitrite and nitrate (NOx) in different cell lines[a]

	SNP (300 μM) effect on release			
Cell line	NOx (M) 12 h	LDH (%) 12 h	NOx (M) 24 h	LDH (%) 24 h
C6	31.95 ± 0.32	5.70 ± 0.45	59.09 ± 0.08	4.10 ± 1.80
HeLa	45.65 ± 0.51	2.90 ± 2.60	76.22 ± 1.10	26.70 ± 2.10
N1E-115	18.60 ± 1.10	46.88 ± 16.30	23.10 ± 0.50	56.48 ± 9.24
U-138	13.40 ± 0.25	4.30 ± 1.60	35.90 ± 3.70	12.60 ± 0.83
IMR-32	4.79 ± 0.89	0.00 ± 0.00	20.75 ± 1.33	3.86 ± 0.64

[a]Cell lines of different origins were cultured and treated with 300 μM SNP, and levels of NOx and LDH were measured as described previously [23]. Cytotoxicity was expressed as the percentage of LDH activity from the plain conditioned medium (0%) to total LDH activity in the medium when the cells were completely lysed (100%).

76 μM at 12 and 24 h, respectively (Table 2). Hence, there is an increased NOx release over time and by dose of the treatment in these cell lines, however the release did not follow a simple linear formula.

5.2 Reduced Levels of NOx Release in SNP-Treated Neuronal Cell Lines

In N1E-115 cells at 100 μM SNP treatment, NOx release was 17 and 25 μM at 12 and 24 h, respectively. However, in these cells at 300 μM, NOx release after SNP treatment was only 19 and 24 μM at 12 and 24 h, respectively (Tables 1 and 2). Similar to the neuroblastoma cells, lower levels of NOx were released in the conditioned medium of PC12 cells than that in astrocytic and glial cell lines at all time points and SNP doses studied (data not shown).

6 SNP-Induced Cell Death

SNP-induced damage in different cell types was assessed by assaying for elevations in lactate dehydrogenase (LDH) levels. In this regard, the studied cell lines varied to different degrees in their sensitivity to SNP. For example, glial C6 cells were found to be about 90% viable with 70 μM NOx release, whereas neuroblastoma N1E-115 cells were about 50% viable with only 15 μM NOx release. Thus, the neuronal cell line N1E-115 was most sensitive to cell death in the face of a low level of NOx production. On the contrary, C6 and U-138 cells (and to some extent HeLa) were resistant to NOx-mediated damage up to 48 h after 300 μM SNP treatment (Tables 1 and 2).

7 NOS in Different Cell Types

In neurons, NO is mainly generated by a calcium-dependent activation of constitutive neuronal NOS [9]. Specifically, neurons express a constitutive type of NOS, and its activity is regulated by stimulation of the *N*-methyl-D-aspartate (NMDA) receptor, muscarinic receptor [26], or an increase in Ca^{2+} levels inside the cells [27]. Additionally, neurons can express iNOS [28]. In glial cells, NO is synthesized in a calcium-independent manner via induction of NOS [29]. Astrocytes express Ca^{2+}-independent iNOS [30]. There are several reports describing the cytokine induction of NOS activity in astrocytoma cells [31]. In addition, the endothelial form of NOS is also present within the CNS and is associated with the brain vasculature [32]. Recently, NO-mediated modulation of synaptic activity by astrocytic P2Y receptors has been shown [33].

7.1 Lack of NOS Activity in U-138, C6, and HeLa Cells

NOS activity was quantified in U-138 cells because they were used as a prototype for the astrocytic cell line. Moreover, in these cells a significant release of NOx occurred over time after SNP treatment with very little cell toxicity. NOS activity was assayed by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. No intrinsic NOS activity was detected in U-138 cells. None of the doses of SNP attenuated or stimulated the enzyme activity when C6 or HeLa cells were incubated with SNP for 48 h.

7.2 Presence of NOS Activity in N1E-115 Cells

NOS activity in the neuroblastoma cell line N1E-115 was assessed as it expresses endogenous NOS. When N1E-115 cells were treated with 100 μM and 300 μM SNP in serum-free dulbecco's modified eagle's medium (DMEM) medium (in vivo), no change in NOS activity was detected. The basal level of NOS activity was very low

after 48 h of serum deprivation. However, when the cells were incubated with SNP in DMEM medium containing 10% fetal bovine serum (FBS), the basal level of NOS activity was significantly elevated (data not shown).

7.3 Inhibition of NOS Activity by SNP

An addition of SNP into a cytosolic preparation of N1E-115 cells elicited a concentration-dependent inhibition of the formation of L-[^{3}H]citrulline. For example, compared with untreated N1E-115 cell extracts, there was a decrease of 38% NOS at 300 μM SNP and 73% NOS at 3 mM SNP. However, when the cytosolic preparations from C6, HeLa, or U-138 cells were incubated with SNP for 1 h, there was no NOS activity observed [23].

8 Effects of Different Agents on NOx Production and LDH Release in Astrocytic Cells

To evaluate the pathway involved in NOx production, the effects of a cytokine, a free radical scavenger, and an antioxidant, an NOS inhibitor were studied [23]. Astrocytes are the major cell type within the CNS, and involvement of astrocytes in AD brain pathology has been well documented, therefore U-138 cell lines were used to study the effects of different agents.

8.1 High Dose of IL-1β Decreased Nitrite Production in C6 Cells When Stimulated with SNP

Cytokines like IL-1β were shown to stimulate NOS expression in various cell types [34, 35]. Incubation of U-138 cells with a low dose of IL-1β in the assay did not stimulate nitrite production. This is in agreement with a study by Feinstein et al. [31], which showed no induction of NOS in another glioma cell line, C6, after treatment with IL-1β. However, in our system, a higher dose of IL-1β (30 ng/ml) showed a decrease in nitrite production when stimulated with SNP compared with the control [23].

8.2 Inhibition of NOx Release with Carboxyl-PTIO Treatment

The compound carboxyl-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) is a water-soluble, stable NO radical scavenger that reacts with free NO radical in a stoichiometric manner. Carboxyl-PTIO was hence used to test the specificity of NO production from SNP. A significant inhibition of

NOx production was observed when U-138 cells were treated with 100 μg/ml carboxyl-PTIO.

8.3 Inhibition of NOx Release with SOD-1 Treatment

NO reacts with superoxide radicals to generate peroxynitrite [9]. As superoxide dismutase (SOD) is a scavenger of superoxide ions and prevents peroxynitrite formation, SOD-1 treatment should decrease NOx release in the medium. In this regard, the addition of SOD-1 to U-138 cells produced a decrease in NOx accumulation.

8.4 L-NAME Treatment Did Not Change the Release of NOx Release

A well-studied NOS inhibitor, L-NAME (L-nitro-arginine methyl ester), was used to study the involvement of NOS in SNP-mediated NOx release in U-138 cells. Incubation of the cells with 300 μM L-NAME did not change the amount of NOx production by SNP. This observation suggests that the action of SNP does not involve NOS. The measurement of LDH demonstrated that, compared with controls, none of the agent described above showed a significant level of toxicity in cell culture.

9 Pathway of NOx Production in Various Cell Types

Different cell types generate varying amounts of NOx from SNP-treated cells, signifying a cell type–specific release of NOx. It remains to be elucidated how cells influence this process, but the following pathway can be suggested from the work of several laboratories (Fig. 2). SNP generates NO after liberating cyanide ions. Peroxynitrite formation results from the reaction of a superoxide ion and NO, which decompose to NO_2 and hydroxyl radicals and subsequently results in the formation of NO_2/ NO_3. As NO was not directly measured in experimental studies but rather is followed through generation of its oxidative metabolites (NOx) that are derived from peroxynitrite, it follows then that more superoxides should lead to an increased peroxynitrite formation. As cellular processes result in the generation of ROS and superoxide ions, these results suggest that the ability of astrocytic and neuroblastoma cells to produce a different level of NOx is due to their different capacity in superoxide generation. Most of the biological effects of SNP are mediated through NO, which activates guanylate cyclase and increases cGMP levels in many cell systems. The mechanism of NO release from SNP is not clear, but several studies [36, 37] indicated that spontaneous release of NO from SNP does not account for the total amount of NO generated from SNP. The dominant site for metabolic activation of SNP to NO in vascular smooth muscle resides in the membrane fractions [38]. As

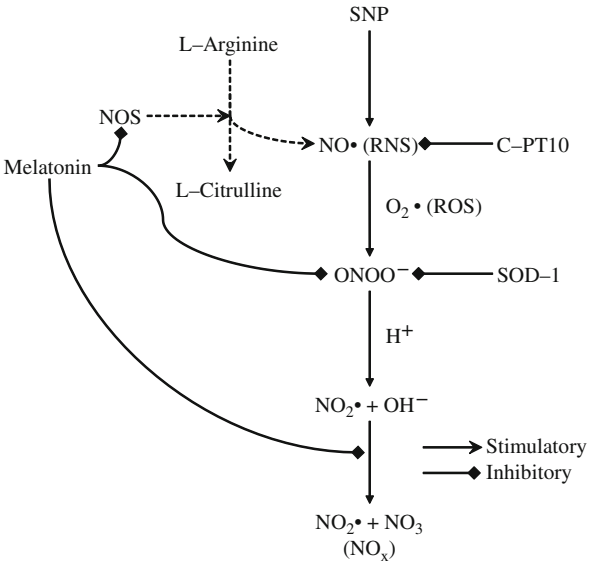

Fig. 2 Generation NOx by SNP in cultured cells. A schematic diagram illustrating the interaction of SNP, NO/its metabolites, and other regulators. SNP chemically releases NO after liberating cyanide ions. Peroxynitrites are formed as a result of the reaction between the superoxide ion and nitric oxide. Peroxynitrites then decompose to NO_2 and hydroxyl radicals, which subsequently result in the formation of NO_2/NO_3 (NOx). As the cellular process results in the formation of ROS including superoxides, astrocytic and neuroblastoma cells produce various levels of NOx due to their different capacity of superoxide generation. The protective effect of NOx against SNP-mediated toxicity differs in cell types, which generate varying degree of NOx from the SNP-treated cells. The generation of NO is regulated by melatonin, SOD-1, and other agents as indicated within the figure and described in the text

our study showed differential release of NOx from different cell types, we hypothesize that the metabolic interaction of SNP with different cell lines may account for this difference in NOx release [23].

