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Molecular mechanisms of oxidative stress in aging: free radicals, aging, antioxidants and disease

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1 INTRODUCTION

The aging process has been shown to result in an accelerated functional decline. The exact mechanisms that cause this functional decline are unclear. The free radical theory of aging, however, has gained strong support because it is able to explain some of the processes that occur with aging and the degenerative diseases of aging. This theory proposes that an increase in oxygen radical production with age by mitochondria produce an increase in cellular damage [1—4].

Indeed, researchers have shown that oxygen utilization by mitochondria of aerobic organisms can generate several reactive radicals, such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and possibly hydroxyl radical ($HO^{\bullet}$; [5—7]).

In addition, nitric oxide (NO·) is also produced by mitochondria [8] and may have implications for the aging process and several disease states associated with aging. Phagocytes are another potent source of oxidant production, and they produce $O_2^{\cdot-}$, H_2O_2, HO·, NO·, and hypochlorous acid (HOCl; [7,9—10]). HOCl is an inflammatory mediator and a strongly oxidizing and chlorinating compound that can form other reactive metabolites, such as nitryl chloride (NO_2Cl) and nitrogen dioxide ($NO_2\cdot$), in the presence of nitrite [11]. Recently, scientists have shown that activated human polymorphonuclear neutrophils convert nitrite into NO_2Cl and $NO_2\cdot$ metabolites, which can significantly contribute to the formation of potentially harmful compounds [12]. The potentially deleterious effects of reactive oxygen, reactive nitrogen, and chlorinating species — for simplicity, referred to as oxidants or radicals — can affect the aging process.

Aerobic organisms are well-protected against oxidative challenges by sophisticated antioxidant defense systems. However, it appears that during the aging process an imbalance between oxidants and antioxidants balance may occur, referred to as oxidative stress. Oxidative stress induced by oxidant species occurs under conditions when antioxidant defenses are depleted or when the rate constants of the radical reactions are greater than the antioxidant defense mechanisms [13]. As we age the defense mechanisms preventing oxidation may decline in specific tissues, and accelerated oxidative damage could, therefore, trigger a deterioration in physiological function. We will critically look at this interrelationship.

Oxidative damage of biomolecules increases with age and is postulated to be a major causal factor of cellular biochemical senescence [14—20]. There is much support for this hypothesis, including the following observations: Studies by Sohal and coworkers using transgenic *Drosophila* in which the antioxidant enzymes, superoxide dismutase (SOD) and catalase (Cat) — two enzymes that scavenge the highly reactive oxidants, superoxide and hydrogen peroxide, respectively — are overexpressed this showed increases in the average and maximum life span and a reduction in oxidative damage [21,22]. Also, several aged species produce a significantly greater amount of reactive oxygen species compared to their younger counterparts [19,20,23]. Moreover, caloric restriction intervention, — that is, a restriction of 60% of the caloric intake of the ad libitum regimen without malnutrition — extends the life span of rodents by 40% [24,25]. In addition, other species such as invertebrates and fish also show remarkable increases in life span with caloric restriction [25—28]. The postulated mechanism is that a reduction in oxygen consumption, with a concomitant reduction in metabolic rate and body temperature in caloric restricted animals, lowered the chronic oxidative stress with age. In addition, caloric restriction also retards a variety of age-related deleterious biochemical and physiological changes, such as the development of cancer and diabetes [25,28]. Furthermore, caloric restriction attenuates protein oxidation, DNA damage, and lipid peroxidation in several housefly and animal models [21—30].

Other evidence that links oxidant generation and aging is provided by the strong inverse correlation between the rate of mitochondrial oxidant generation

and the maximum life span among different species, i.e., animals with high mitochondrial metabolism have a short life span since they have higher oxygen consumption and therefore higher oxidant production [27,28]. In addition, there is a strong correlation between maximum life span of a species and its SOD activity (the first defense against reactive oxygen species). For example, the activity of human SOD is approximately 16-fold greater than that of mice. The increase in SOD activity may provide more protection against superoxide radicals. This would strongly suggest that life span may, in part, depend on the activity of this enzyme [27,31].

We will review several potent sources of oxidant formation and the potentially deleterious effect of oxygen, nitrogen, and chlorinating species on cellular biomolecules. The mitochondria and phagocytes will be highlighted in relationship to biological aging and selected diseases of aging.

2 SOURCES OF OXIDANTS

The oxygen molecule in its diatomic ground state ($^3\Sigma g^- O_2$) is essential for the production of energy. By itself it qualifies as a radical species since it has two unpaired electrons, each of which is located in a different π antibonding orbital. These two electrons also have parallel spins, i.e., they both share the same spin quantum number. Consequently, ground state oxygen is sparingly reactive: so far to oxidize O_2 another molecule by accepting an electron pair, both electrons would have to possess antiparallel spins relative to the unpaired electrons in O_2, according to Pauli's exclusion principle. This criterion is seldom met in a typical electron pair, so oxygen tends to accept electrons one at a time.

In vivo, multielectron reduction of O_2 is carried out by a coordinated series of enzymes that reduce O_2 1-electron at a time. If O_2 accepts a single electron, the electron must enter an antibonding orbital producing the superoxide radical $O_2^{\cdot -}$. Two-electron reduction of O_2, with the addition of $2H^+$, generates hydrogen peroxide (H_2O_2). Most $O_2^{\cdot -}$ is metabolized by SOD to H_2O_2 and oxygen. In reactions catalyzed by free transition metals such as Fe^{2+}, $O_2^{\cdot -}$ and H_2O_2 can generate the extremely reactive hydroxyl radical (HO·). This molecule is believed to be the major cause of damage to proteins, lipids, and DNA. Oxidative damage to these biomolecules seems to depend on hydrogen peroxide and a reduced transition metal. Therefore, molecules that contain transition metals, such as aconitase (a Krebs cycle enzyme), are likely to undergo oxidative damage [17,18,32—34].

2.1 Mitochondria

The main function of mitochondria is energy production. During oxidative phosphorylation, however, highly reactive oxygen radicals are generated. It has been estimated that the release of reactive intermediates accounts for about 2% of the oxygen consumed during respiration. One major site of oxidant production

occurs in the mitochondrial electron transport chain in which O_2 is reduced to H_2O (Fig. 1). In this process, electrons from NADH are donated to complex I (NADH dehydrogenase complex), and electrons from succinate are donated to complex II (succinate dehydrogenase complex). Ubiquinone, also known as coenzyme Q or UQ, accepts electrons from both complexes and is sequentially reduced, one electron at a time, to ubisemiquinone and ubiquinol. Ultimately, electrons are transferred through complex III (UQ-cytochrome c reductase), cytochrome c, and complex IV (cytochrome-c oxidase) with the result of reducing O_2 to H_2O. Mitochondrial electron transport, however, is imperfect and results in the production of $O_2^{\cdot-}$ from the one-electron reduction of O_2. Some speculate that this electron comes from the free radical ubisemiquinone: instead of accepting another electron and proton to form ubiquinol, one of the electrons may leak from ubisemiquinone and reduce O_2 to $O_2^{\cdot-}$. Enzymatic dismutation of $O_2^{\cdot-}$ then leads immediately to H_2O_2, another important biological oxidant [7].

How much $O_2^{\cdot-}$ and H_2O_2 is generated during mitochondrial respiration? In state four respiration, which is characterized by a high degree of reduction of the electron carriers and a limiting supply of ADP, H_2O_2 production is at its maximum. It has been estimated that under these conditions the release of reactive intermediates accounts for 2% of the oxygen consumed during respiration [5].

Ultimately how much of this mitochondrial H_2O_2 is derived from dismutation of $O_2^{\cdot-}$ in the electron transport chain? Measurement of $O_2^{\cdot-}$ production cannot be accomplished in intact mitochondria because it is dismutated by mitochondrial SOD (mSOD). However, by isolating submitochondrial fractions and by removing mSOD by sonication and washing, it is possible to detect electron transport chain $O_2^{\cdot-}$ production. In experiments done in the 1970s, it was found that submitochondrial particles produce from 4- to 7- nmol $O_2^{\cdot-}$/min per mg of protein, resulting in $O_2^{\cdot-}/H_2O_2$ ratios of 1.5—2.1 [6]. Since two $O_2^{\cdot-}$ anions dis-

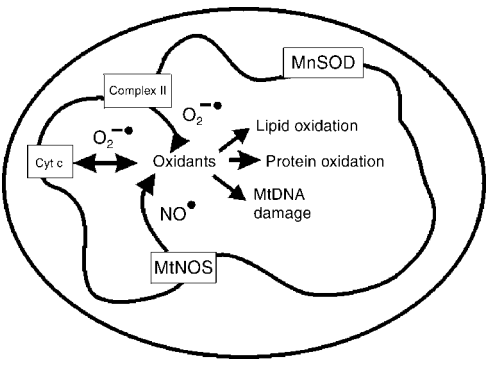

Fig. 1. Mitochondrial oxidant production; MnSOD: manganese superoxide dismutase; MtNOS: mitochondrial nitric oxide synthase.

mutate to form one H_2O_2 molecule, these results indicate that $O_2^{\cdot-}$ is almost certainly a precursor of mitochondrial H_2O_2 (Fig. 1).

The enzyme nitric oxide synthase (NOS), which produces the free radical gas $NO^{\cdot}$ from L-arginine, has been primarily investigated in endothelial cells. However, recent experiments have been performed which indicate that NOS in mitochondria produce NO· (Fig. 1). First, several studies using immunohistochemical techniques showed that skeletal muscle expresses endothelial-type nitric oxide synthase (ec-NOS). These studies also show that there is a strong correlation of ec-NOS expression to mitochondrial content visualized histochemically by succinate dehydrogenase [35]. In addition Bates et al. [36,37] have demonstrated localization of NO· synthase in mitochondria isolated from heart, skeletal muscle, kidney, and brain using a monoclonal antibody against the ec-NOS. Recently, mitochondria have been unambiguously identified as sources of NO·, using electron paramagnetic resonance with spin-trapping techniques. Giulivi et al. isolated NOS from Percoll-purified rat liver mitochondria [38]. Several different mitochondrial preparations, such as toluene-permeabilized mitochondria, mitochondrial homogenates, and a crude preparation of NOS, were incubated with the spin trap *N*-methyl-D-glucamine-dithiocarbamate-Fe II which produced a signal ascribed to the NO· spin adduct [8]. The intensity of the signal increased with time, protein concentration, and L-arginine, and decreased with the addition of the NOS inhibitor N^G-monomethyl-L-arginine. Kinetic parameters, molecular weight, requirement of cofactors, and cross-reactivity to monoclonal antibodies against macrophage NOS suggest similarities to the inducible form. However, the constitutive expression of this NOS enzyme and its membrane localization may indicate a distinctive isoform [8]. These findings suggest that mitochondrial NOS and NO· production may not only have an important role as a cellular transmitter, messenger, or regulator, but also as an active player in oxidative metabolism [8,34—38]. Since NO· and $O_2^{\cdot-}$ react to produce another very reactive oxidant, peroxynitrite ($ONOO^-$), it is clear that mitochondria are a major source of free radicals and oxidants.

2.1.1 Mitochondria and Aging

Several studies have investigated if there is an age-associated increase in the generation of oxidants by mitochondria. An experiment using mongolian gerbils (*Meriones unguiculatus*) found that $O_2^{\cdot-}$ and H_2O_2 production increased with age, especially in isolated mitochondria and submitochondrial particles from the aged heart [39]. In another study using whole hepatocytes of older rats, it was found that older hepatocytes had a decrease in the mitochondrial membrane potential of 30% and an increase in mitochondrial hydrogen peroxide generation of 23%. H_2O_2 levels within the liver cells were also increased [40]. Furthermore, another study on intact, isolated rat hepatocytes reached the same conclusion: cellular oxidant generation by mitochondria increases with age [41].

Similarly, experiments using intact muscle mitochondria from house flies has

shown that the rate of H_2O_2 generation progressively increases 2-fold as the house fly ages [42]. On the other hand, an experiment which attempted to maintain proper physiological substrate concentrations during in vitro mitochondrial incubations detected no difference in H_2O_2 generation by rat heart mitochondria when comparing 24-month-old rats (senescent adults) and 6-month-old rats (young adults) [43].

The enhanced generation of oxidants by older mitochondria may itself be caused by oxidative damage to mitochondrial membranes and proteins. In one experiment, when isolated mitochondria were exposed to oxidant generators like glutaraldehyde (an intermolecular cross-linking agent) or 2,2-azobis-di-hydrochloride, the mitochondria generated H_2O_2 at an increased rate [42]. This evidence, along with the above studies, paints a vicious cycle of mitochondrial oxidant damage and oxidant generation.

In connection with such findings, Miquel and his colleagues [44,45] have widely promulgated the mitochondrial mutation theory of aging. In this theory, senescence is linked to mutations of mitochondrial DNA (mtDNA) in differentiated cells. As discussed earlier, isolated mitochondria from aged animals have an increased production of reactive oxygen species compared to young animals. This observation may be of key importance because it is likely to increase the rate at which proteins and DNA are oxidized. Since these mutations occur in postmitotic cells and mtDNA lacks excision and recombination repair mechanisms, it has been postulated that these mutations would lead to problems in replication, leading to a decline in physiological performance and the pathogenesis of many age-related diseases [44,45]. In addition, mtDNA is not protected by histones or DNA-binding proteins and, therefore, is directly exposed to a high steady-state level of reactive oxygen and nitrogen species. Thus, oxidative modification and mutation of mtDNA may occur with great ease. Mutations in mtDNA can lead to the production of less functional respiratory chain proteins, resulting in increased free radical production and possibly more mtDNA mutations. This vicious cycle operates in different tissues at variable rates and leads to differential accumulation of oxidatively modified mtDNA. This may ultimately reduce energy output and contribute to the common signs of normal aging.

In this context, one study by Yakes et al. [46] compared whether mtDNA accumulates more oxidative DNA damage relative to nuclear DNA; it also investigated whether there were differences in repair between nuclear and mtDNA. Specifically, the researchers investigated the formation and repair of H_2O_2-induced DNA damage in a 16.2 kb mitochondrial fragment and a 17.7 kb fragment flanking the β-globin gene. Fibroblasts treated with a relatively high dose of hydrogen peroxide exhibited 3-fold greater damage to the mitochondrial fragment compared with the nuclear fragment. Furthermore, damage to the nuclear fragment was completely repaired within 1.5 h; whereas, no DNA repair in the mitochondrial fragment was observed [46]. These data suggest that mtDNA is more susceptible to oxidants, and mitochondrial repair mechanisms may be nonexistent or less efficient.

2.2 Phagocytes

Phagocytic cells are another major source of oxidants. Neutrophils and other phagocytes attack pathogens using a mixture of oxidants – for example, $O_2^{\cdot-}$, $NO\cdot$, H_2O_2, and HOCl. The process of phagocytosis begins when the neutrophil travels to a site of infection as directed by certain chemotactic signals that are generated at the infection site. When foreign microbes, which may be bound by serum-derived glycoproteins (i.e., opsonized), perturb the plasma membrane of a neutrophil, a dormant pyridine-nucleotide-dependent oxidase is activated. This pyridine nucleotide oxidase is believed to be a reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) or a reduced nicotinamide adenine dinucleotide oxidase (NADH oxidase), whose action might involve a b type cytochrome [7,10]. This process initiates a "respiratory burst", which lasts for 15 to 20 min and reduces O_2 to $O_2^{\cdot-}$ (a one-electron reduced product) and H_2O_2 (a two-electron reduced product). It also serves as the first source of oxidant production in phagocytes.

Simultaneously, the plasma membrane of the phagocyte invaginates around the foreign particle, surrounding it and subsequently pinching off to become a phagosome. Also during this time period, degranulation occurs: granular lysosomes migrate toward the phagosome, fuse with it, and empty their granular contents into it; after fusion with lysosomes, the phagosome becomes a phagolysosome. The contents of the lysosomes primarily include digestive enzymes like acid hydrolases, neutral proteases, and alkaline phosphatases. These lysosomes also contain cationic proteins, lipopolysaccharides, lactoferrin, myeloperoxidase (MPO), and biopolymers that might be involved in bactericidal reactions. This entire process occurs extremely rapidly taking no longer than a few minutes [7,10].

Inside the phagolysosome, the heme containing enzyme MPO forms an enzyme-substrate complex with H_2O_2 that can then catalyze the two-electron oxidation of halides like Cl^-, Br^-, and I^- by H_2O_2. The oxidation of the halides, particularly Cl^-, forms a toxic agent with potent antimicrobial properties. In the case of chloride, the oxidation product is HOCl, which subsequently kills the ingested microorganism. The H_2O_2 needed for this process is generated in the respiratory burst and can be detected in the phagosome [9]. Furthermore, a recent study on the MPO-hydrogen-peroxide-chloride system showed that MPO also generates Cl_2 gas and that human neutrophils employ chlorine gas as an oxidant during phagocytosis [47].

2.2.1 Phagocytes and aging

Currently it is unclear if there are age-associated trends in oxidant production by phagocytes. Age-dependent oxidant generation is relevant because it is very common for older individuals to suffer from inflammatory-related conditions such as arthritis. Some studies have indicated that oxidant production is a function of

age; in fact, several experiments have shown that older macrophages produce a decreased amount of $O_2^{\cdot-}$, H_2O_2, NO· and other oxidants [48–50]. In a study on rat peritoneal macrophages by Alvarez et al., production of $O_2^{\cdot-}$ was decreased by 50% and H_2O_2 by 75% in older macrophages [49]. NO· production was also reduced to 40% with age [48]. In a study done on human monocytes by Alvarez et al., they found that superoxide production was age and sex dependent. It decreased 45% in men and 70% in women during aging [51].

Other studies have shown that oxidant production is age-dependent, but in these studies the generation of reactive oxygen species appeared to increase with age. In a study by Lavie et al., they found a 2-fold increase in oxidant production by senescent peritoneal macrophages after being stimulated by latex and zymosan [52]. Also, a study on patients with obliterative atherosclerosis of the lower legs showed an increase in superoxide anion production by polymorphonuclear leukocytes (PMNLs) under basal conditions. After stimulating these PMNLs with formyl-methionin-leucyl-phenylalanine and calcium ionophore, the researchers found that the PMNLs of the atherosclerotic patients had an increased ability to release myeloperoxidase and elastase than PMNLs of healthy, middle-aged subjects [53]. Moreover, in a recent study there was an increased concentration of hydrogen peroxide, following stimulation by formyl peptide, in individual neutrophils from older volunteers (ages 65 and older) compared to neutrophils from younger volunteers (aged 21–34 years). By analyzing enzyme kinetics, the researchers concluded that the age-associated accumulation of H_2O_2 in stimulated neutrophils could be accounted for by impaired glutathione peroxidase (GPX) [54]. Further studies are warranted to investigate whether age causes an increase or decrease in oxidant production by PMNLs and whether aging-associated human diseases significantly affect oxidant production.

3 AGING AND ANTIOXIDANT DEFENSES

The possibility that oxidative stress and aging is mainly due to a decline in antioxidant defenses is still not clear. In fact, most researchers would argue that antioxidant defenses are, in general, not different between young and old animals. We will briefly discuss the general trends of several major organs, the effects of age on each of the major antioxidant systems, and the effect of selected antioxidants on age. This topic has been extensively reviewed by Matsuo [55], and we will discuss some of the general findings on the antioxidant enzymes – SOD, Cat, and GPX. We will also discuss the major trends involving the changes, if any, in water soluble and lipid soluble antioxidants, mainly focusing on vitamin C, GSH, and vitamin E, realizing that there are a multitude of other known and possibly unknown antioxidant defenses which may change with age.

SOD is a cytosolic (copper/zinc) and a mitochondrial (manganese) isoenzyme, which dismutates the superoxide radical to oxygen and hydrogen peroxide. The majority of the studies, which investigated total SOD activity in the brains of rats and mice, have found little change in the enzyme's activity with age [55].

(One study, in fact, actually found an increase in mitochondrial SOD activity in the brain of rats with age, which is not what one would expect if decreased antioxidant enzymes were a major factor in aging) [56]. Furthermore, the majority of the studies investigating the liver of mice also showed no change in total SOD. In contrast, many studies in rats showed a decrease in liver SOD activity [55]. This discrepancy is unclear, but could give an indication of variable adaptations between species. Most studies show that SOD activity in the heart of both mice and rats does not change with age [55]. Skeletal muscle SOD, however, does increase with age in old rats compared to either young or middle-aged rats (Table 1, [57—60]). Some of the inductions of SOD with age could reflect a chronic adaptation to the increase in superoxide production.

Gluthathione peroxidase is the major hydrogen peroxide scavenger enzyme, and it is found in both the cytosol and mitochondria. Together with its substrate GSH (discussed later), they form a formidable defense against hydrogen peroxides and lipid peroxides. Rat brain shows no change with age in this important reactive oxygen species scavenging enzyme [55]. Both the liver and the heart GPX show about an equal number of studies reporting either no change or a decrease in enzyme activity; thus, there is no consistent pattern of change with this enzyme [55]. Again, skeletal muscle GPX activity increased with age in rats, indicating an adaptive change to reduce oxidative stress in this tissue (Table 1 [57]).

Another H_2O_2 metabolizing enzyme is Cat. It metabolizes hydrogen peroxide to oxygen and water. This enzyme is primarily found in the peroxisomes, but it is also located, less abundantly, in mitochondria. The literature shows no consistent trend in changes of Cat activity in brain tissues with aging; an equal amount of studies show an increase or a decrease in activity [55]. This may reflect difficulties in the measurement of Cat activity. Cat activity in the liver seems to decline consistently in aging rats. In striking contrast, Cat activity increases in the heart and skeletal muscle of aging rats [55,57]. For example, Leeuwenbugh et al. found that skeletal muscle Cat activity increased by a large margin of 150% comparing 4.5-month-old rats with 26.5-month-old rats, and there was a 40% increase com-

Table 1. Activities of antioxidant enzymes in deep vastus lateralis (DVL) skeletal muscle of young and old rats [56].