10 Mechanism of NOx Production from SNP

The specificity of NOx production from SNP was tested by three different agents: (i) an NO scavenger, (ii) an NOS inhibitor, and (iii) a superoxide scavenger (Fig. 2). When cells were treated with carboxy-PTIO, a characterized and stable NO radical scavenger, a significant inhibition of NO production was observed. Treatment of U-138 and N1E-115 cells with L-NAME, a well-studied NOS inhibitor, did not change the amount of nitrite production by SNP. This observation suggests that the action of SNP does not involve NOS. SOD, which enhances the biological half-life of NO by removing peroxide ions, did not increase nitrite accumulation in our study. The mechanism of the interaction of SOD and NO is not fully understood.

However, it is known that SOD increases the generation of NO from L-arginine in the presence NOS. The mechanism by which SOD increases NOS-mediated NO signal cannot be fully explained by dismutation of $O_2^{-\bullet}$, and the effect of SOD on free NO was different from the effect of SOD on NO that is generated by NOS.

11 Levels of NOx and Cellular Viability

The level of NOx is important for cellular viability, and this determines how each cell line differs in its ability to handle SNP-mediated toxicity. For example, C6 cells were found to be the most and N1E-115 cells the least viable after a similar level of SNP-mediated toxicity. Although the toxic effect of SNP has been suggested to be due to the formation of peroxynitrite generated via the interaction between NO and superoxide, this could also potentially involve the accumulation of either cyanide, NO, or any of these combinations. Nonetheless, these results clearly demonstrate that the neuronal cell lines studied were more vulnerable to NO-mediated cytotoxicity than were astrocytic or glial cell lines, and this finding is of considerable potential in vivo relevance.

12 Protective Cellular Mechanism to Reduce the SNP-Induced Toxicity

Activated astrocytes and macrophages in vivo produce a high amount of NO. It is believed that some protective mechanism may be operative in these cells to avoid the SNP-induced cyanide toxicity. Differential susceptibility of neurons and astrocytes to NO might be due to their different content of reduced glutathione (GSH). The GSH content of neurons has been reported to be drastically decreased after peroxynitrite exposure, whereas the GSH content of the astrocytes was not affected [39]. It has also been reported that neurons containing NOS are more resistant to NO toxicity [18] by an unknown mechanism. Endogenous NO is synthesized from L-arginine by NOS in the various cells described. The astrocyte and microglial cells play an important role in brain pathology and are in close contact with neurons. Elevated NO production from astrocytic cells could potentially damage the surrounding neurons that are more vulnerable to NO-mediated toxicity and may occur during disease processes. In this regard, the role of Aβ in the induction of NOS activity in astrocytic cells has been investigated: Aβ was able to induce the expression of iNOS and NO production in C6 cell lines in the presence of various cytokines [40]. These activated astrocytes may cause neuronal damage via the indirect NO mechanism and thereby impact or drive pathologic processes.

13 Relationship Between the Enzymatic and Nonenzymatic Pathways of NO Release

The release of NOx from SNP in cell culture appears to proceed via a nonenzymatic pathway from the following observations. First, the SNP-derived NOx release was not inhibited by cotreatment with L-NAME, an NOS inhibitor. Second, most of the cell lines described herein (except N1E-115) lack a basal level of NOS activity. Third, other inducers of NOS (such as IL-1 and LPS) have not been found to exert any significant effect on SNP-mediated release of NOx. Although nonenzymatic, the release of NOx involves the intermediate step of NO because an NO scavenger, such as carboxy-PTIO, inhibits it. Additionally, the formation of peroxynitrite with superoxide is critical in final NOx release and is supported by the inhibitory effect of superoxide dismutase (Fig. 2). We have also investigated the relationship between the enzymatic and nonenzymatic pathways of NO release. Our results demonstrate that nonenzymatic release of NOx could inhibit the activity of NOS, resulting in a decreased accumulation of NOx. This could be due to the inhibition of NOS by ROS generated by SNP. This assertion is in agreement with previous reports showing a feedback inhibition of NOS activity with excess NOx. In addition, it is supported by our previous results on differentiated PC12 cells [23].

14 Cellular Participation for the Generation of NOx

According to our working model, a direct interaction of the released NO with superoxide anions and other ROS leads to the formation of nitrite. As ROS are a natural by-product of normal metabolism, cell type–dependent generation of ROS could directly influence the nitrite measurement in these cells. As the half-life of NO is approximately 30 s and that of SNP can be several hours, the effect of SNP over 48 h may be exclusive of NO production but secondary to other metabolites such as KCN. We have not measured the ratio of nitrite to nitrate production, but rather have focused on the release of total NOx. Depending upon the aqueous phase examined, nitrite production usually is significantly increased over nitrate production during the release of NO. These results suggest that NOx release does not solely depend on the growth rate of different cell lines and that SNP-mediated damage caused by the combination of NO and ROS results in severe damage to the neuronal cell lines. As there is a buildup of ROS during normal aging and particularly in AD, the selective loss of neuronal cells should be expected from the apparent lack of any efficient neuroprotective mechanism against superoxides, free radicals, and other ions.

15 Role of Melatonin in the Inhibition of NOx Release

Melatonin hormone, a well-characterized antioxidant, could play an important protective role in aging and AD based on cellular and animal studies [41, 42]. Inhibition

of NO production may be a further means whereby melatonin reduces oxidative damage under conditions, such as in ischemia/reperfusion and sepsis, where NO seems to be important in terms of the resulting damage [43]. Previously, we have shown that melatonin can promote neuronal differentiation [41, 44]. We have additionally explored whether the treatment of cultured cells with melatonin can reduce the release of free radicals and other ROS. Indeed, in neuroblastoma cells, the release of NOx as mediated by SNP was significantly inhibited by treatment with melatonin [23]. These results suggest that SNP-mediated NOx release was mediated by superoxide ions and/or free radicals that, in turn, can be inhibited by melatonin (Figs. 1 and 2). We have also characterized the effects of different neuroprotective compounds, including melatonin [35, 44], to reduce and/or prevent the formation of this cascade pathway.

16 Relationship Between NO Metabolites, Antioxidants, and AD

These results suggest that the release of cell type–specific ionic metabolites could be the target for generation of toxic products, like peroxynitrite, which lead to cell injury, damage, and, eventually, to death. It is conceivable that in neurodegenerative disorders epitomized by AD, the likely increased release of superoxides and other ROS can potentially initiate a feedback loop involving the formation of toxic products that are capable of producing further insult and damage to cells. Thus, the ROS-scavenging function of compounds exemplified by melatonin, along with their neuroprotective and neurodifferentiating roles, can potentially be used for the prevention of neurodegenerative disorders such as AD. The mechanism(s) underpinning such actions warrant further study as such pathways are likely shared among neurodegenerative diseases and provide potential for a common therapeutic/preventative strategy. In conclusion, studies described herein demonstrate that the exposure of neuronal cells to SNP makes them more vulnerable to injury. Furthermore, astrocytic and neuronal cell cultures can be used as valuable model systems to study the involvement of nitric oxide metabolites, peroxynitrites, superoxides, and other reactive oxygen species in health, aging, and neurodegeneration.

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Free Radical–Mediated Damage to Brain in Alzheimer's Disease: Role of Acrolein and Preclinical Promise of Antioxidant Polyphenols

Charles Ramassamy, Madeleine Arseneault, and Dang Thanh Nam

Abstract Brain aging is associated with accumulation of oxidation-induced damage, likely due to the imbalance between antioxidant defenses and intracellular generation of reactive oxygen species (ROS). Alzheimer's disease (AD) is the most frequent neurodegenerative disease with multiple causes, and aging is considered as the major risk factor for the development of this disease. From early stages, oxidative damage is strongly implicated in the pathophysiology of this disorder. Lipid peroxidation generates various by-products such as F2α-isoprostane, 4-hydroxynonenal, malondialdehyde, and acrolein with the latter being the most reactive. In the neuroblastoma SK-N-SH cell line, our results show that acrolein can induce cell toxicity through a nonapoptotic pathway. Moreover, acrolein can alter the redox state by depleting glutathione levels. Considering the role of oxidative stress and the toxic effect of by-products of lipid oxidation, intake of compounds with antioxidant activities such as polyphenolic compounds may be beneficial in the prevention of AD. In this chapter, we will review the role of free radical–mediated damage in AD and in transgenic mouse models and present the main intracellular target of polyphenolic compounds underlying their potential neuroprotective effect.

Keywords Apolipoprotein E · Protein carbonyls · 4-HNE · Glutathione · Catechins · NF-kB

1 Introduction

1.1 Free Radical–Mediated Damage to Brain During Aging and in Alzheimer's Disease

Brain aging is associated with accumulation of oxidation-induced damage, likely due to the imbalance between antioxidant defenses and intracellular generation of

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reactive oxygen species (ROS). The overall rationale for implication of oxidative stress in aging brain is based on the presence of high levels of unsaturated fatty acids, which are vulnerable to oxidation (particularly high in 20:4 and 22:6 fatty acids); on high amounts of oxygen consumed by the brain (about 20% of the total amount used in the body); on lower levels of antioxidants; and on the high concentrations of transition metals such as Fe^{2+}, which are key catalysts of oxidation-induced damage.

In brain, oxidative damage to DNA occurs continuously resulting in damaged nucleotides and strand breaks. It has been estimated that 10,000 oxidative interactions occur between DNA and endogenously generated free radicals per human cell per day. One of the most widely studied base lesions is 8-hydroxy-2′-deoxyguanosine (8-OHdG), a hydroxyl radical–damaged guanine nucleoside. Several studies have demonstrated that the level of 8-OHdG is elevated in old brains, with a fourfold increase when compared with young brains [1]. In mitochondria, higher levels of oxidative damage and DNA mutations have been ascribed to location of the DNA near the inner mitochondrial membrane sites where superoxide anions are mainly formed.