	GPX DVL	CAT DVL	SOD DVL
Young	4.25 ± 0.29	15.8 ± 2.2	1960 ± 143
Adult	5.70 ± 0.34[a]	29.4 ± 3.8[b]	2190 ± 118
Old	5.88 ± 0.36[a]	39.6 ± 4.2[b]	2510 ± 120[a]

Values are means ± SE; GPX: glutathione peroxidase; SOD: superoxide dismutase (units/g wet weight); CAT: catalase ($K \times 10^{-2}$/g wet weight); GSH: glutathione μmol/g wet weight; Young = 4.5 months; Adult = 14.5 months; Old = 26.5 months. [a]$p < 0.05$, Adult or Old vs. Young; [b]$p < 0.001$, Adult or Old vs. Young. (Adapted from [57].)

paring middle-aged animals (14.5 months) with the very old animals ([57] Table 1).

Vitamin C is a critical water soluble antioxidant interacting with GSH and vitamin E in maintaining a reduced intracellular environment [61]. This antioxidant shows a consistent decline with age in various species. For example, researchers from Sweden determined that the nucleus accumbens in 3-, 6- and 18-month-old Sprague-Dawley rats revealed a significant age-related decrease in basal extracellular vitamin C concentration [62]. Several studies show that there is a significant decline in vitamin C levels in the liver of rats [63,64]. Vitamin C content also decreases with age in toad skeletal muscle [65]. One complexity is that these data are difficult to translate to humans since humans lack the enzymes to synthesize vitamin C and are dependent on acquiring vitamin C from their diets. Many epidemiological studies clearly show that inadequate vitamin C levels correlate highly with diseases such as cancer and cataracts [14], providing cogent evidence that this antioxidant has strong anti-aging properties.

GSH (L-γ-glutamyl-L-cysteinyl-glycine; GSH) is a tripeptide (Fig. 2) found in virtually all animal cells. It is an important reducing agent that maintains enzyme activity and functions to maintain compounds like dehydroascorbate (vitamin C) and α-tocopherol (vitamin E, Fig. 3) in the reduced state.

GSH has several characteristics that determine its metabolism and antioxidant function. One is that glutamate and cysteine peptide linkage is linked through a γ-carboxyl group of glutamate instead of the more common α-carboxyl peptide linkage [66—68]. This unique characteristic makes the γ-carboxyl bond resistant to all peptidases except γ-glutamyltranspeptidase, which is bound to the external surface of the cell membrane. Therefore, GGT does not effect the intracellular breakdown of GSH [66—68]. Most importantly, the moiety of GSH is the cysteinyl reactive thiol group, which is responsible for many of the antioxidant functions of GSH metabolism (Fig. 2).

These characteristics, along with the fact that GSH is the most abundant non-protein thiol source and antioxidant in the cell, give GSH multiple functions for antioxidant defense, such as the following:

1) GSH provides a substrate for GSH peroxidase wherein GSH is used to reduce hydrogen and organic peroxides to water and alcohol;
2) GSH conjugates with harmful exogenous and endogenous toxic compounds;
3) GSH reduces disulfide linkage of proteins and other molecules maintaining glycolytic and antioxidant enzymes in the reduced state;

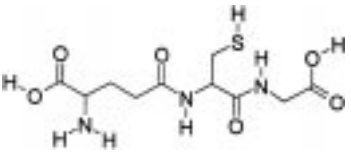

Fig. 2. Glutathione.

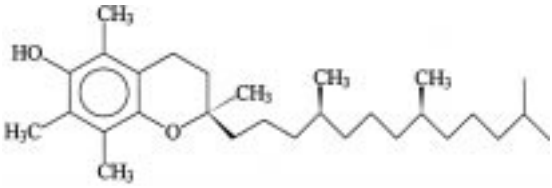

Fig. 3. Vitamin E.

4) GSH is a major thiol source maintaining essential redox status of the cell;
5) GSH is a major nontoxic storage form of cysteine providing a vehicle for transport between organs; and
6) GSH plays an important role in the reduction of ribonucleotides to deoxyribonucleotides [66—68].

There are several papers that discuss the effect of age and GSH levels. We will discuss some of the findings in brain, liver, heart, and skeletal muscle tissues. One study investigated GSH and several major antioxidant enzymes. In contrast to what one might expect, a significant increasing pattern of GSH content was found in the mice cerebellum and brain stem with age [69]. They also reported that mouse brain Cat activity did not show a significant change in any of the regions of the brain except in the cerebellum; whereas, SOD and GPX activity increased with age in most regions of the brain, suggesting that overall antioxidant defenses were not impaired with increasing age in mouse brain [69]. Most studies are in agreement that there is no significant decline in GSH concentration in the brain. Other reports found an increase [70] in GSH in the brain, while another found no change in GSH content in brains of senescent rats [71]. These studies reported increases in plasma, heart, and liver GSH concentration in old rats [70,71]. There are, however, some reports that show a decrease in brain GSH levels [72,73]. These studies found no change or an increase in liver GSH concentration [63,70,72,73]. Liver GSH is critical since it supplies GSH to all tissues. The liver contains the highest GSH concentration and continuously exports GSH to extrahepatic tissues.

GSH levels in skeletal muscle have also been investigated with age. Leeuwenburgh et al. found no difference in GSH concentration in deep vastus lateralis muscle and a significant increase in GSH content in the soleus muscle (Table 2 [57]), indicating that aging does not result in a significant decline in this antioxidant and that there are fiber-specific adaptations of the GSH system in skeletal muscle with age (Table 2).

These studies at least indicate that there is no overall decline of this important antioxidant. Some studies report increases in GSH content such as in skeletal muscle [57]; while others show reduction in GSH content such as in the eye lens [63]. Thus, in most tissues GSH is unlikely a limiting antioxidant for proper antioxidant protection with aging. In contrast, studies investigating subcellular components and changes of GSH content with age have found that GSH may decline

Table 2. Content the antioxidant glutathione (GSH) in deep vastus lateralis (DVL) and in soleus skeletal muscle of young and old rats (56).

	GSH DVL	GSH Soleus
Young	1.94 ± 0.21	2.10 ± 0.13
Adult	1.91 ± 0.16	2.64 ± 0.06
Old	1.76 ± 0.15	2.88 ± 0.11[a]

Values are means ± SE; GPX: glutathione peroxidase; SOD: superoxide dismutase (units/g wet weight); CAT: catalase (K × 10^{-2}/g wet weight). GSH: glutathione μmol/g wet weight. Young = 4.5 months; Adult = 14.5 months; Old = 26.5 months. [a]$p < 0.001$, Adult or old vs. young (adapted from [57]).

with aging. One such study investigating GSH in rat cerebral cortex synaptosomes as a function of age found a decrease in GSH content [74]. Another study investigating mitochondrial GSH content showed a general decline in GSH levels with aging in rats and mice in the liver, kidney, and brain [75]. This would suggest that there may be differences in GSH content among compartments within cells during the aging process. These changes may not have been detected when looking at the overall levels of GSH in tissues in some of the previously selected studies. Therefore, there is a need to investigate specific organelles within the cell to determine changes in antioxidant levels.

The lipid soluble antioxidant vitamin E refers to at least eight structural isomers of tocopherol and tocotrienols [76—83]. Among these, RRR-α-tocopherol possesses the highest antioxidant activity. Vitamin E is often used to attenuate oxidative stress in many pathophysiological conditions [77—83]. For example, in an often-cited study by Stephens et al., patients with angiographically proven symptomatic coronary atherosclerosis who receive α-tocopherol treatment have a significantly diminished risk for cardiovascular disease. These patients show a substantial reduction in the rate of nonfatal MI after 1 year of vitamin E treatment [84].

As an antioxidant, vitamin E is important because of its ability to convert several free radicals such as superoxide, hydroxyl, and lipid peroxyl radicals into "repairable" radical forms [13,83]. Moreover, vitamin E is the primary antioxidant in cell membranes and often acts as a chain breaking antioxidant attenuating further lipid peroxidation [13,61,83]. Also, vitamin E has a relatively long biological half-life and has limited side effects even when administered in high doses [80—82]. When vitamin E scavenges a radical, a vitamin E radical is formed. This vitamin E radical is not capable of scavenging additional radicals but can be "recycled" back to its native state by several other antioxidants such as vitamin C [13,61,83]. Thus, investigators often use both vitamin E and vitamin C in an attempt to achieve maximal antioxidant protection.

We will discuss the major trends that occur during aging and their effects on vitamin E levels. One study investigated the antioxidant enzymes (SOD, Cat and

GPX activities) and vitamin E concentration in rats. They found little change in these antioxidant defense systems throughout the life span of the rats. In fact, vitamin E concentration in lung and liver tissues increased with age [85]. Moreover, concentrations of tocopherols in selected areas of rat brains increased significantly with age in the medulla and spinal cord, with no changes in other regions of the brain [86]. Heart vitamin E did not decline in this study [86]. De et al. found that plasma levels of vitamin E and ascorbic acid decreased in serum, but there were no changes in liver concentration [64]. In another study, serum vitamin E increased markedly with age, but there was little change in membrane vitamin E content in ad libitum fed rats [87]. Sawada et al. reported no significant changes in blood vitamin E levels with age in rats [88].

In general, there is no dramatic overall decline with age of the major antioxidant defense systems or a decline in vitamin E concentration in ad libitum fed animals. In fact, some tissues actually show significant increases of vitamin E in senescence animals and upregulations of antioxidant enzymes, for example, in skeletal muscle [57]. Vitamin C shows a general decline with aging, which may not be relevant to humans since vitamin C concentration in humans is entirely dependent on dietary vitamin C intake. Lower concentrations of vitamin C in most tissues with age could partly explain the increase in oxidative damage which occur during in vitro oxidative challenges. Researchers often challenge tissues of young and old rats with free radicals in vitro and these studies often show that tissues from old animals are more susceptible to free radical damage than tissues from young animals [39,89,90].

Another potentially protective antioxidant in brain tissues is melatonin, and it is probably more influential than vitamin C in affecting the outcome of in vivo oxidative challenges in the brain. The brain rapidly takes up this pineal hormone, which declines with advancing age. Melatonin is more effective than GSH in scavenging the highly toxic hydroxyl radical in in vitro experiments, and it also has been found to be more efficient than vitamin E in neutralizing the peroxyl radical [91]. Besides melatonin, there may be a plethora of other potent antioxidants and antioxidant systems present. Alterations in these systems could have far greater relevance than vitamin E, vitamin C, and the primary antioxidant enzyme systems.

4 PROTEIN OXIDATION AND AGING

During the aging process, protein oxidation is increased in a wide variety of human and animal tissues. The exact pathways for oxidative cellular damage are poorly understood because the reactive metabolites are very short-lived and difficult to detect directly in vivo. The quantification of oxidative damage to proteins has been studied almost exclusively by assessing the total carbonyl content [92,93]. The oxidants responsible for carbonyl formation within the proteins in vivo are believed to be radicals, such as, hydroxyl radicals. Indeed, hydroxyl radicals can be generated by metal-catalyzed oxidation systems [18,89,94,95], and

different metal-catalyzed oxidation systems convert several amino acid residues to carbonyl derivatives [18,89,94,95].

The assessment of protein oxidation using the reactive protein carbonyl assay does provide a good indication of overall oxidation; however, it also reflects covalent adduct formation from lipid peroxidation products [96]. Moreover, several oxidative pathways and products from lipid peroxidation and glycooxidation can generate carbonyls from proteins [96—99]. These pathways consist of both oxidative and nonoxidative pathways besides metal catalyzed oxidation, and they are efficient in generating reactive protein-carbonyls [96—99]. The oxidants and nonoxidant modifications are generated by HOCl, peroxynitrite, aldehydes, glucose, ribose, and the strongly oxidizing enzyme myeloperoxidase [96—99].

There is a large amount of literature available which clearly shows a consistent increase in protein-bound carbonyls with advanced age and in several degenerative diseases of aging. We will discuss some of these findings. For example, protein-bound carbonyls are present at low levels in dermal fibroblasts isolated from young to middle-aged humans, but they are increased 2-fold in fibroblasts from people over 60 years of age [100]. Furthermore, there are age-related increases in the carbonyl content of proteins in human brain [101], gerbil brain [102], rat hepatocytes [103], and in flies [104]. These studies show a consistent pattern of increasing protein oxidation in old age.

Recently, other radical species, besides the hydroxyl radical, have been implicated in oxidative protein damage. Radicals, such as NO· are implicated in several disease states such as cardiovascular disease and neurodegenerative diseases, but few studies to date have investigated the role of NO· on protein oxidation and aging. Since NO· is the only biological molecule produced in high enough concentrations to out-compete SOD for superoxide — consequently reacting with superoxide to form $ONOO^-$ — it could be a very relevant compound in aging.

Some studies investigating the deleterious effects of NO· metabolites and aging suggest that certain proteins are oxidized selectively in vivo. This may be possible because $ONOO^-$, formed from NO· and superoxide, reacts relatively slowly with most biological molecules, which makes it a selective oxidant [105—107]. $ONOO^-$ modifies tyrosine in proteins and generates 3-nitrotyrosines, a fairly specific marker detectable in vivo. For example, the level of nitration of the SERCA2a isoform of calcium-ATPase in sarcoplasmic reticulum vesicles isolated from rat skeletal muscle increases with age; there are approximately one and four nitrotyrosine residues per young and old Ca-ATPase, respectively [108]. In addition, nitration was undetectable in a closely related form of the protein [109], which strongly suggests that certain calcium-ATPases are selectively modified by reactive nitrogen species. In vitro studies suggest that this level of protein oxidation may alter the function of SERCA2a in vivo [109]. These observations raise the possibility that specific proteins accumulate oxidative damage during aging. Therefore, it is plausible that an increases in NO· and/or superoxide production with age, in mitochondria could become deleterious to mitochondrial respiratory enzymes.

In a recent study, very specific and sensitive analytical methods were used to measure the levels of some of these "fingerprints" of oxidative damage in tissue proteins of aging rats [110,111]. We found that specific markers for protein oxidation, such as o-tyrosine (a marker for hydroxyl radicals) and 3-nitrotyrosine (a marker for reactive nitrogen species) do not increase in skeletal muscle, heart, and liver of aging rats. This suggests that proteins damaged by hydroxyl radical and reactive nitrogen do not accumulate with aging in these tissues. This was surprising because previous studies using non-specific measures had suggested that proteins damaged by hydroxyl radical accumulate in the tissues of old animals. This study does strongly suggest that hydroxyl radical and reactive nitrogen species damage proteins during biological aging; however, the accumulation of these amino acids may have been prevented by removal mechanisms for these markers in these specific tissues [110]. Thus, proteolytic degradation of intracellular proteins may account in part for the relatively constant level of amino acid oxidation products seen in this study. Also, since the average level of protein oxidation in tissue was measured, it may have missed detecting a marked increase in protein oxidation in a few selected proteins or in specific organelles such as the mitochondria.

Protein oxidation and protein nitration has been also detected in Progeria's disease and in several other human disease states. Fibroblasts obtained from patients with diseases of accelerated aging (Progeria or Werner's syndrome) have dramatically higher levels of protein carbonyls [100] compared to that of age-matched controls. Leeuwenburgh et al. [112] and Beckman et al. [113] found that nitrated proteins are abundant in low density lipoproteins (LDL) and plaque isolated from patients with atherosclerosis. In these studies it is unclear whether peroxynitrite or other reactive nitrogen species were responsible for tyrosine nitration. Peroxynitrite is a source of hydroxyl radical-like species, but also of nitrogen dioxide. It is nitrogen dioxide that most likely has the ability to directly oxidize proteins and other macromolecules in vitro. Other pathways that generate reactive nitrogen species are mediated by myeloperoxidase and may be responsible for tyrosine nitration [11,12].

Peroxynitrite has also been implicated in neurodegenerative diseases and the generation of carbonyls from side-chain and peptide-bond cleavage. It also nitrates tyrosine residues. In brain tissue from patients with Alzheimer's disease, scientists found increased 3-nitrotyrosine in neurons including neurofibrillary tangles, whereas 3-nitrotyrosine was undetectable in the cerebral cortex of age-matched control brains [114]. In Alzheimer's brain, using in situ 2,4-dinitrophenylhydrazine labeling linked to an antibody system, Smith et al. describes protein-bound carbonyl reactivity [115]. Tissues from disease-related, intraneuronal lesions and other neurons showed significant increases in reactive carbonyls. In striking contrast, carbonyls were not found in neurons or glia in age-matched control cases. They concluded that oxidative stress is a key element in the pathogenesis of Alzheimer's disease. These findings strongly suggest that reactive oxygen species and possibly peroxynitrite are involved in the oxidative damage of

Alzheimer's disease.

In subjects with Parkinson's disease, protein carbonyls were assessed in post-mortem brain tissue and age-matched controls. In brain areas associated with Parkinson's, such as the substantia nigra, caudate nucleus, and the putamen, there was a significant increase in protein-bound carbonyl levels [116]. Other markers of protein oxidation are also affected with amyotrophic lateral sclerosis. Researchers investigated amyotrophic lateral sclerosis and found elevated levels of free 3-nitrotyrosine [117].

In summary, these results suggest that levels of oxidized proteins increase with age and in several neurodegenerative aging-associated diseases. Metal-catalyzed oxidation reactions could be partly responsible for protein oxidation; however, there are multitudes of other oxidative reactions, which can generate protein carbonyls and other oxidative modifications in proteins and amino acids. Reactive nitrogen species could nitrate proteins and have major physiological consequences on normal protein function. Thus, reactive metabolites from oxygen and nitrogen metabolism could be active players in aging and the degenerative diseases of aging.

5 REPAIR AND TURNOVER OF OXIDATIVELY DAMAGED PROTEINS

Oxidative damage repair mechanisms are a critical component in maintaining intracellular homeostasis. Oxidative DNA damage, which forms unnatural adducts, can be removed by a variety of mechanisms including endonucleases and glycosylases [83,118]. Protein damage will be recognized by specific proteases and degradation will follow [17,18,83]. Oxidized lipids can be repaired or removed by the GSH-GPX systems or by phospholipases [13,83,118,119]. We will briefly discuss the interplay between several factors underlying the accumulation and removal of oxidized proteins and oxidized amino acids in the cell.

In general, the level of oxidized proteins in a tissue reflects the balance between the relative rates of protein oxidation and clearance. For example, lens or collagen proteins turn over extremely slowly and thus, should accumulate products of oxidative damage over time. In contrast, intracellular proteins in liver and muscle turnover continuously with half-lives ranging from a few hours to 7—10 days, since a mechanism exists for the continuous removal of these oxidized proteins. In general, one can predict that the accumulation of oxidized proteins is dependent upon the balance between pro-oxidant, antioxidant, and removal mechanisms. The interactions between several of these components are depicted schematically (Fig. 4).

Isolated mitochondria from old animals showed increased production of reactive oxygen species compared to young animals. This observation may be of key importance because it is likely to increase the rate at which proteins are oxidized. Thus, the significant age-related increases in the generation of superoxide and hydrogen peroxide by mitochondria in various tissues may have led to an

increased accumulation of oxidized proteins observed by many researchers. Also, species-specific differences in metabolic rate may exist and could also influence the formation of oxidized macromolecules differently. Other factors affecting protein oxidation include changes in tissue specific antioxidant defenses with age (discussed in previous sections), which either, decline modestly, show no change, or increase. A final factor that may affect the accumulation and the removal of oxidized proteins are several proteolytic systems.

Starke-Reed, Stadtman and co-workers [17,103] have performed several pioneering studies addressing how proteins are oxidized and subsequently proteolytically degraded and how these systems may change with age. They found that with age there is less efficient removal of oxidized proteins through proteolytic cleavage, which may cause the accumulation of protein carbonyls with aging [17,103]. Several proteolytic enzymes responsible for degrading oxidized proteins decline with age in tissues [17,103]. These proteases rapidly degrade oxidized enzymes but do not effect native unoxidized enzymes. Several multicatalytic proteases provide

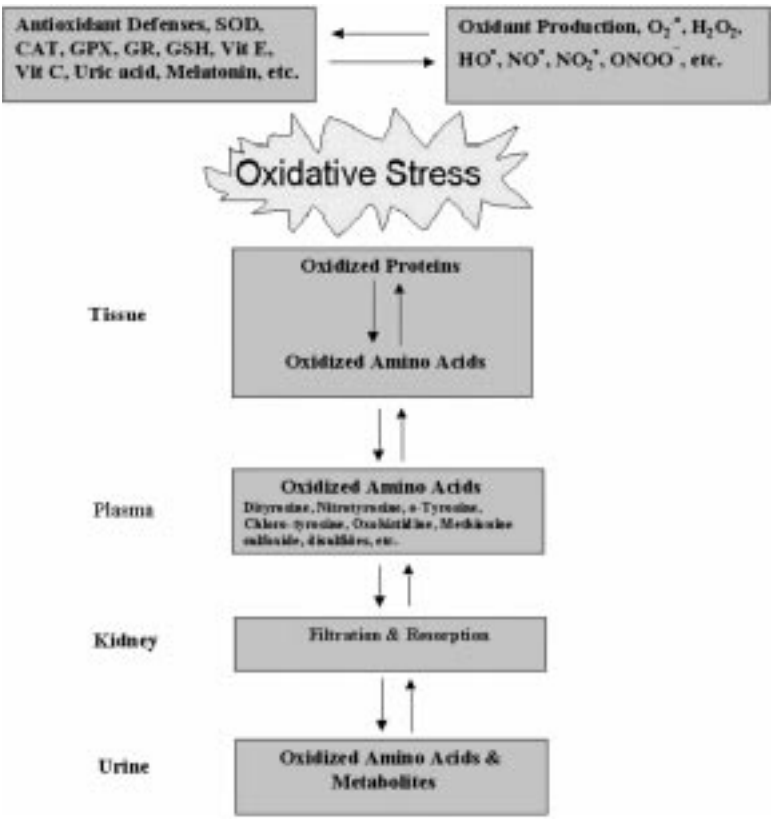

Fig. 4. Several distinct aspects of free radical biology eventually determine the accumulation of oxidized proteins: 1) oxidant production, 2) antioxidant protection, 3) oxidative damage, 4) repair of oxidative damage, and 5) removal of oxidative damage.

major intracellular pathways for protein degradation [120—129]. Recently, Cuervo and Dice discussed various proteolytic systems, such as ubiquitin-proteasome, calpain pathways, and multiple lysosomal pathways and the effect of age [120].