It is now well established that lipid peroxidation is elevated in brain with age. Lipid peroxidation yields a large number of compounds such as malondialdehyde (MDA), 4-hydroxynonenal (HNE), F2-isoprostanes, and acrolein. The most widely studied are the active aldehydes and isoprostanes. A set of isoprostanes that can be formed in brain from peroxidation of docosahexanoic acid is the neuroprostanes [2]. HNE is the major product formed from peroxidation of omega-6-conjugated fatty acids, such as arachidonic acid and linoleic acid [3]. HNE is biologically active causing gene induction such as for those encoding for antioxidant and cytoprotection; it can react with many biological molecules including various amino acids, proteins, and bases in DNA. MDA was increased in the cytoplasm of neurons and astrocytes in normal aging but was rarely detected in normal young subjects. In hippocampus, neuronal and glial MDA deposition was marked in the CA4 region but mild in CA1 [4]. Moreover, the lipid peroxidation products MDA, HNE, and acrolein have been reported to react with DNA and proteins to produce further damage in aged brains (see review by [5]).

Accumulation of oxidized proteins in brain is widely considered as a hallmark of aging [6–8] with an increase of nearly twofold and fourfold in human and rats, respectively [6]. Oxidation-induced damage to proteins can affect virtually all amino acids, with sulfur-containing amino acids and aromatic amino acids being the most susceptible [9]. The amount of oxidized protein is influenced not only by its rate of formation but also by its rate of degradation. In a functioning system, oxidized proteins are degraded by the proteasomal/protease system, which is responsible for removing damaged proteins that form insoluble aggregates (see review by [10]). During aging, proteasome activity also declines [10], and this could contribute to the elevation of oxidized proteins. Age-related modifications of multiple biomolecules by oxidative damage may affect brain health and function in the long run. These changes observed in normal aging are exacerbated in various neurodegenerative disorders such as in AD, amyotrophic lateral sclerosis, Huntington's disease, or

Parkinson's disease. In this chapter, we will focus on the role of oxidative damage in AD.

AD is the most frequent neurodegenerative disease with multiple causes, and aging is considered as the major risk factor. According to the Alzheimer's Society, the number of AD cases could reach 34 million by 2025 [11]. Its prevalence doubles approximately every 5 years after the age of 60, with 1 in 10 individuals over 65 years and nearly half of those over 85 years being affected by the disease. With the anticipated demographic shift to an aging population, AD presents one of the greatest threats to the future of the health care system. AD is characterized by a gradual decline in cognitive and memory loss. Neuropathologically, AD is associated with numerous biochemical changes such as the accumulation of extracellular neuritic plaques of the amyloid-β peptide, intracellular neurofibrillary tangles, cholinergic alterations, neuronal metabolic insults with glutamate-induced excitotoxicity, proliferation of astrocytes, microglial activation, synaptic and neuronal loss, and elevation of oxidative stress.

AD is multifactorial with a complex combination of genetic and nongenetic components. The early-onset familial form represents only a small fraction of all AD cases ($\leq$5%) and typically presents itself with age of onset younger than 65 years, whereas the nongenetic or sporadic form represents the majority of AD cases. After three decades of intensive research, at least four genes and hundreds of potential susceptibility loci have been identified. To date, mutations on three genes have been reported to cause the early-onset familial form of AD. These include the genes coding for the amyloid precursor protein (*APP*) on chromosome 21, for presenilin 1 on chromosome 14 (*PSEN1*), and for presenilin 2 on chromosome 1 (*PSEN2*) [12–14]. While these AD-causing mutations occur in three different genes located on three different chromosomes, they all share a common biochemical pathway; that is, the altered production of the amyloid-β peptide (Aβ), which leads to neuronal death and dementia. Aβ is released after the cleavage of APP by β- and γ-secretases, respectively. β-Secretase has been identified as an aspartic protease, and γ-secretase can cleave APP at the C-terminal end of Aβ at different sites, giving rise to Aβ peptides that are 39–43 amino acids long. The exact location of C-terminal cleavage is critical due to the generation of the amyloidogenic peptide $A\beta_{1\text{-}42}$, which is correlated with AD development.

On the other hand, the late-onset form of AD has one common genetic risk factor, which is the ε4 allele of the apolipoprotein E gene (*APOE*) located on chromosome 19q13 [15, 16]. The increased risk with the ε4 allele of the apolipoprotein E gene has been consistently replicated in a large number of studies across many ethnic groups. Unlike the mutations in the known early-onset familial form genes, the ε4 allele of the apolipoprotein E gene is neither necessary nor sufficient to cause AD but instead operates as a genetic risk factor by decreasing the age of onset in a dose-dependent manner. The ε4 allele is also a risk factor for atherosclerosis [17, 18], HIV disease progression [17, 18], and additional neurologic disorders including cerebral amyloid angiopathy (CAA) and CAA-associated cerebral hemorrhages [19], tauopathies and dementia with Lewy bodies [20], Parkinson's disease [21], and multiple sclerosis [22]. These observations suggest that the ε4 allele may be associated

with acceleration in the development and progression of several neurodegenerative diseases. ApoE is a plasma glycoprotein with a molecular mass of 34 kDa, synthesized mainly by liver, neurons, and astrocytes in brain and other cell types including macrophages and monocytes. A polymorphism of ApoE in human serum has been described by isoelectric focusing, which determined the three major isoforms of ApoE (ApoE2, ApoE3, and ApoE4). A single locus with three alleles (E2, E3, and E4) is responsible for this pattern. The apoE2, apoE3, and apoE4 isoforms differ in amino acid sequence at two sites (residues 112 and 158) with apoE2 containing cysteine residues and apoE4 containing arginine residues at both sites while apoE3 contains cysteine and arginine at positions 112 and 158, respectively. APOE is a major apolipoprotein and a cholesterol carrier in brain [17]. In brain, apoE–lipoprotein particles are produced primarily by astrocytes and deliver cholesterol and other essential lipids to neurons through members of the low-density lipoprotein receptor (LDLR) family [22, 23]. Aβ assemblies, particularly Aβ oligomers, are highly toxic to neurons. Synthetic Aβ42 oligomers decrease the viability of cultured neurons 10-fold more than Aβ42 fibrils and 40-fold more than unaggregated peptides [24]. ApoE4 augments Aβ42 oligomer-induced neurotoxicity to a larger extent than ApoE3 [25].

Recently, it was shown that the sortilin-related receptor 1 (*SORL1*) is genetically associated with sporadic AD in several populations [26] but not in familial AD [27]. SORL1 is a member of the LDLR family, which binds apolipoprotein E. SORL1 functions as a sorting and trafficking protein, guiding APP into the recycling endosome pathways that result in reduced Aβ production [28]. A large body of evidence indicates that oxidative stress is involved in the pathogenesis of AD. Oxidative stress in AD patients occurs due to various factors such as genetic factors (the inheritance of the ε4 allele of apoE) [29–33], mutations (*APP*, *PSEN-1*, *PSEN-2* genes), environmental causes, lifestyle-related factors, and certain health conditions such as diabetes, brain injury, and hypercholesterolemia [34]. Oxidative stress is found in various transgenic mouse models of AD, as well as in tissues and fluids from patients with AD (living and postmortem brains) and cognitive diseases such as mild cognitive impairment (MCI) and Down syndrome. Oxidative stress affects AD patients at protein [35], nucleic acid [36], and lipid [37] levels. For instance, isoprostanes, derived from free radical oxidation of docosahexaenoic acid, are increased in brain cortex [38]. Interestingly, F2-isoprostanes, prostaglandin-like compounds derived from free radical–catalyzed peroxidation of arachidonic acid, are also elevated in plasma, urine, and cerebrospinal fluid from AD patients [39]. Moreover, we and others have demonstrated that the levels of lipid peroxidation in AD brain is dependent on the ApoE genotype and level [29, 31, 32, 40, 41].

Protein carbonyls and 3-nitrotyrosine, which are the markers of protein oxidation, are also elevated in AD [42, 43]. Protein carbonyls are present in both tangles-bearing and non-tangles-bearing neurons [6, 44] and in frontal lobe and hippocampus [45, 46]. Nitrotyrosine and dityrosine cross-linked proteins are elevated eightfold and threefold, respectively, in the hippocampus and neocortical regions of AD brain compared with age-matched controls [47]. By using redox proteomics, specific elevation of oxidatively modified proteins such as α-enolase, heat shock cognate 71 (HSC 71), creatine kinase BB (CK BB), glutamine synthase (GS), and

ubiquitin carboxy-terminal hydrolase L-1 (UCHL-1) have been identified in the hippocampus and the parietal lobe of the AD brain [48, 49].

It has been found that levels of multiple oxidized bases from nuclear and mitochondrial DNA in AD brain were significantly higher in frontal, parietal, and temporal lobes compared with that in control brain regions [50]. Moreover, mitochondrial DNA had approximately 10-fold higher levels of oxidized bases than nuclear DNA. These data are consistent with higher levels of oxidative stress in mitochondria. DNA from temporal lobe showed the most oxidative damage, whereas cerebellum was only slightly affected in AD brains. These results suggest that oxidative damage to mitochondrial DNA may contribute to the degeneration of some areas as observed in AD. DNA repair mechanisms have a critical role in protecting the genome. Several studies have shown a decline in repair of 8-OHdG in AD [51, 52]. RNA oxidation is also an important event in the pathogenesis of AD as up to 50% of mRNAs purified from AD frontal cortices were oxidized [53, 54]. Interestingly, studies on either human samples or experimental models coincidentally suggest that oxidative RNA damage is a feature in vulnerable neurons at the early stage of AD, indicating that RNA oxidation actively contributes to the onset or the development of AD [53, 54].

Further evidence of oxidative stress in AD is the modification of antioxidant activity in the brain. In early-stage as well as in late-stage AD, activities of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and glutathione reductase are lower in brain [55]. We have demonstrated that catalase activity and glutathione level are apoE genotype dependent [32]. The glutathione transferase activity is decreased in several regions of AD brain including hippocampus [56]. Notably, some studies highlight a role for free radical–mediated injury to brain regions from early stages of AD, as lipid, protein, as well as DNA and RNA oxidation are elevated in MCI [36, 37, 57], a condition that is a transition phase between control and AD. MCI suffers from a decline in cognition without signs of dementia, with activities of daily living relatively unaffected. Pathologically, MCI has also been characterized by using magnetic resonance imaging technology to show measurable atrophy in the hippocampus and entorhinal cortex [58], both neurodegenerated areas in AD. Increased levels of HNE and acrolein in different brain areas were also described in the hippocampus/parahippocampal gyrus and cerebellum from MCI compared with that in control subjects [59]. Both by-products of lipid peroxidation are known to be neurotoxic and can affect neuronal functions [60, 61]. These studies establish oxidative damage as an early event in the pathogenesis of AD that can serve as a therapeutic target to slow the progression or perhaps the onset of the disease.