The existence of specific mitochondrial proteolytic systems that can recognize and degrade oxidatively damaged proteins is currently under investigation [121]. Investigators have found that mitochondria have their own proteolytic system, probably because the mitochondria possess both an inner and outer membrane, making them quite impermeable (especially the inner membrane) to cytosolic proteases and peptidases [121]. The presence of a mitochondrial proteolytic system indicates the need for removal of oxidized proteins and amino acids from the mitochondria. There is little known about the removal of oxidized proteins from mitochondria, but it is likely that small peptides and unnatural oxidized amino acids are removed. It is feasible that unnatural oxidized amino acids in mitochondria, such as *o,o′*-dityrosine (see section 6), are recognized and proteolytically removed, after which they are released into the plasma and then excreted into the urine. One indication of this possibility is provided by a study of Guilivi and Davies [129].

These scientists found that one such oxidation product, *o,o′*-dityrosine produced in red blood cells, was released after being exposed to a continuous flux of hydrogen peroxide [129]. The proteasome macroxyproteinase appears to be responsible for *o,o′*-dityrosine release during the selective degradation of oxidatively modified proteins [129]. We will now discuss how to monitor specific unnatural amino acids as markers for protein oxidation and turnover.

We have used gas chromatography and mass spectrometry (GC-MS) for the determination of unnatural oxidized amino acids, such as dityrosine. This approach could be very useful in monitoring protein oxidation and turnover. This analytical technique can detect trace amounts of unnatural oxidized amino acids, and it aids in identifying and elucidating the structure of unknown, new molecules. This approach can monitor stable end products such as *o*-tyrosine, *m*-tyrosine, *o,o′*-dityrosine, 3-chlorotyrosine and 3-nitrotyrosine using a combination of stable isotope dilution GC-MS [47,110—112,130].

Several oxidized amino acids and their oxidative pathways are depicted in Fig. 5. The hydroxyl radical (HO·) converts phenylalanine to *o*-tyrosine and *m*-tyrosine ([130]; Fig. 5). Another important free radical mechanism involves protein oxidation by tyrosyl radicals. Tyrosyl radicals can be generated by hydroxyl radicals, as well as by peroxidases and other heme proteins in the presence of hydrogen peroxide and tyrosine [130]. This reaction generates tyrosyl radicals which can cross-link tyrosine-residues on proteins to form protein-bound *o,o′*-dityrosine, an unnatural isomer and stable end-product of protein oxidation. Another potential mechanism for protein oxidation involves NO•, a long-lived radical that plays a critical role in cellular signaling and cytotoxic host defense mechanisms [131]. The interaction of NO• with superoxide yields peroxynitrite. Protonated peroxynitrite rapidly decomposes to generate several other reactive nitrogen species, including the nitronium ion (NO_2^+) and nitrogen dioxide ($NO_2^{\cdot}$) [132]. Reactive

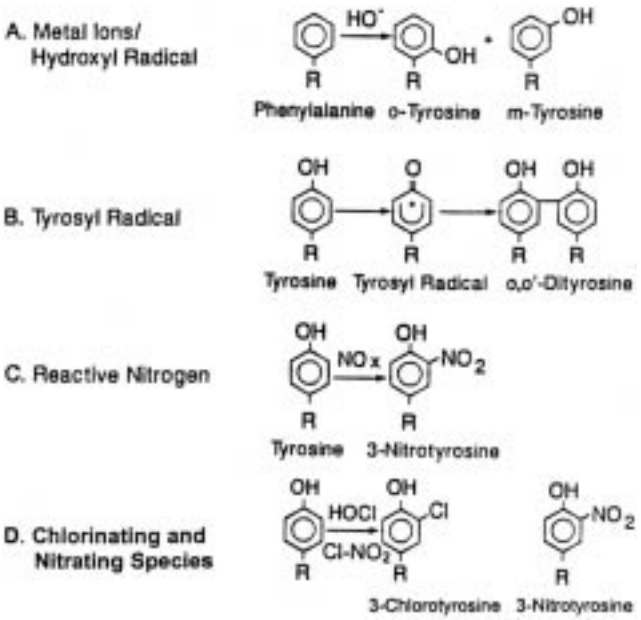

Fig. 5. Formation of unnatural amino acids. Reaction pathways for the oxidation of protein-bound aromatic amino acids by (**A**) hydroxyl radical ($HO^{\bullet}$), (**B**) tyrosyl radical, (**C**) reactive nitrogen intermediates (NO_x), such as peroxynitrite, and (**D**) hypochlorous acid (HOCl) and the recent discovered gas nitryl chloride ($Cl\text{-}NO_2$). $HO^{\bullet}$ reacts with phenylalanine residues in proteins to generate *o*-tyrosine and *m*-tyrosine (**A**), unnatural tyrosine isomers. Following a hydrogen abstraction, tyrosine becomes a highly reactive tyrosyl radical (**B**). Two tyrosyl radicals can cross-link to generate *o,o′*-dityrosine (**B**). The interaction of nitric oxide ($NO^{\bullet}$) with superoxide yields peroxynitrite (ONOO-). Protonated peroxynitrite rapidly decomposes to generate several other reactive nitrogen species, including the nitronium ion (NO_2^+) and nitrogen dioxide ($NO_2^{\bullet}$) to generate 3-nitrotyrosine (C). Hypochlorous acid generates 3-chlorotyrosine and nitryl chloride ($Cl\text{-}NO_2$) forms both 3-nitrotyrosine and 3-chlorotyrosine.

nitrogen species derived from NO● generate 3-nitrotyrosine in vitro and in vivo (Fig. 5). Moreover, scientists have recently shown that nitrite and HOCl present in physiological concentrations generate several nitrating and chlorinating species [11,12]. These in vitro studies demonstrate that the intermediate product nitryl chloride ($CL\text{-}NO_2$) may well be of greater physiological importance than peroxynitrite ($ONOO^-$) in protein modification [11,12]. Moreover, interactions of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite may also be relevant in oxidative injury [133,134] (Fig. 5).

It is unclear yet if the unnatural amino acids generated by these oxidative pathways could be incorporated into cytosolic or mitochondrial proteins and thus interfere with normal physiological functions. The structures of the unnatural amino acids are very similar to their native forms making this a strong possibility (Fig. 5). However, these products have been detected in biological tissues under normal and pathophysiological conditions. For example, Wells-Knecht et al.

found that there were no significant changes in levels of *o*-tyrosine in human eye lens with age. In contrast, there was a significant increase in dityrosine in lens proteins of approximately 33% between the age of 1 and 78 [135]. Also, Leeuwenburgh et al. found increases in skeletal muscle and heart dityrosine content in mice with age and showed that caloric restriction attenuated this increase [136].

3-nitrotyrosine has been detected in a variety of disease states such as cardiovascular disease and in neurodegenerative diseases [112—114,117]. 3-chlorotyrosine was detected by Hazen and Heinecke in human atherosclerotic lesions indicating that chlorinating reactions catalyzed by the myeloperoxidase system of phagocytes were one pathway for protein oxidation in vivo [137]. Thus, these specific products can be used as markers for specific oxidation pathways in vivo.

In a recent study, we supplemented rats with specific antioxidants and found a significant reduction of protein-bound *o,o′*-dityrosine in skeletal muscle by approximately 50%. In contrast, antioxidant therapy minimally affected *o*-tyrosine levels in this tissue. Levels of the oxidized amino acids in urine samples mirrored those of skeletal muscle proteins. The antioxidant therapy produced a striking 50% decline in *o,o′*-dityrosine levels but barely changed *o*-tyrosine levels. Quantification of the levels of oxidized amino acids in urine may thus serve as an integrated, noninvasive measure of oxidative stress in vivo because they change in parallel with levels of protein-bound oxidized amino acids in skeletal muscle (Fig. 4) [138].

6 PHYSIOLOGICAL RELEVANCE OF OXIDATION

Since oxidative stress has been shown to have important implications in the aging process, it is beneficial to understand the role oxidants play in several selected physiological processes. That is, how do reactive oxygen species modify the function of a biochemical system? and what mechanisms are involved in this change of function?

One interesting example concerns the effects of oxidation on cell surface receptors. Research on the transferrin receptor regulation process showed that free radical-induced oxidative stress generated by menadione, a known free radical inducer, was capable of rapidly downmodulating the membrane transferrin receptor due to blocking the recycling of the receptor on the cell surface. It is believed that the mechanism underlying such a change is related to the thiol group oxidation of cytoskeletal proteins and the disruption of calcium homeostasis, both of which are caused by menadione [139].

In another study on receptors, it was found that human erythrocyte insulin receptor processing was similarly impaired by menadione-induced oxidative stress. Insulin-induced downregulation is a model used to investigate cell surface regulative phenomena and how such processes are affected by the plasma membrane and the cytoskeleton. It was found that free radical-induced damage decreased the insulin-induced downregulation process, as measured by binding assays. In addition, there were slight alterations in the ultrastructure of the plas-

ma membrane due to oxidative damage, but there were significant alterations in the cytoskeletal protein assembly. It is hypothesized that modifications in specific cytoskeletal elements by oxidative stress could lead to the observed decrease in the insulin-induced downregulation process. This hypothesis is supported by the fact that there were changes in the electrophoretic pattern of cytoskeletal proteins like spectrin [140].

In another study, it was observed that oxidative-induced stress to K562 cells altered the microfilament system and the microtubular network. The researchers hypothesized that oxidative imbalance modified the cytoskeleton, leading to impairment of the expression of receptors [141]. These cases are theorized to be specific examples of a more general mechanism in which the expression of cell surface receptors and their recycling are impaired by oxidant-induced modification of the cytoskeleton and intracellular molecules.

Moreover, modification of tyrosine residues in receptor molecules has been shown to impair signaling pathways. For example, a specific modification, such as nitration of a tyrosine residue would compromise one of the most important mechanisms of cellular regulation, the cyclic interconversion between the phosphorylated and unphoshorylated form of tyrosine [142]. This possibility is underscored by the demonstration that nitration of tyrosine residues in model substrates prevents the phosphorylation of these residues by protein tyrosine kinases [143,144]. It is postulated that the nitration of tyrosine residues is an irreversible process and can, therefore, lock the enzyme into a relatively inactive form.

A specific example concerns the platelet-derived growth factor (PDGF) receptor. This receptor has five known tyrosine autophosphorylation sites. Mutations in specific tyrosine residues in the receptor — e.g., tyrosines 1009 and 1021 — prevent the binding and activation of phosopholipase C-γ (PLC-γ), an important signaling protein. If PLC-γ does not bind to the tyrosine residues, then the inositol phosopholipid signaling pathway is not activated [145]. Thus, a site-specific modification of a single amino acid by an oxidant could result in the decline of a protein's activity.