2 Markers of Free Radical–Mediated Damage in Transgenic Mouse Models

All of the transgenic mouse models are in fact models of dominantly inherited forms of AD. Therefore, extrapolation to the late-onset form of AD should be made

carefully. Some strains develop Aβ plaques, some accumulate neurofibrillary tangles (NFTs), and some do both. Mice overexpressing mutant human APP develop Aβ plaques at about 10–12 months of age but do not develop NFTs. Mice that express both human mutations APP and PS1 (APP-PS1) accumulate Aβ plaques at a younger age but still do not develop NFTs. On the other hand, accumulation of NFTs is achieved in mice carrying mutant human tau genes that are associated with frontotemporal dementia characterized by NFT formation but not senile plaques. These mice do not develop Aβ plaques. The development of both Aβ plaques and NFTs have been achieved recently in a triple transgenic mouse model expressing APP, PS1, and a mutant tau protein [62]. Oxidative damage has been examined in each of these transgenic mouse models. Some of these studies are summarized in the Table 1. Most of the animal studies of oxidative damage associated with AD pathology have used the Tg2576 strain, which expresses the human APP gene containing the *Swedish* mutation associated with autosomal dominant AD. These mice develop Aβ plaques and memory impairment from 10 to 12 months of age [63], and the deposition of Aβ plaques is associated with an increase in cortical SOD and glutathione peroxidase [64]. In general, oxidative damage is greater in mice with single APP or PS1 mutation than in wild-type mice, and even higher in double transgenic mice expressing APP-PS1. The role of NFT accumulation in oxidative damage has been examined to a limited extent. Oxidative markers are also elevated in 3 × Tg

Table 1 Markers of oxidative stress in some transgenic mouse models of Alzheimer's disease

Strains and models	Markers of oxidative damage and localization	References
APP (Tg2576)	Elevation of F2-isoprostanes in hippocampus and in plasma but no elevation in cortex of aged animals	[66, 137]
	Increase of HNE and antioxidant enzymes (SOD and HO-1) in vicinity of Aβ plaques	[138]
	No increase of carbonyl levels in cerebral cortex of aged animals	[139]
APP (Tg2576)-PS1DeltaE9-PGE2 EP2 receptor–/–	Decrease of F2-isoprostanes and F4-neuroprostanes in neuronal and non-neuronal cells	[140]
PDGF-APP (Tg2576)	No elevation of lipid peroxidation at 3–4 and 12–15 months of age	[141]
Thy1-APP751 (SL)	Elevation of HNE levels in brain at 3 months in female and at 12 months in both genders, that is, before and after plaque deposition	[141]
Thy1-APP751 (SL)	Reduction of the activity of CuZn-SOD	[141]
APP-PS1	Elevation of 3-nitrotyrosine (3-NT), carbonyls, 4-HNE in APP/PS1 neurons in culture by day 3, and F2-isoprostanes	[140, 142, 143]
Tau	Carbonyls, 8OHdG	[144]
Apolipoprotein E	Elevation of lipid oxidation, reduction of α-tocopherol	[33]

mice [65]. In the Tg2576 mice model, a serial examination in brain, plasma, and urine indicates an elevation of F2-isoprostane at 8 months of age, before the formation of senile plaques, with further increase between 12 and 18 months of age [66]. Several studies have explored the role of antioxidants by treating mice with α-tocopherol or vitamin E. Vitamin E can attenuate both oxidative damage and Aβ plaque accumulation if it is initiated early in life [67]. The hypothesis that oxidative stress promotes Aβ deposition is strengthened by the findings obtained from Tg2576 crossed with mice deficient in α-tocopherol transfer protein with increased cerebral oxidative damage and accelerated Aβ deposition [68, 69]. The evidence for increased oxidative stress in transgenic mice is variable and suggests that, in addition to the transgene expression, other factors such as diet may be as important in producing oxidative damage.

3 Acrolein as a Potential Inducer of Oxidative Stress and Its Role in AD

Lipid peroxidation generates various by-products such as F2α-isoprostane, 4-HNE, MDA, and acrolein [3] with HNE and MDA being the most abundant and acrolein the most reactive. Each of these by-products of lipid peroxidation has been shown to be elevated in AD brain. Acrolein is an α, β-unsaturated aldehyde with two functional groups that can participate in chemical reactions: the aldehyde group and the carbon–carbon double bond (Fig. 1). Acrolein is the strongest electrophile among the unsaturated aldehydes and therefore displays strong reactivity with nucleophile compounds [3]. Moreover, as acrolein is a potent alkylating agent, it may react with matrix tissue or cell surface proteins, causing alteration of structure and function of matrix proteins as it can pass through the intact cell membrane with facility. As an

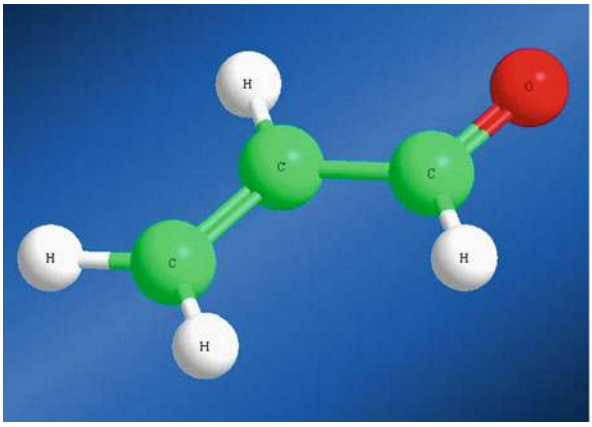

Fig. 1 Molecular structure of acrolein

endogenous toxin, this makes it especially insidious, and acrolein produced can thus spread from the dying cell of origin to damage or kill adjacent cells.

On hippocampal cell culture model, acrolein has been shown to be toxic in a time- and concentration-dependent manner and is more toxic than HNE at 5 μM concentration. Within 6 h of treatment, 0.5 μM acrolein led to 25% decrease in neuron survival, and all cells were dead at a concentration of 10 μM, whereas within 24 h of treatment, 2.5 μM acrolein led to nearly complete neuronal death [60]. However, the level of toxicity depends on neuronal culture. For instance, on cortical neuron cultures, treatment with 10 μM acrolein for 6 h led to an approximately 20% decrease in neuronal survival, and all cells were dead at a concentration of 25 μM [61]. On neuroblastoma SK-N-SH cell line, our results show that acrolein can induce cell toxicity from 10 μM after 48 h (Table 2). The mode of cell death induced by acrolein is dependent on the dose and on cell type. It has been shown that acrolein activated early-stage processes in the mitochondrial pathway of apoptosis, such as Bax translocation to mitochondria, cytochrome c release, caspase-9 activation, and translocation of apoptosis-inducing factor to the nucleus [70]. It is not clear whether acrolein initiates cell death by apoptotic mechanisms or by cellular necrosis. We observed that with 5 μM concentration, acrolein did not induce apoptosis in SK-N-SH cells (Fig. 2). Several mechanisms could be implicated in the neurotoxic mechanism of acrolein such as oxidative stress. Therefore, we have examined the effects of acrolein on the intracellular levels of GSH. Our results show that the intracellular levels of glutathione (GSH) were rapidly depleted by acrolein with a biphasic effect (Table 3). Altogether, these results indicated that toxicity is likely due to depletion of cellular thiols and thus in alteration of the redox state of SK-N-SH cells. This mechanism could be implicated in the pathophysiology of AD. In addition, protein-bound acrolein has been detected in plaque deposits in atherosclerosis [71] and this protein colocalized with NFTs and neuritic plaques in AD brains [72]. Lovell et al. [61] were the first to demonstrate that a significant increase of acrolein levels in vulnerable brain regions of AD, in hippocampus

Table 2 Effects of acrolein dose and time (hours) on the viability of SK-N-SH cells

	Acrolein concentration				
Parameters	0 mM	1 μM	5 μM	10 μM	15 μM
48 h	100.00	105.44	95.86	83.75*	68.11**
Percentage of SEM (%)	2.74	2.51	3.00	3.44	7.08
N Number of samples	9	9	9	9	9
72 h	100.00	94.49	80.56	52.20*	34.39**
Percentage of SEM (%)	2.84	2.68	2.71	6.69	7.61
N Number of samples	16	25	27	27	23

SK-N-SH cell viability was analyzed after 48 and 72 h of treatment in the presence of different concentrations of acrolein using the Resazurin test. Results are presented as percentage of control (taken as 100%). Data are means ± SEM of at least three experiments performed in quadruplicate.

*$p < 0.05$ and **$p < 0.01$ compared with control without acrolein by a Dunnett *t*-test.