There are many other physiological effects of reactive oxygen species. For example, the free radical gas $NO^{\bullet}$ has been shown to have a wide variety of biological effects, including the ability to act as an inhibitor of mitochondrial electron transport [146]. Furthermore, $NO^{\bullet}$ binds reversibly to cytochrome oxidase and can completely inhibit mitochondrial oxygen consumption [146]. Another example concerns mitochondrial aconitase, a key enzyme in the citric acid cycle, which is a major target of superoxide and peroxynitrite mediated disruption of the (4Fe-4S) prosthetic group. This results in significant losses of aconitase activity [32—34]. Interestingly, aconitase activity declines as a function of age [147]. Therefore, oxidation may be a mechanism by which enzyme function declines with age.

7 LIFE-PROLONGING INTERVENTIONS

Since free radical reactions are postulated as a major cause of aging and degenerative diseases, much research has been done to develop life-prolonging interventions to slow down these chemical reactions. We will discuss several intervention therapies and their effect on attenuating oxidative stress and increasing life span.

7.1 Dietary restriction

Laboratory observations that rats on caloric-restricted diets live longer has been confirmed in a series of studies that show an inverse relationship between caloric intake and life span in mice. Restricting the caloric intake of rats by 30 to 60 percent has led to remarkable increases in the average life span and the maximal life span by similar amounts [148,149]. In one study, after decreasing the caloric intake of rats by 40% while maintaining normal levels of micronutrients, the average life span of the rats increased by 40% and the maximum life span by 49% [150].

In another study, groups of mice were fed different amounts of calories beginning one month after birth. There was a direct proportional relationship between the degree of caloric restriction (up to the point of frank starvation) and the increase in average and maximal life spans. Restricting calories in middle-aged mice (12 months) also resulted in an increased life span, but to a lesser degree. This finding refuted the contention that caloric restriction increased life spans by extending the developmental period [151].

A study was done comparing the concentrations of markers for oxidative damage (*o,o′*-dityrosine and *o*-tyrosine) between mice that had access to unlimited calories with those that were restricted to 60% of the caloric intake of the ad libitum regimen. In mice fed ad libitum, levels of *o,o′*-dityrosine increased with age in cardiac and skeletal muscle but not in liver or brain, and levels of *o*-tyrosine did not rise with age in any of the tissues examined. Caloric restriction prevented the increase in *o,o′*-dityrosine levels in cardiac and skeletal muscle but this did not influence *o*-tyrosine levels in any of the four tissues. Thus, caloric restriction was shown to attenuate an increase in certain aging markers vs. a regimen of ad libitum caloric intake [136].

Why does reducing caloric intake result in longer life spans and decreased oxidative damage? First, it is important to note that 2—3% of the oxygen consumed by mammals is believed to result in the generation of superoxide anion and H_2O_2. Since decreased food intake results in proportionally less oxygen being utilized in mitochondrial respiration, reducing caloric intake will also result in less free radical generation. In support of this theory, there have been repeated findings that dietary restriction depresses body temperature, and a decrease in body temperature indicates a reduced rate of oxygen consumption [152]. For example, in caloric restricted mice housed at room temperature (20—22°C), it was found

that their body temperature cycles from ~37°C to 23°C to 27°C daily [153]. The body temperature in caloric restricted rats also decreases, but by lesser amounts [154]. Moreover, a study by Lane et al. found that a 30% reduction in calories (compared with age-matched monkeys feeding ad libitum) decreased core (rectal) body temperature in rhesus monkeys by ~0.5°C [155].

Harman, who proposed the free radical theory of aging in 1954, believes that food restriction increases life span by decreasing free radical reaction initiation rates [156]. Since less free radicals are generated, there will be less injury to the mitochondria and their DNA, which overall should reduce aging and age-associated diseases. For example, one study on the effects of caloric restriction on the rates of mitochondrial superoxide anion generation and hydrogen peroxide production in the brains of mice showed that $O_2^{\cdot-}$ and H_2O_2 generation were reduced in the caloric restricted rats by 50% or more [157].

Alternatively, caloric restriction may cause more efficient electron transport chain coupling, resulting in less leakage of electrons and therefore a decreased generation of oxidants. Moreover, the beneficial effects of caloric restriction also involve increased antioxidant defenses. Lifelong caloric restriction increased the expression of SOD and Cat in liver tissue from 18-month-old rats; that is, there were higher mRNA levels and higher rates of nuclear transcription for SOD and Cat in the dietary restricted animals [158]. Similarly, another study found that dietary restriction (40% restriction of intake) in male Fischer F344 rats increased the activities of SOD by 24—38% and Cat by 64—75% in liver at 21 and 28 months of age. Also, at 28 months of age, GPX activity in the liver of caloric restricted rats was 37% higher than rats fed ad libitum [159]. These findings indicate an overall upregulation of antioxidant defenses.

Others believe that decreased food intake serves to lower the metabolic rate, which has been shown to be inversely proportional to life span in studies on mammals and cold-blooded animals. Two studies have found that the metabolic rate decreased in rhesus monkeys and rats after a lengthy period of caloric restriction [160, 161], but such findings were not confirmed in a third study [162]. In fact, in a study in which the metabolic rate of rats on an ad libitum diet was kept the same as those on restricted diets, the researchers found major increases in life span among the rats on a restricted diet without a decrease in metabolic rate [150,163].

Another hypothesis holds that caloric restriction increases longevity by slowing the rate of cell division in many tissues. Given that cancer is defined as the uncontrolled proliferation of cells, decreased cell division provides an explanation why the incidence of late-life lymphomas, breast, and prostate cancers is dramatically reduced in caloric restricted rats [28,164,165]. Alternatively, some argue that the longevity-increasing effects of caloric restriction are due to the fact that caloric restriction reduces glucose levels. Decreased blood glucose levels result in less sugar being accumulated on long-lived proteins, which reduces the overall deleterious buildup of glycoxidation products [166].

7.2 Chronic adapted exercise

Another potent life-prolonging intervention is regular physical exercise. Numerous studies have been done which show that regular exercise reduces aging changes and increases average life expectancy modestly (approximately 9% in rats) [167], but this leaves the maximum life span unchanged. On the other hand, acute exercise may result in increased oxidative stress since more O_2 is consumed. More oxygen consumption results in an increased generation of oxidants from electron leakage in the mitochondrial electron transport chain. In fact, overtraining is known to be injurious to organisms and can result in muscle damage, in the form of decreased mitochondrial respiratory control, increased levels of markers of protein oxidation, and the loss of the structural integrity of sarcoplasmic reticulum [168].

Protection from exercise-induced oxidative stress has been achieved in rats by diligent endurance training. Quintanilha and Packer, working with Bantin-Kingman female rats, found that endurance training raised the levels of antioxidant enzymes in both skeletal and cardiac muscle [169]. Leeuwenburgh et al. found that a 10-week exercise training program increased GPX and SOD activities in the deep portion of the deep vastus lateralis muscle (DVL) in young rats [170]. In another study, Leeuwenburgh et al. found that endurance trained rats had a 33% increased GSH content in DVL. They also found that trained rats had a 62% higher GPX activity and 27% higher SOD activity [171]. Therefore, although exercise results in increased oxidant levels, long-term exercise counters this effect by increasing the activity of antioxidants and antioxidant enzymes.

The following is a typical example of the moderating effects of physical exercise on aging. This study examined the effect of exercise on the tendons of male Sprague-Dawley rats, from 5 to 23 months of age. After training these rats in a treadmill, the researchers compared their tail tendons to those of a sedentary group. Overall, the tail tendon collagen of the exercise group had a lower thermal stability, and other biomechanical parameters with respect to maximum stress were also lowered in the trained group. Thus, it was concluded that physical exercise deterred aging effects on rat connective tissue [172].

Previous studies have found that male rats given access to voluntary wheel running showed an increase in average life span. However, since the male runners did not increase their food intake, it was not possible to determine whether the longevity-increasing effect was due to less energy being available for cell growth and proliferation or whether another exercise effect was involved. A study by Holloszy resolved the issue by studying female rats, who increase their caloric intake in response to wheel running. The female runners ate approximately 20% more (37% more from 5 to 10 months of age) than the sedentary rats, with both groups attaining similar peak body weights. The runners had a statistically significant increase in average life span without an increase in maximal life span, which shows that exercise can increase the average life span of rats independent of the concomitant decease in energy availability for cell activities [173].

7.3 Antioxidant therapy

Since free radical damage to biological molecules has been implicated as the primary cause of aging, many attempts have been made to attenuate free radical reactions through exogenous antioxidants. By abating these harmful reactions, scientists theorized that aging-associated diseases and the aging process itself would be slowed down, ultimately extending life span. Table 3 summarizes a selection of interventions and their effect on median and maximum life span [21,22,29,174—181] (Table 3).

Antioxidant enzymes, such as SOD, GPX, and Cat, have been shown to have higher levels of activity in longer lived strains of species. For example, such findings were obtained in studies of *Neurospora crassa* [178], *Drosophila melanogaster* [179], and *Caenorhabditis elegans* [180]. Therefore, genetically manipulating the antioxidant enzyme defense systems could prolong life (Table 3). Indeed, studies with *Drosophila* in which both SOD and Cat were overexpressed resulted in extension of the life span by a maximum of one-third and a reduction in protein oxidative damage [21,22]. Cat alone was ineffective in increasing life span [175].

Deprenyl, a MAO-B inhibitor, may be effective for creating symptoms of Parkinson's disease. Treatment of old male rats with deprenyl caused a significant increase in average life expectancy by 34% compared to saline-treated control animals (Table 3). This may be due to an upregulation of SOD and Cat activities, which may have provided protection of catecholaminergic neurons [176,177].

Table 3. The effect of antioxidants and antioxidant enzymes on median and maximum life span.

Antioxidants of diet (%)	Increase in life span (%)		Species
	Median	Maximum	
2-Mercaptoethylamine			
0.05%	12.8		Mice
1.00%	29.2		
Santoquin (0.5%)	18.1		Mice
Tocopherol-*p*-chloro-phenoxyacetate	13.0	13.0	Drosophila
d-Tocopherol	31.4	23.2	Nematodes
Vitamin E	16.8	15.4	Rotifer
Sulfhydryl agent	28.0		Rotifer
N-acetylcysteine		26.6	Drosophila
Antioxidant Enzymes			
Catalase only	No change		Drosophila
Catalase and Superoxide Dismutase	33.0+		Drosphilia
Deprenyl	34.0+[a]		Rats

[a]Deprenyl induces SOD and Cat antioxidant enzymes activity; 34.0% mean increase in [176,177] life span + specific strain maximum. (Adapted from [29,21,22,174—181].)

7.3.1 Natural and synthetic antioxidants

Besides antioxidant enzymes, there are a large number of antioxidant compounds that attenuate free radical reactions by breaking propagation chain reactions. The results, however, of these compounds are difficult to interpret since some of the antioxidants have unknown fates and perhaps potentially adverse effects.

Many antioxidant compounds have been shown to increase the average or mean life span of an organism. For example, in a study on the effect of adding *N*-acetylcysteine (NAC, an antioxidant and cysteine source to increase GSH levels) to the diet of *Drosophila melanogaster,* it was found that NAC results in a dose-dependent increase in median and maximum life span. Flies fed 1 mg/ml NAC food live 16.6% longer; those fed with 10 mg/ml NAC food live 26.6% longer [181].

Also, a study by Harman on vitamins C and E found that vitamin C did have some life-extending effects, and vitamin E produced a marginal increase on median life span with no influence in maximum life span [182]. Other studies using synthetic antioxidants achieved similar results: a synthetic antioxidant, butyl hydroxytoluene (BHT) was examined by Clapp et al. [183]. These researchers found that both sexes of BALB/c mice receiving 0.75% by weight BHT showed extended life spans. However, BHT at levels above 0.5% by weight have been shown to be toxic because at this level BHT adversely interferes with mitochondrial respiration [184]. A study examining a free radical scavenger, thiazolidine-4-carboxylic acid, found that intake at concentrations greater than 400 μM resulted in a significant increase in the median life span, but not the maximum life span. And Harman found in 1968 that adding 1.00% by weight of 2-mercaptoethylamine to the diet of LAF_1 mice, shortly after they were weaned, resulted in increases in the average life span by 29.2%; on average these mice lived to 31.6 months vs. the previous average of 24.5 months [185].

Unfortunately, some of these studies have a shortcoming in that they lack food intake data. Without monitoring food intake, some of the increases in life span may have been due to the effect of caloric restriction and not that of the antioxidant. In studies where food intake was monitored, there was an associated decrease in body weight of up to 10—15% with the intake of various antioxidants [185,186]. Why antioxidants have this effect is not clear: some hypothesize that antioxidants adversely effect mitochondrial function, decreasing ATP formation by uncoupling aspects of mitochondrial respiration. Perhaps under these circumstances both body weight and superoxide anion and hydrogen peroxide levels will be decreased.

Regarding mice, there are a number of antioxidant compounds that will increase the average life span; for example, 2-mercaptoethylamine [185] and ethoxyquin [186]. However, there are only three known compounds that have been shown to increase the maximum life span in mice; they are 2-mercaptoethanol (2-ME) [187], and two pyridine derivatives [188,189]. The study on 2-ME by Heidrick et al. is the only study of the three that carefully monitored body weight

and food intake. It showed that dietary supplementation at the level of 0.25% by weight of 2-ME increases average life span by 13% and maximum life span by 12%, without reducing caloric intake. Also, the total incidence of tumors was decreased 29% in the group taking 2-ME [187]. In contrast to mice, antioxidant supplementation increased mean, as well as maximum life spans in *Drosophila* [190,191] and nematodes [192].

However, why do so few antioxidants fail to raise the maximum life span in mouse models? Many scientists believe that these antioxidants are not added in significant concentrations to slow down the free radical damage to mitochondria or perhaps are not added in optimal combinations.

In addition to natural and synthetic antioxidants, there are a number of pharmacological spin traps, which can serve as antioxidants. Spin traps are nitrones or nitroso compounds that react with free radicals to form stable nitroxides, which are then quickly reduced to hydroxylamines (Fig. 6).

Nitroxide derivatives of spin traps such as *N-tert*-butyl-α-phenylnitrone (PBN) have been shown to have dramatic anti-aging effects and serve as excellent inhibitors of free radical reactions (Fig. 7). In one study on protein oxidation, old gerbils were given twice-daily intraperitoneal injections of PBN over a 2-week period. Their performance in running a radial-arm maze test was compared with that of young gerbils; at 32 mg/kg PBN the old gerbils performed nearly as well as the young gerbils. This result was accompanied by reversed age-related oxidative damage: levels of protein carbonyls dropped, glutamine synthetase and neutral protease activity increased, and unoxidized to oxidized ratios of proteins rose. Most impressive was the pronounced effect PBN had on the short-term memory of the older gerbils: untreated older gerbils made twice as many short-term memory errors as young gerbils, but treated older gerbils performed almost as well as the younger gerbils. The effect was also reversible: after stopping the treatment, glutamine synthetase and neutral protease activity fell and oxidation of proteins increased [193]. It is believed that these pronounced effects are due to the free radical scavenging effect of PBN and the inhibitory effect PBN has on the initiation of free radical reactions (Fig. 7).

Two subsequent studies with PBN were unable to confirm such results [194,195], but a third study did partially confirm the results of Carney et al.

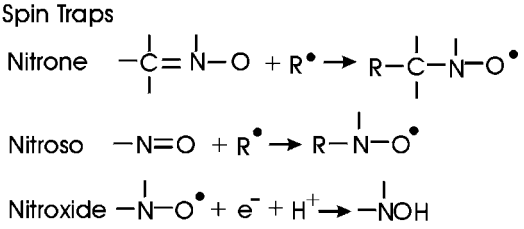

Fig. 6. Nitroso compounds and nitrones can react with free radicals to form nitroxides, which subsequently can be reduced to hydroxylamines [24].

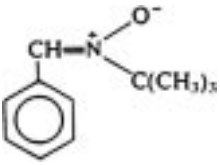

Fig. 7. N-tert-butyl-α-phenylnitrone (PBN) [265].

These scientists confirmed that protein carbonyl content decreased in gerbil brain cortex, but similar effects were not observed in the gerbil heart or the mouse brain cortex. Thus, the effects of PBN on protein carbonyls are variable depending upon tissue and species [196]. PBN has also been found to reverse age-related cognitive deficits in aged rats [197]. Lastly, it was found that daily intraperitoneal injections of 30 mg/kg of PBN in a strain of senescence-accelerated mice (SAM-P8) dramatically increased their mean life span: the control mice had a 50% mean survival rate of 42 weeks, but the PBN group's mean survival rate was increased to 56 weeks [198].

8 ANTIOXIDANTS IN HUMAN HEALTH AND DISEASE

Free radical damage to biological systems have been implicated in a large number of aging-associated diseases, from eye disorders such as cataracts and retrolental fibroplasia to cardiovascular disease and diabetes. Since there is such a myriad of aging-associated diseases, this review will limit its focus to cardiovascular disease and neurodegenerative diseases and the effect of antioxidant therapy.

8.1 Cardiovascular disease

Since cardiovascular disease is the primary cause of death in the USA, it is of chief importance to understand the etiology of atherosclerosis. Atherosclerosis is a disease of the arteries in which the innermost parts of the vessels, the intima, thicken. One of the primary types of thickening consists of fatty, slightly raised, narrow, yellow streaks. These streaks are rich in foam cells, which are distorted cells with a high lipid concentration that come from endogenous smooth muscle cells and from macrophages. These fatty streaks are likely the precursors of fibrous plaques, which have the effect of obstructing the arterial lumina. These plaques are also composed of various cellular debris, cholesterol crystals, and lipid deposits. They cause disease by limiting blood flow to the body organs. Heart attacks (myocardial infarction) and strokes (cerebral ischaemia) result when an arterial lumen is totally occluded, often with a thrombus forming at the plaque site.

What role does cholesterol play in developing atherosclerosis? The leading theory holds that the vascular endothelium produces lesions when damaged by mechanical and chemical reactions. Localized injured areas have increased

permeability, resulting in a localized increase in the subendothelial space in concentrations of serum components such as LDL. With increased serum concentrations, more monocytes will attach to the subendothelial space, infiltrate, and develop into macrophages. Activated monocytes and macrophages cause damage to the endothelial cells by secreting $O_2^{\cdot-}$, H_2O_2, HOCl, $NO^{\cdot}$ and hydrolytic enzymes. Other damaging compounds are continuously being produced in the subendothelial space by the reaction of O_2 with polyunsaturated substances present there [199–201].

Macrophages normally possess some LDL receptors, but if the LDL is peroxidized then the LDL is recognized by another type of receptor on the macrophage, the acetyl-LDL receptors or scavenger receptors. When bound to these receptors, LDL is rapidly engulfed by the macrophages resulting in a high intracellular concentration of cholesterol, which tends to convert the macrophage into a foam cell. In addition, since macrophages, smooth muscle cells, and arterial endothelial cells are known to oxidize LDL, this process of macrophage ingurgitation is increased [201].

Since oxidants have been implicated as a major cause of atherogenesis, it is reasonable to conclude that therapies which decrease the oxidation rate of serum components and vessel wall lipids and/or the permeability of the endothelial space should have a beneficial effect. Numerous studies have been conducted to test if the above hypothesis is correct, and much work is currently being done in this area. A few of the more significant and interesting results are reviewed below.

For example, a study on vitamin E supplementation found that men and women who supplemented their diets with 100 mg of vitamin E for two years or more had approximately a 40% decrease in the risk of coronary heart disease [202,203]. A study on vitamin C (ascorbate) found that human vascular endothelial cells, enriched with vitamin C, lowered their ability to modify LDL. The data also showed that extracellular vitamin C strongly inhibited metal ion-dependent, endothelial cell mediated atherogenic modification of LDL [204]. Vitamin C may also be important in the health of blood vessels due to its interaction with $NO^{\cdot}$. $NO^{\cdot}$ has been shown to have vasodilatory effects, but it may be inactivated by reactive oxygen species such as superoxide radicals. Researchers have found that in essential hypertensive patients, impaired dilation of the blood vessels was significantly improved by vitamin C. Interestingly, this effect was reversed by N^G–monomethyl-L-arginine, a potent inhibitor of nitric oxide synthase [205] (Fig. 8).

Another example concerns the drug probucol; it is used clinically to lower blood cholesterol levels, but it is also a potent antioxidant. In a recent experiment, atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits regressed when treated with probucol, and this anti-atherogenic effect was far greater than expected merely from its cholesterol-lowering capability. Thus, the researchers suggested that its antioxidant properties contributed to its anti-atherogenic effect [206]. A study devoted to determining if probucol would decrease vascular $O_2^{\cdot-}$ production confirmed that it does decrease vascular

superoxide production in cholesterol-fed rabbits. Probucol treatment, in cholesterol-fed rabbits, normalized both O_2^{-} production and endothelium-dependent relaxation to acetylcholine. Thus, the researchers suggested that probucol may prevent O_2^{-} induced inactivation of endothelium-derived NO· [207].

Another recent study done on antioxidants and their anti-atherogenic effects concerns the Chinese herb, Salvia miltiorrhiza Bunge (SMB), which is widely used for the treatment of atherosclerosis-related disorders. The water-soluble polyphenolic antioxidant, Salvianolic acid B (Sal B), was isolated from the roots of this plant and was found to scavenge 1,1-diphenyl-2-picrylhydrazyl radicals and inhibit LDL oxidation more effectively than probucol. In an experiment using rabbits fed on a high cholesterol diet, endothelial damage at 6 weeks was found to be reduced by 53% in the SMB group. "SMB treatment also reduced the atherosclerotic area in the abdominal aorta significantly by 56% and cholesterol deposition in the thoracic aorta by 50%" [208].

Lastly, very exciting results have been obtained in rabbits undergoing diet supplementation with flaxseed oil. Flaxseed is a rich source of omega-3 fatty acids and lignans and is known to have antioxidant effects since it suppresses the production of oxygen free radicals by PMNLs and monocytes. Flax seed also supresses interleukin-1 (IL-1), tumor necrosis factor (TNF), leukotriene B4 (LTB4), and platelet activating factor (PAF). Since these compounds are known to stimulate PMNLs to produce oxygen free radicals, suppressing their production will also decrease levels of free radicals. In a well-designed experiment, flaxseed (type I flaxseed) reduced hypercholesterolemic atherosclerosis by 46% without significantly lowering serum lipids [209]. In a follow-up study on type II flaxseed (2—3% α-linolenic acid), it was found that this type of flaxseed reduced the development of atherosclerotic plaque by 69% [210].