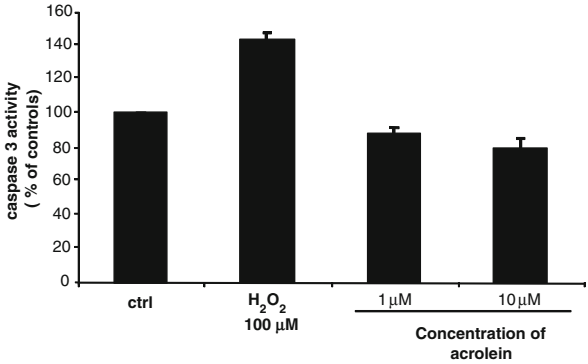

Fig. 2 Effect of acrolein on the activity of caspase-3 in SK-N-SH cells. Caspase-3 activity was analyzed in SK-N-SH cells after 24 h of treatment with acrolein

Table 3 Effects of acrolein dose and time (hours) on glutathione level in SK-N-SH cells

	Acrolein concentration				
Parameters	0 mM	1 μM	5 μM	10 μM	15 μM
30 min	100.00	88.22	52.90	35.75	30.15
Percentage of SEM (%)	4.07	4.48	10.24	13.81	17.58
N Number of samples	24.00	12.00	12.00	12.00	12.00
1 h	100.00	99.75	77.61	55.86	41.87
Percentage of SEM (%)	2.55	3.85	4.39	5.41	8.72
N Number of samples	24.00	12.00	12.00	12.00	12.00
2 h	100.00	103.55	89.91	56.72	33.34
Percentage of SEM (%)	3.82	3.43	3.67	4.07	9.75
N Number of samples	24.00	12.00	12.00	12.00	12.00
4 h	100.00	97.94	84.81	59.22	49.83
Percentage of SEM (%)	1.97	2.35	4.74	3.07	9.16
N Number of samples	24.00	12.00	12.00	12.00	12.00
14 h	100.00	135.31	178.32	138.78	31.26
Percentage of SEM (%)	1.87	2.13	1.80	7.34	14.96
N Number of samples	24.00	12.00	12.00	12.00	12.00

Glutathione levels were determined using the monochlorobimane probe after different times of treatment. Results are expressed as percentage of control (taken as 100%). Data are means ± SEM of at least three experiments performed in quadruplicate.

and amygdala, and Williams et al. have shown that the levels of acrolein are significantly higher in hippocampus and cerebellum from early AD cases with no difference between control and MCI [59]. However, in the middle temporal gyrus from early AD and MCI patients, the levels of acrolein were significantly higher compared with those in control. Interestingly, it has been shown that the activity of glutathione-S-transferase (GST), the enzyme responsible for the detoxification of reactive aldehydes such as acrolein, was significantly decreased in hippocampus and not in cerebellum from AD brains [56]. These results suggest that the AD brain does not retain the ability to detoxify reactive aldehydes in hippocampus, an affected

region in AD, whereas the brain can detoxify these aldehydes in the cerebellum. Immunohistochemical analysis revealed that the protein-bound acrolein was mainly localized in neurons and rarely seen in glial cells in contrast with protein-bound crotonaldehyde, which was mainly seen in glial cells and rarely in neurons [73]. As acrolein consumes the reduced form of GSH, these observations suggest that this aldehyde may reinforce oxidative stress in neuronal cells in AD brain. Oxidative stress in turn activates NF-κB, which promotes the transcription of proinflammatory cytokines and NO enzymes, thus it is likely that acrolein could contribute to neuronal death via inflammatory pathways. The interaction between acrolein and the Aβ peptide has been suggested as it has been reported that acrolein facilitated the formation of amorphous planar Aβ aggregation [74]. The Aβ–polyacrolein aggregates consisted of uniformly sized planar structures that appeared polygonal, but without acrolein, spherical particles appear during fibrillogenesis. These results suggest that acrolein could contribute directly or indirectly to the pathophysiology of AD.

4 Dietary Antioxidants and Risk of AD: Mechanisms of Action

The contribution of polyphenols (PLs) to the antioxidant capacity of the human diet is much larger than that of vitamins [75]. PLs are found in all plant foods and may contribute to the beneficial health effects of fruits and vegetables. They can be divided in several subclasses including non-flavonoid compounds and flavonoids. An increasing number of in vitro and in vivo studies show that PLs from nutrition can counteract some specific aspects of the neurodegenerative and neuropathologic processes observed in AD. The beneficial intake of nutritional flavonoids and polyphenols components is also supported by recent epidemiologic studies. The antioxidant activities of PLs are attributed to many factors including the ability to inhibit pro-oxidant enzymes, upregulate enzymes involved in drug metabolism, form complexes with pro-oxidant redox-cycling cations, such as iron or copper, and to scavenge ROS/NOS by direct hydrogen ion donation. Many PLs display higher antioxidant activity than vitamins C and E as a result of their hydroxyl groups. For instance, the free radical scavenger property of catechin compounds increases with the number of hydroxyl groups [76]. It is now recognized that the biological activity of PLs likely arises from more specific interactions with cellular targets than from their antioxidant activities. For example, quercetin has been shown to upregulate enzymes involved in antioxidant defense such as γ-glutamylcysteine synthase, the rate-limiting enzyme in glutathione synthesis [77].

A large range of PLs were thought to protect cells against oxidative damage through their antioxidant property, which was evident in in vitro experiments with cell cultures or cell-free systems using various methods. Though this mode of action remains the focus for many polyphenols, recent data indicate that the protective effect of PLs may extend beyond their antioxidant activity.

For instance, green tea is rich in flavonoids (30% of dry weight of a leaf) [78] and the main compounds are epigallocatechin gallate (EGCG), (–)-epigallocatechin

(EGC), (–)-epicatechin (EC), and (–)-epicatechin-3-gallate (ECG) [79]. These flavonoids display antioxidants properties in the order EGCG > ECG > EGC > EC [80]. Green tea polyphenols have shown beneficial effects in animal models of stroke/cerebral ischemia, AD, and Parkinson's disease. Neuroprotection in ischemia by EGCG may be mediated through reducing iNOS expression, peroxynitrite formation, and preservation of mitochondrial complex activity and integrity [81, 82]. There are substantial in vitro studies that describe the protective effect of different catechins against the Aβ-induced damage [83–85]. Different mechanisms could be involved in this neuroprotective effect, as in neuronal cell culture, EGCG could promote the non-amyloidogenic α-secretase pathway [85], and EC could reduce the formation of amyloid β-fibrils [86]. In primary neuronal cells derived from a transgenic mouse model overexpressing APP with the mutation *Sweden*, EGCG significantly reduced Aβ peptide generation ($A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$) by 38% [87] with purified EGCG being more potent than green tea. Green tea catechins, especially ECGC, also modulate a number of signaling pathways such as MAPK [88], protein kinase C [85], and phosphatidylinositol-3-kinase (PI-3 kinase)-Akt [89], and these modulations may mediate some of the neuroprotective mechanisms of EGCG. In neuronal cell line and primary cell culture models, EGCG prevented the decline in ERK1/2 induced by 6-hydroxydopamine or oxidized low-density lipoproteins [90, 91]. MAPK is also involved in the regulation of the expression of proapoptotic and antiapoptotic genes. EGCG-treated SH-SY5Y neuroblastoma cells have decreased expression of proapoptotic genes Bax, Bad, cell cycle inhibitor Gadd45, Fas ligand, and tumour necrosis factor–mediated apoptosis ligand TRAIL [90]. EGCG, at doses of 1–10 μM, protected against Aβ peptide and 6-hydroxydopamine–induced cell death by activation of protein kinase C [85], which plays a central role in neuronal cell survival, and loss of its activity is frequently observed in neuronal insults such as in the presence of Aβ peptide accumulation and other neurotoxins. EGCG could also prevent LPS-induced neuronal cell death as well as the expression of inflammatory proteins, inducible nitric oxide synthetase and cyclooxygenase-2 [92]. Recently, Wang et al. reported that the consumption of grape seed extract containing catechins prevents Aβ deposition and attenuates inflammation in brain from APP(Swe)/PS1 transgenic mice [93]. Finally, the administration of EGCG could prolong the inhibition of the acetylcholinesterase activity induced by huperzine A [94]. In summary, green tea and its active component EGCG exert several intracellular mechanisms relating to neuroprotection.

Curcumin is a low-molecular-weight molecule with potent antioxidant and anti-inflammatory activities. The yellow curry spice is part and parcel of Indian vegetables. When fed to aged APP(Swe)/PS1 transgenic mice with advanced amyloid accumulation, curcumin reduced Aβ levels and plaques. Interestingly, curcumin also blocked Aβ aggregation and fibril formation in vitro with an $IC_{50} = 0.8$ μM [95], and this property could be implicated in the reduction of amyloid plaque burden observed in vivo after curcumin treatment in APP(Swe)/PS1 transgenic mice. Curcumin is also a good inhibitor of expression of inflammatory cytokines, Cox-2 and iNOS, likely by inhibition of JNK/AP-1 and NF-κB mediated gene transcription [96]. All of these factors (IL-1, TNF-α, Cox-2, iNOS, JNK, NF-κB) are also

implicated in Aβ toxicity [97, 98]. Moreover, curcumin could chelate the redox active metals such as iron and copper [99]. Begum et al. reported that when curcumin was fed to APP(Swe)/PS1 transgenic mice, indices of oxidative stress (i.e., oxidized proteins and IL-1β) were significantly reduced [100]. Remarkably, recent evidence has demonstrated that curcumin is a potent inducer of heme oxygenase-1 (HO-1) in vascular endothelial cells [101]. HO-1 induction occurs through the antioxidant response element (ARE) [102]. Recently, Ma et al. demonstrated that mice fed with the combination of fish oil and curcumin for 1 month had significant results on Y-maze, and the combination induced higher inhibition of JNK and tau phosphorylation [103]. Curcumin was also investigated as an inhibitor of glycogen synthase kinase-3β (GSK-3β). GSK-3 is a serine/threonine kinase, a key enzyme involved in glycogen synthesis. It is involved in a variety of cellular processes ranging from glycogen metabolism, insulin signaling, cell proliferation, and neuronal function. Its high expression in brain is associated with a variety of neurologic disorders such as AD. There are several GSK-3 inhibitors being developed for AD (see review by [104]). Curcumin was found to fit within the binding pocket of GSK-3β via several attractive interactions with key amino acids. Hence, curcumin was found to potently inhibit GSK-3β with an $IC_{50} = 66.3$ nM [105]. Altogether, curcumin, a highly lipophilic compound, can protect cells against Aβ toxicity by preventing Aβ peptide aggregation and by reducing plaque burden through its antioxidant and anti-inflammatory activities. Moreover, curcuminoids and all individual components also possess a pronounced acetylcholinesterase inhibitory activity [106]. These various pharmacological activities of curcumin indicate that curcumin is a promising compound in the development of disease-modifying drugs to prevent and/or cure AD.

Resveratrol (*trans*-3, 4, 5-trihydroxystilbene) is the most relevant and the main biologically active non-flavonoid found in grapes and red wine. A number of studies have demonstrated the antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic effects of this compound [107, 108]. Several epidemiologic studies indicate an inverse correlation with wine consumption and incidence of AD [109–111]. At cellular levels, resveratrol could protect PC12 cells against Aβ-induced toxicity and accumulation of intracellular ROS [112]. The inhibition of Aβ secretion by resveratrol could be implicated in this neuroprotective effect, as the secretion of Aβ is reduced in two cell lines, HEK 293 and N2a, transfected with APP695 and treated with resveratrol [113]. This effect was not mediated by β- and γ-secretase activities but may be through the elevation of degradation of Aβ peptide. Other neuroprotective mechanisms involve the modulation of NF-κB/Sirt1 pathways, as in vitro and in vivo studies have shown that resveratrol is a specific activator of Sirt1 [114, 115]. This property could be implicated in the protective effect against Aβ involving the inhibition of the NF-κB activity [116]. In cultured PC12 cells, resveratrol could also upregulate HO-1 gene expression via the activation of NF-E2-related factors 2 (NRf2) and protect against H_2O_2-induced cell death [117]. APP(Swe)/PS1dE9 transgenic mice fed for 9 months beginning at 3 months of age with 2% grape seed extract containing 592.5 mg/g total phenolic displayed a significant reduction of brain Aβ burden and microglia activation [118]. In summary, in

addition to its antioxidant effects, the efficacy of resveratrol and grape seed extract against Aβ toxicity involves several pathways. Thus, resveratrol and phenolic compounds from grape seeds are promising compounds in delaying the development of AD. Their bioavailability needs to be addressed due to its rapid metabolization in liver and intestinal epithelials cells. Moreover, the efficacy of these compounds in the treatment of AD will also depend on the extent to which resveratrol metabolites become bioavailable.

Several dietary supplements with either spinach, strawberry, or blueberry extracts have been reported to reduce some neurologic deficits in aged animal models [119–121]. In blueberries (*Vaccinium ashei reade*), catechin is the major flavonoid found with 387 mg/100 g fresh weight, epicatechin ranged from 34 to 129 mg/100 g fresh weight, and total anthocyanins ranged from 84 to 113 mg/100 g fresh weight [122]. It has been estimated that 0.543–1.69 mg/l of total anthocyanins was present in human serum after a consumption of 100 g blueberries containing 1.20 g total anthocyanins, and the maximum level was reached 4 h after the consumption. Interestingly, a significant positive correlation between serum anthocyanin content and postprandial antioxidant status has been observed [123]. This absorption could have some positive effects in the brain through different processes as it has been demonstrated in different animal studies. Thus, dietary supplementation for 8 weeks with blueberry extract reversed cognitive deficits in the Morris water maze performance test in 19-month-old rats [124]. These results could be correlated with the presence of different classes of polyphenols in brain areas associated with cognitive performance after blueberry supplementation. Thus, several anthocyanins (cyanidin-3-*O*-β-galactoside, cyanidin-3-*O*-β-glucoside, cyanidin-3-*O*-β-arabinose, malvidin-3-*O*-β-galactoside, malvidin-3-*O*-β-glucoside, malvidin-3-*O*-β-arabinose, peonidin-3-*O*-β-arabinose, and delphinidin-3-*O*-β-galactoside) were found in the cerebellum, cortex, hippocampus, or striatum from the 19-month-old rats supplemented with 2% blueberry extract for 8–10 weeks [125]. These findings indicate that some polyphenolic compounds are able to cross the blood–brain barrier and localize in various brain regions important for learning and memory.

It is now well established that the effect of blueberry extract on cognitive functions might involve more than its antioxidant actions. Thus, aged rats with blueberry extract diet had significantly lower levels of NF-κB than those of aged rats on a control diet [126]. These results are in accordance with the known effect of flavonoids on cell signaling such as on the activity of NF-κB [127, 128]. Additional evidence has been observed in a recent study with the double APP(Swe)/PS1 transgenic mouse model, in which genetic mutations promote the production of the Aβ peptide the hallmark AD-like senile plaques in several regions. When these mice were supplemented with blueberry extract (2% of diet) from 4 months and continued until 12 months of age, their performance in a Y-maze test, a cognitive performance test, was similar to that of non-transgenic mice and significantly better than that of nonsupplemented transgenic mice [129]. However, examination of the brains of these mice revealed that blueberry extract supplementation did not affect Aβ peptide production or deposition or the number of plaques. These data suggest that the impairment of cognitive functions observed in these transgenic mice may not

necessarily be the result of deposition of the Aβ peptide. In these mice supplemented with blueberry extract, the activities of hippocampal ERK as well as striatal and hippocampal protein kinase C are higher than those in transgenic mice supplemented with a control diet. Both protein kinase C and ERK have been shown to be involved in early and late stages of memory formation [130, 131]. These results indicate that blueberry extract supplementation might prevent cognitive deficits through neuronal signaling pathways. Diet supplemented with blueberry extract could also protect the brain against apoptosis, as rats receiving blueberry extract had significantly lower caspase-3 activity in the ischemic hemisphere [121]. Taken together, these studies demonstrate that blueberry extract–supplemented diets could protect neuronal loss and prevent the decrease of cognitive functions against different insults through the antioxidant and antiapoptotic activities and regulation of some cell signaling pathways.

Pomegranates (*Punica granatum* L.) contain a very high level of polyphenols compared with other fruits and vegetables [132], and the most important polyphenols are ellagic acid, punicalagin, and hydrolyzable tannins such as ellagitannins and gallotannins. Recently, the administration of pomegranate juice (PJ) to APP(Swe)/PS1 transgenic mice from 6 to 12.5 months of age exhibited improvements in cued and spatial learning tasks compared with sugar-water control [133]. Additionally, PJ-treated mice had a significantly reduced burden of plaque load and soluble $A\beta_{1\text{-}42}$ in hippocampus.

Grape juice is also a rich source of flavonoids that include catechins, epicatechins, quercetin, anthocyanins, and proanthocyanidins [134]. When aged Fisher 344 rats were given 10 or 50% grape juice from 19 to 21 months of age, their performance motor functions in rod walk and cognitive performance in the Morris water maze were improved [135].

5 Conclusions

Over the past 10 years, studies from transgenic mouse models indicate that the elevation of oxidative damage is a relatively early event in the pathogenesis of AD. Data from MCI patients confirm that advanced oxidative damage occurs early in the pathogenesis of AD, before the onset of dementia. It is now well established that oxidative damage could play a critical role in AD. For instance, acrolein, a by-product of lipid peroxidation, could elicit neurofilament-L aggregation [136]. Further studies are also required to understand the effect of ROS on basic cellular and molecular functions of the various nerve cells in the brain and how this in turn affects the physiopathology of neurodegenerative diseases.

Some epidemiologic studies suggest that increased consumption of antioxidants or PLs from food can delay or suppress processes that underlie age-related cognitive decline and may reduce the risk to develop AD. PLs from fruits and vegetables seem to be potential agents in neuroprotection by virtue of their ability to influence and modulate several cellular processes such as signaling pathways, proliferation, apoptosis, redox balance, differentiation, and so forth. Although PLs are abundant in

fruits, vegetables, tea, wine, and medicinal plants, more detailed studies are required to determine their absorption, bioavailability, and ability to cross the blood–brain barrier. In view of their multiple biological activities, PLs hold great promise as potential therapeutic/prophylactic agents in neurodegenerative diseases.

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An Epigenetic Model for Susceptibility to Oxidative DNA Damage in the Aging Brain and Alzheimer's Disease

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Abstract Epigenetics and oxidative stress are two cellular mechanisms that appear to be independent from each other and associated with distinct cellular processes. Epigenetics is associated with developmental regulation of gene expression, mainly through the process of methylation of genetic promoter regions. On the other hand, oxidative stress is a cellular process of disruption in the homeostasis between cellular antioxidant systems and reactive oxygen and nitrogen species leading to cell malfunction or death. The oxidative stress process is linked to a myriad of diseases and is a consequence after toxicologic challenges. In this chapter, we summarize results from our laboratories that identify developmental stage and toxicologic challenges that result in a novel mechanism in which toxicologic exposure during development alters the normal pattern in markers of oxidative stress and epigenetic regulation. The concordance of these two alterations results in pathologic changes similar to those seen in Alzheimer's disease. Thus, we postulate a novel mechanism in which epigenetics and oxidative stress are at play in disease, and the occurrence of one of these processes will alter the occurrence of the other.

Keywords Epigenetics · Cytosine methylation · 8-hydroxy-2′-deoxyguanosine · DNA damage · DNA repair · Lead · Alzheimer's disease

1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with clinical manifestations of profound memory loss and dementia that are highly associated with aging. AD pathology is characterized by senile plaques and neurofibrillary tangles (NFTs), combined with massive neuronal loss, mainly in the hippocampus and association regions of the neocortex. The major constituents of senile plaques are

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39–42 amino-acid peptides, snipped from a larger protein called amyloid precursor protein (APP) [1–6]. In addition to plaques and tangles, there is growing evidence of increased oxidative stress as a component of the AD clinical spectrum. Particularly, increased levels of 8-hydroxy-2′-deoxyguanosine (oxo8dG), an oxidized form of the base guanine, have been found in DNA from affected brain regions in AD [7, 8]. It is still unresolved if increased oxidative damage to DNA plays a role in the neurodegenerative cascade or is just an epiphenomenon of the neurodegenerative process. Although some familial cases of AD have served to identify genes that play a major role in AD development [9], most of the AD cases (90%) are sporadic with no direct association with the familial cases. This coupled with the knowledge of the differential susceptibility and course of illness, as well as the late age onset of the disease, suggests that epigenetic and environmental components play a role in the etiology of late-onset AD (LOAD). This chapter will summarize current literature and results from our studies pointing to the possibility of early-development toxicologic exposures that can render neurons prone to amyloid-β (Aβ) accumulation and increased oxidative damage to DNA. This research falls in line with the knowledge that the majority of AD cases occur in the elderly; however, it is still unresolved whether AD is a disease of old age or whether it has earlier beginnings. Epidemiologic studies have shown that people with dementia are more likely to have had low scores on intelligence tests when they were children compared with those of people without dementia [10].

2 Epigenetics and AD

Epigenetics is a major mechanism that accommodates gene-expression changes in response to gene–environment interactions [11, 12]. Epigenetics refers to modifications in gene expression that are influenced by DNA methylation and/or chromatin structure, RNA editing, and RNA interference without any changes in DNA sequences [13]. DNA methylation and histone deacetylation are known to occur shortly after DNA synthesis and could be modified by diverse physiologic or pathologic factors altering gene expression for the lifetime of the organism.

Most research work on epigenetics has been focused on its role in susceptibility to cancer development [14]. However, there is evidence of epigenetic aberrations in mental illnesses, namely in fragile X disease and Retts syndrome [15–17]. Fragile X disease is associated with an expanded (>250 copies) number of hypermethylated CGG repeats 5′ of the FMR1 gene that results in downregulation of the gene [15, 16]. Disease severity in fragile X is directly correlated with the extent of methylation in the 5′ region of the FMR1 gene [18]. Retts syndrome, on the other hand, is linked to mutations in the gene encoding the methylated cytosine binding protein ($MeCP_2$) [17]. The $MeCP_2$ recruits a variety of proteins that form a complex thus repressing gene expression [19]. Both fragile X and Retts syndrome are responses to well-established alterations to a single gene. However, recent work in autism spectrum disorders suggests a major epigenetic component to the origin of the disease, indicating some contribution of differential methylation, but not to a single gene [20].

DNA methylation is one of the most studied aspects of epigenetic modifications. The addition or removal of methyl groups from cytosines can impact gene expression and alter cell and organism function. For example, hypomethylation of the membrane-bound catechol-*O*-methyltransferase (MB-COMT) gene has been implicated in both schizophrenia and bipolar disorder [21]. On the other hand, hypermethylation of the RELN (reelin) gene has been shown to be associated with schizophrenia [22]. In addition to DNA methylation, other mechanisms linked to epigenetic regulation have been found to play a role in neuronal function, as demonstrated by the use of inhibitors of histone deacetylases to ameliorate deficits in a wide range of psychiatric and neurologic conditions [23].

Whether development of LOAD is rooted in epigenetic changes during development is unknown. However, recent data lends support to the possibility for an epigenetic impact in AD. Structural genomics studies have demonstrated that more than 200 genes might be involved in AD pathogenesis [24]. In addition, the AD population exhibits a higher absolute genetic variation rate of 40–60%, and AD patients differ in their genomic architecture from patients with other forms of dementia [24]. Analysis of DNA methylation patterns in genes with a potential role in AD etiology identified an age-specific epigenetic drift associated with unusual methylation patters in LOAD, supporting a potential role of epigenetic effects in the development of the disease [25]. Additionally, genes that are genetically associated with LOAD (PSEN1, APOE) showed the largest interindividual variance in DNA methylation, with the APOE gene exhibiting the most variably methylated sequences. APOE presented a bimodal methylation pattern, with a hypomethylated CpG-poor promoter and a fully methylated 3′-CpG-island, containing the sequences for the ε4-haplotype, the only established genetic risk factor for LOAD [25]. Interestingly, the gene MTHFR, coding for methylenetetrahydrofolate reductase, showed a significant interindividual epigenetic variability. Alteration in MTHFR expression can influence homocystine levels, which may contribute to LOAD predisposition [25]. The sporadic nature of the disease, the differential susceptibility and course of illness in males and females, as well as the late age onset of the disease add to the hypothesis that epigenetic and perhaps environmental components play a role in the etiology of LOAD [25]. On the other hand, the genetics of AD have revealed that early (<60 years old) onset AD (EOAD) is associated with mutations in either APP or the presenilins [26–29], whereas the risk to develop LOAD is linked to the apolipoprotein E (ApoE) polymorphism [30]. Twin studies often used to confirm the inheritance pattern of a disease have shown an estimated concordance well below 100% (20–80%) for AD, suggesting that LOAD is a complex non-Mendelian disease [31–33].

3 Oxidative Stress and AD

In addition to the established pathology of amyloid plaques and neurofibrillary tangles in brain of AD sufferers, there is a growing body of evidence indicating changes in the redox status of AD brains. This is supported by findings of increased levels of

oxidative damage markers in every major cellular macromolecule (proteins, lipids, and DNA) [34]. Also, alteration in expression of antioxidant systems lends support to a role for free radical damage in AD pathology [35]. Due to their postmitotic nature, damage to DNA in neurons could be highly detrimental to their function and viability. Guanine (G) has the lowest oxidation potential of the DNA bases; thus, oxo^{8}dG is the most prevalent form of oxidative base modifications produced [36–38]. Some evidence suggests that in addition to its mutagenic properties, presence of 8-oxodG in DNA can alter binding of transcription factors and can impact epigenetic signaling [39, 40].

The following sections in this chapter rely on the aforementioned evidence of epigenetics and oxidative stress as major players in risk and pathology of AD and explore the interactions between DNA methylation and DNA oxidation to propose a mechanism that attempts to explain some of the pathologic findings evident in LOAD. This model proposes AD as a disease in which alteration in the methylation profile of genes can modify susceptibility to DNA damage resulting in neurodegeneration.

4 Exposure to Lead and the Developmental Basis of AD

Based on evidence of an inverse relationship between birth weight and the incidence of cardiovascular disease, Davis Barker enunciated a hypothesis known as the fetal or developmental basis of adult disease (Barker hypothesis). This hypothesis states that many adult diseases might have a developmental origin [41–44]. A large body of subsequent clinical and experimental data has lent support to this hypothesis, as it has been shown that diseases of the cardiovascular system, hypothalamic–pituitary–adrenal (HPA) axis and diabetes can also be affected by nutritional imbalances during fetal development [45–47]. Some clinical and animal studies suggest that certain deficiencies in the CNS in adults may have origins in alterations during development. Memory impairment in adult animals and diseases such as schizophrenia have been linked to infection, fetal malnutrition or hypoxia in early life [48–51]. In addition to supporting the developmental basis of disease, these observations serve to propose a new concept regarding some adult diseases that emphasizes the role of environmental factors acting in the periconceptual, embryonic, fetal and infantile phases of life [52]. The impact of such influences early in life may be partially mediated through epigenetic mechanisms that involve DNA methylation.

Given the recognized effects of lead (Pb) exposure in children, important work has been performed to understand the consequences and the mechanisms linked to Pb exposure in this susceptible population [53]. Pb is known to produce cognitive and behavioral deficits in children [54, 55] with the added risk of being ubiquitous in distribution. In relation to risks associated with Pb exposure in adult populations, several population-based case-control studies have found that chronic occupational exposure to Pb as well as other metals is associated with the incidence of Parkinson's disease [56]. Other studies point to a relation between high blood and bone Pb levels

and increased risk of amyotrophic lateral sclerosis (ALS), suggesting that Pb exposure plays a role in the etiology of the disease [57]. These early studies provided hints as to the possible connection between Pb exposure and neurodegenerative disease, then a seminal work looked at tibia bone Pb levels in 529 former organolead workers and its relationship to ApoE genotype, a known risk factor for LOAD. Results led to the conclusion that the persistent CNS effects of Pb are more toxic in individuals with at least one ApoE ε4 allele [58]. The link between past adult Pb exposure and neurodegeneration was further established by the same research group using brain magnetic resonance imaging (MRI) [59] and was consistent with their previous work showing an association between Pb exposure and longitudinal cognitive decline. Whereas these studies focused on adult occupational exposure to Pb, it is not known if the workers they studied had been previously exposed to Pb as children.

Animal studies from our laboratories strongly suggest that exposure to Pb during development can be a risk factor that promotes the pathogenesis of AD. Rodents exposed to Pb during fetal development and lactation were monitored for the lifetime expression of the APP gene. We observed that APP mRNA expression was transiently induced in neonates but exhibited a delayed overexpression 20 months after exposure to Pb had ceased. Furthermore, the increase in APP gene expression in old age was accompanied by an elevation in APP and its amyloidogenic Aβ(1–42) product [60]. In addition to rodent studies, analysis of brains from aged-primates (23 years old) that had been exposed to Pb early in development showed evidence of expression of AD-related genes (APP, BACE1 [beta-site APP cleaving enzyme 1]). Furthermore, developmental exposure to Pb altered the levels, characteristics, and intracellular distribution of Aβ staining and amyloid plaques in the frontal association cortex [61]. Given the ample evidence that oxidative damage is a component of AD pathology [8, 62–64], we opted to assess levels of the oxidative DNA marker 8-oxo-dG in both models of developmental Pb exposure. We found a similar accumulation in 8-oxo-dG in the same brain regions of aged primates and rodents developmentally exposed to Pb [61, 65]. Thus, our model of early exposure to Pb consistently yields a model of increased APP expression with concomitant Aβ deposits, in addition to increased oxidative damage to DNA of affected brain regions. There are alternative hypotheses to elucidate the origin of this latent buildup of oxidized DNA. Increases in Aβ could lead to the generation of reactive oxygen species promoting the formation of 8-oxo-dG; alternatively, epigenetic modulation in the methylation pattern of cytosines could interfere with the repair or oxidation potential of adjacent oxidized guanines [66].

5 Epigenetics, the Environment, and Load

DNA methylation of cytosines is a major epigenetic event that can influence the regulation of gene expression and has been linked to the process of gene imprinting in mammals. Alterations in 5-methylcytosine patterns on the promoters of genes

are the first level of regulation of gene expression in development, differentiation, carcinogenesis, and aging. The methylation of cytosine can sometimes serve as a heritable code by the selective action of some methylases that act on cytosine nucleotides in a CG sequence. Methylation occurring predominately at this symmetrical CG dinucleotide, due to the preferences of DNA methyltransferase for a hemi-methylated substrate, maintains specific heritable patterns of methylation [67].

DNA methylation patterns are mainly established in utero, and it has been established that the fetal environment may alter such patterns leading to sustainable changes in gene expression that endure for a lifetime [68]. In terms of the brain, this environmentally dependent modulatory period may continue into postnatal development. Genome-wide demethylation patterns are also observed shortly after fertilization and followed later by a new wave of methylation of the CG sequences [69]. Thus, the process of methylation and demethylation appears to be a controlled programmed event providing cells with a broad developmental potential and a mechanism that widens the means to regulate the expression of genes and to transmit information beyond the one stored in the genetic code.

Animal studies have clearly demonstrated that early-age environmental stimuli can alter methylation patterns leading to gene expression changes that may result in modified behaviors or increase disease risk in adulthood. It was shown that maternal grooming changed the methylation pattern and expression of the glucocorticoid receptor (GR) in the hippocampus in rat offspring, leading to permanent changes in their stress response [70]. In a different approach, it was shown that modification of the maternal diet throughout pregnancy led to a decrease in the methylation of the GR and the peroxisomal proliferator-activated receptor (PPAR) genes, which was consistent with their elevated mRNA expression in the offspring after weaning [71].

In addition to behavioral and nutritional imbalances, chemical exposure can also interfere with the status of DNA methylation. One way in which environmental agents or occupational exposure could interfere with DNA methylation is by disrupting the enzymes that conduct such reactions. In vitro studies show that the addition of cadmium (Cd) to hepatic nuclear extracts inhibited DNA methyltransferase [72]. More recently, it was reported that subchronic exposure to Cd inhibited DNA methyltransferase activity in cultured cells, and chronic exposure enhanced the activity of the DNA methyltransferase. The Cd effect in DNA methyltransferase translated into altered levels of methylation of DNA, suggesting that the action of Cd on DNA methylation may be responsible for its carcinogenic properties [73]. In addition to DNA methylation, environmental agents could also disrupt chromatin restructuring and produce long-term alterations in gene expression. Reports that examined sperm chromatin structure in monkeys found alterations at environmentally relevant blood Pb levels, and other studies have shown decreases in the level of protamine–DNA interactions that may alter sperm chromatin condensation [74, 75]. However, very few studies have been conducted on DNA methylation in the brain, and none have examined the potential of environmental agents to disturb this process.

6 DNA Methylation and DNA Oxidation

In CpG dinucleotides, the cytosine is the preferred base for DNA methylation, and the guanine is the site for oxidative damage. 8-Oxo-dG is widely used as a biomarker of oxidative DNA damage. In the absence of exogenous DNA-damaging reagents, endogenously formed metabolic reactive oxygen species (ROS) are able to create 10^5 8-oxo-dG daily in cells [76]. Oxidative DNA damage is primarily repaired by the base excision pathway, which is initiated by a DNA glycosylase that recognizes the modified base [77]. Oxoguanosine DNA glycosylase 1 (OGG1) is the major repair enzyme to recognize and remove 8-oxo-dG. Oxidative DNA damage and possible deficiencies in Ogg1 are considered to be a central factor in the process of aging and aging-related diseases such as AD [78, 79].

Results from our studies in rats and primates show that developmental Pb exposure increases Aβ levels as well as 8-oxo-dG levels in old age [61, 65]. Aβ is known to induce functional disturbances in vivo through its pro-oxidant and neurotoxic properties [80, 81]. Aβ promotes the formation of ROS, and the use of antioxidants can prevent Aβ-elicited neurotoxic cascades [82–85].

Few studies have addressed the epigenetic phenomenon of DNA methylation and DNA oxidative damage simultaneously, and little is known about how DNA methylation and DNA oxidation interact with each other. Researchers using synthetic DNA oligonucleotides have found oxidation of guanine in CpG dinucleotides reduced the methyl group binding domain (MBD) binding to this site [40]. When 5-methylcytosine is oxidized to 5-hydroxymethylcytosine, its affinity to MBD is greatly reduced to the same level as unmethylated cytosine. Methylated CpG has also been found to account for decreased transcription factor binding to the promoter region [86, 87].

To model the interactions between methylation and oxidation, we synthesized oligonucleotides that resembled the binding site for the transcription factor Sp1. In some oligos, the cytosine in a CpG was replaced by methylcytosine and the guanine by an 8-oxo-dG. A third oligos containing both methylcytosine and 8-oxo-dG adjacent to each other was also prepared. We then conducted DNA binding and repair studies. Expectedly, we found that presence of either 5-methylcytosine or 8-oxo-dG dramatically suppressed Sp1 DNA binding; however, the combination of both had an effect greater than that of either alone [88]. Likewise, the repair of 8-oxo-dG was greatly diminished when the 8-oxo-dG was preceded by 5-methylcytosine (Fig. 1). These experiments show that the methylation status of a gene can greatly impact gene expression and DNA repair. On the other hand, oxidized DNA will inhibit DNA methylation of an adjacent cytosine [89, 90].

Though substitutions in synthetic DNA oligonucleotides show the interplay between oxidative damage and methylation of DNA, this relationship can also be seen in cells. Studies with oxidant-transformed cell lines have shown unusual changes of methylation patterns of several genes [91, 92]. This suggests the oxidative DNA damage and DNA methylation interact with each other, which may consequently alter the methylation patterns and transcriptional activity of affected genes.

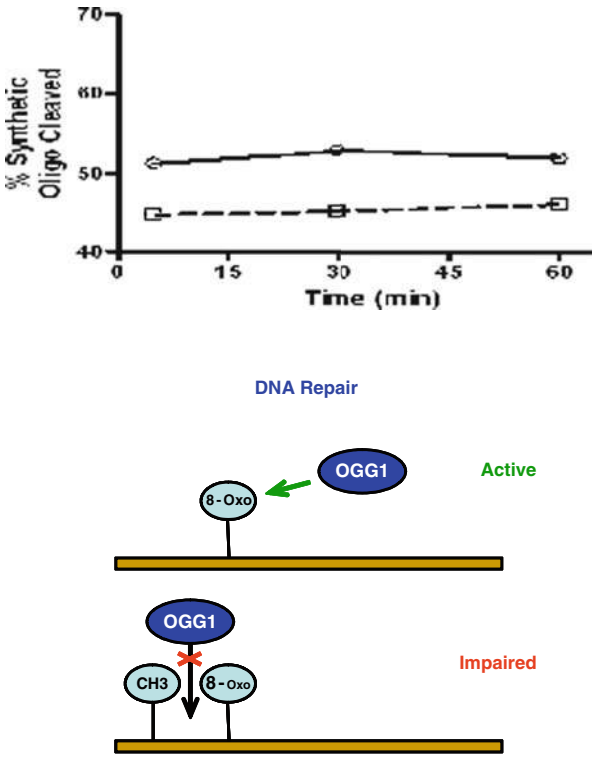

Fig. 1 Structural modifications in CpG dinucleotides and their impact on DNA repair: DNA repair glycosylase (Ogg1) activities were evaluated in oligonucleotides containing a 5-methylcytosine (5-mC) and/or an adjacent 8-oxo-dG. An unmodified oligonucleotide was used as control. The *top panel* illustrates results for assessments of Ogg1 activity; the *bottom panel* illustrates the biological consequences of these modifications. Presence of a methylated cytosine next to an oxidized G reduced the activity of Ogg1, preventing repair of oxidative damage to DNA. The *solid line* represents time-associated repair when only 8-oxo-dG is present; the *dashed line* represents repair when 5-mC is adjacent to the oxidized G. Methods for Ogg1 activity have previously been published [60, 93]. The values presented were derived from three to four experiments

In considering methylation and oxidation, it is important to make some important distinctions. Oxidation is a dynamic process that can occur any time there is oxidative stress, but it is typically high early and late in life. Methylation on the other hand is poorly understood and is presumed to occur during early development and sustained for the rest of life. Although there are DNA methylating enzymes, we are not aware of the existence of a demethylating enzyme in mammalian systems; however, methyl group donors available in the diet can alter the methylation pattern in favor of hypermethylation. The natural mechanisms that underlie hypomethylation are not understood. Furthermore, it is important to recognize that even if a substance is not a pro-oxidant, it can still lead to an accumulation of oxidative damage through alterations in methylation patterns that impact the repair of adjacent oxidized guanines.

In our model, environmental agents such as heavy metals can inhibit the enzymes that maintain or methylate DNA. In this scenario, the exposure to such metals has to occur during a developmental period when such methylation patterns are being established; however, though it is assumed that methylation patterns are maintained for life, it is also plausible that they are dynamically changing. To evaluate this possibility, we quantified the content of methylcytosine on three positions of the APP promoter in monkeys that were 6, 12, and 23 years old. We found that they all maintained their methylcytosine content, with the exception of one position that decreased with age [88]. Although this experiment examined only a few sites out of possibly thousands, it suggests that both stable methylcytosine patterns and alternating content are possible; however, how such selective effects are imparted is still unknown.

7 Summary and Conclusions

The Pb exposure model was used to test the hypothesis that the origins of AD begin early in life and that environmental exposure can determine future susceptibility to disease. In this chapter, we summarize our results with Pb exposure; however,

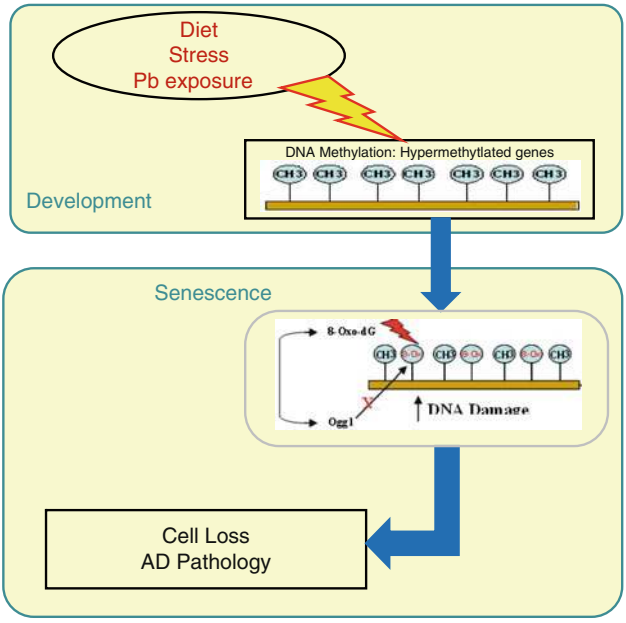

Fig. 2 Epigenetic modifications during development and their impact on gene expression, DNA damage, and neurodegeneration in the aging brain: Exposure to Pb (or other perturbations) early in life may also enhance the methylation of some genes. The epigenetically modified genes may be more susceptible to oxidative stress later in life. Epigenetic modulations of 5-methylcytosine residues impair the capacity to repair adjacent oxidized guanine bases thereby rendering neurons more susceptible to damage. These events enhance neurodegeneration in the aging brain

this model is not restricted to Pb and could be applied to nutritional deficiencies, stress, chemical exposure, or any other perturbation that interferes with epigenetic programming of gene expression. In summary, our model proposes that exposure to Pb early in life has a dual effect on methylation of genes in terminally differentiated neurons. Genes that are hypermethylated early in life face an aging-related build-up in oxidative stress, because methylated cytosine will reduce the ability of the DNA repair enzyme Ogg1 to recognize and remove oxidized adjacent guanines. The reduced capacity to repair DNA can exert greater damage on neurons and result in cell loss. Thus, this model suggests that early events that modify normal genetic processing can build in susceptibility to disease later in life via an interaction between exposure, epigenetics, and oxidative stress. Figure 2 describes the proposed model of epigenetic changes and increased susceptibility to 8-oxo-dG accumulation seen after developmental Pb exposure and in AD.

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