8.2 Neurodegenerative diseases

Since the brain is one of the body's most metabolically active organs — consuming oxygen at a rate of 35 ml/min/kg, compared to the heart's oxygen consumption rate of 59 ml/min/kg [211,212], it is highly susceptible to damage from free radical processes. The brain is also unique in that it generates oxidants in ways that are foreign to other body systems. For example, metabolism of excitatory amino acids and neurotransmitters generates reactive oxygen species, and the constant use of oxygen by neural mitochondria results in high superoxide levels by processes dis-

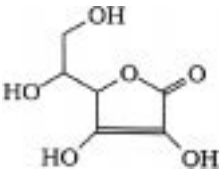

Fig. 8. Vitamin C [265].

cussed earlier in this paper [213]. Recently, it has been found that endogenous guanidino compounds like guanidinoglutaric acid can form highly reactive species such as superoxide and hydroxyl radical in aqueous solution [214].

The brain also appears to be particularly vulnerable to free radical damage because it lacks or has localized many of the antioxidant enzymes. For example, SOD is localized primarily in neurons [215], and GSH and GPX are localized in astrocytes [216,217]. Also, there appears to be very little activity of Cat in the brain [218].

The hypothesis that age-associated, chronic neurodegenerative diseases like Alzheimer's disease and Parkinson's disease are related to oxidative stress is well-supported by many findings. First, there are numerous reports in the literature that patients with neurodegenerative diseases have damaged mitochondria coupled with high levels of oxidative damage [219—221]. Second, there are reports that in the cerebrospinal fluid there is free, nonprotein bound Fe^{3+} [222]. The brain normally has particularly high levels of iron in the globus pallidus and substantia nigra [223,224], which is believed to be due to the necessity of iron for the correct binding of neurotransmitters and receptors. However, in Alzheimer's disease there are increased neuronal iron concentrations due to increased neurofibrillary tangles [225]. In Parkinson's disease, it was discovered that the total iron content of the substantia nigra was 77% higher than other brain regions [226]. These findings are significant because nonprotein bound Fe^{3+}, through the Haber-Weiss reaction, can be reduced by superoxide to become Fe^{2+}. Fe^{2+} can then, in turn, react with hydrogen peroxide to form the highly reactive hydroxyl radical. These effects are believed to contribute to high levels of oxidative stress and the concomitant rapid peroxidation of highly abundant unsaturated brain lipids [227,228].

8.2.1 Alzheimer's disease

The role reactive oxygen species play in the etiology of Alzheimer's disease is currently an area of intense research. It is well-known that Alzheimer's disease is clinically associated with the development of amyloid plaques. It is believed that these plaques are caused by the inproper folding and processing of amyloid β-precursor protein (AβPP). Aggregation of AβPP may involve free radicals [229], and it was found that AβPP can itself generate peptidyl free radicals [230]. These findings are supported by the discovery that the synthetic, in vitro formation of amyloid plaques from AβPP can be accelerated by the presence of oxygen; in fact, these amyloid plaques themselves appear to stimulate the production of reactive oxygen species [231].

At this point in time, much work is being done in developing antioxidant therapies for Alzheimer's disease. For example, researchers discovered that melatonin can protect neurons against AβPP toxicity and resistance to proteolysis. It was found that melatonin interacts with Aβ-40 and Aβ-42 to inhibit the formation of β-sheets and amyloid fibrils, the formation of which determines the toxicity

and proteolytic resistance of AβPP [232]. Researchers in Germany are examining the protective effects of vitamin E and estrogen to limit oxidative stress in the long run [233]. Also under investigation in idebenone, a synthetic free radical scavenger which traps electrons, lazaroids (21-aminosteroids), pyrrolpyrimidines, NO· blockers, selegiline [234], and α-lipoic acid [213].

There is also evidence that extracts of ginkgo biloba are useful in the treatment of Alzheimer's disease. It has previously been demonstrated that extracts of ginkgo biloba confer give protection for neuronal cells against conditions of impaired oxidative phosphorylation [235]. This herb also can act as a free radical scavenger [236], and it has the capability to prevent peroxidation of lipid membranes by oxidants [237]. Recently, researchers in Germany conducted a clinical, double-blind, placebo-controlled study on 20 outpatients given oral doses of 240 mg/day of a specially manufactured ginkgo biloba extract, EGb 761, for three months. The purpose was to determine if the extract could stabilize cognitive performance or delay the progression of Alzheimer's related dementia. Using various psychometric tests, there was found to be a statistically significant improvement between the baseline values and final values for these tests among the group being actively treated with EGb 761 [238]. Another study on EGb 761 achieved similar results: in this year-long study, one group of Alzheimer patients took 40 mg of EGb 761 three times a day, while a second group of patients received a placebo. On the "Geriatric Evaluation by Relative's Rating Instrument" test, the ginkgo group improved daily living and social behavior by 37%, compared to 23% by those in the placebo group. Also, over the course of the study, the condition of only 19% of the ginkgo group patients deteriorated, compared to 40% of the placebo group. In conclusion, the researchers summarized that EGb 761 could stabilize and, in some cases, improve the cognitive performance and the social functioning of Alzheimer patients for 6 months to 1 year [239].

8.2.2 Parkinson's disease

Dopaminergic neurons are uniquely vulnerable to damage and disease. Their loss in humans is associated with diseases of the aged, most notably, Parkinson's disease. Parkinson's disease involves the loss of dopaminergic neurons, especially in the midbrain area called the substantia nigra. Oxidative stress is known to play a major role in the destruction of these neurons. For example, since Fe^{+3} is increased in the substantia nigra [226] and hydrogen peroxide is also produced during dopamine metabolism in the dopaminergic neurons [240], there is the possibility of Fe^{3+} catalyzed production of hydroxyl radicals which will result in significant damage to these neurons. Hydroxyl radical production is also increased when the mitochondrial respiratory chain dysfunctions, as has been found in diverse tissues of Parkinson's patients [241]. Other free radicals are generated when dopamine undergoes autoxidation or is enzymatically oxidized by monoamide oxidase [242]. Hydrogen peroxide produced in such reactions could then react with the free iron to form hydroxyl radicals, which react to damage

biomolecules and could lead to the loss of dopaminergic neurons. Another source of neurotoxicity may be the release of copper ions in the presence of L-DOPA; it has been demonstrated that L-DOPA and dopamine can damage DNA by oxidizing it in the presence of copper ions and hydrogen peroxide [243]. Furthermore, research has shown that there is an increased production of superoxide in the mitochondria of the substantia nigra along with increased levels of activity of SOD [244]. In fact, the severity of Parkinson's symptoms has been correlated with the degree to which malondialdehyde [245] and hydroperoxides are increased and GSH levels are decreased [246,247].

Moreover, many studies have been done on the toxic metabolite 1-methyl-phenyl pyridium (MPP+) of the neurotoxin 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP). In monkeys and other animal models, MPP+ induced Parkinson's disease-like symptoms and caused neuronal loss in the substantia nigra [248,249]. It was also found that MPP+ inhibits the mitochondria's respiratory chain complex I [250,251]. This intensively researched animal model for Parkinson's disease is being actively used to examine the role oxidants play in MPTP toxicity.

The loss of the dopaminergic neurons results in decreased dopamine production in various regions of the brain, including the cortex, nucleus accumbens, striatum, and thalamus [252]. Since these areas are involved in controlling voluntary movement, loss of dopamine results in improper signaling which causes the characteristic clinical symptoms: jerky movements, trembling of the hands and lips, muscle rigidity, body tremors, a shuffling gait, and eventually loss of the ability to control voluntary movements [253].

Many of the antioxidant therapies employed in the treatment of Alzheimer's disease are also being used to treat Parkinson's disease, e.g., melatonin. Low doses of 6-hydroxydopamine (6-OHDA) are known to induce apoptosis of undifferentiated and differentiated PC12 cells by generating free radicals, and this system has been proposed as an experimental model for Parkinson's disease. Significantly, melatonin was found to prevent apoptosis by 6-OHDA in neuronal cells, perhaps by scavenging free radicals and increasing the mRNA levels and the activity of antioxidant enzymes [254]. Melatonin and its precursor, *N*-acetylserotonin (normelatonin), have also been found to protect human neuroblastoma SK-N-MC cells and primary cerebellar granular neurons against oxidative stress [255]. Another study investigated the effects of melatonin on rescuing dying cells (100% tau+ neurons), including tyrosine hydroxylase immunopositive dopamine neurons. Apotosis was prevented in these cell lines, and this effect was dose and time dependent and was mimicked by other antioxidants such as 2-iodomelatonin and vitamin E. Melatonin also prevented the usual 50% loss of dopamine neurons caused by neurotoxic injury induced by 1-methyl-4-phenylpyridine. These remarkable results indicate that melatonin possesses a tremendous ability to rescue neurons from cell death [256] (Fig. 9).

Studies using other experimental models of Parkinson's disease found that inhibition of monoamine oxidase B (MAO-B), the enzyme responsible for the oxidation of dopamine, reduces oxidative stress on the dopaminergic neurons and

can prevent neuronal degeneration [257]. In mutant mice lacking the gene for MAO-B, researchers found that these mice were resistant to the neurodegenerative effects of MPTP [258]. Moreover, in a double-blind study using selegiline (deprenyl) to inhibit MAO-B, it was established that selegiline can delay the need to be treated with L-DOPA by 10 months [259]. More recent studies have confirmed this antioxidant effect. For example, a study examining the effect of tocopherol and deprenyl on the progression of Parkinson's disease discovered that deprenyl (10 mg per day) but not tocopherol (2000 IU per day) delays the onset of disability associated with early Parkinson's disease [260].

Also under much investigation are thiol antioxidants, such as GSH, *N*-acetylcysteine (NAC), and dithiothreitol (DTT). These antioxidants and metal chelators, along with vitamin C, have been shown to prevent dopamine autooxidation; they also act to inhibit dopamine-induced apoptosis [261]. Other potential antioxidant treatments include α-lipoic acid [213], lazaroids [262], bromocriptine [263], and estrogen. Recently, estrogen replacement therapy has been shown to be protective against the development of Parkinson's disease-associated dementia [264] (Fig. 10).

9 SUMMARY

1. Major discoveries in free radical biology have been made in the past few decades substantiating the free radical theory of aging. Moreover, a large amount of scientific papers and a multitude of scientific disciplines have established a strong relationship between free radical biology and pathophysiology.
2. We discussed several major sources of free radical oxidant production in vivo and their relationship to aging and several degenerative diseases of aging. The mitochondria and phagocytes are potent sources of oxidant formation and may cause damage to DNA, lipids, and proteins generating unnatural structures.
3. Broadly, the altered biomolecules may play a critical role in the development of degenerative diseases and the aging process itself. Specific protein modifications can alter enzyme function or cell signaling ability. Furthermore, certain altered biomolecules that accumulate can affect protein function.
4. Scientific evidence has shown that several interventions such as dietary restriction, chronic exercise, and antioxidant therapies can attenuate the aging process and disease states.

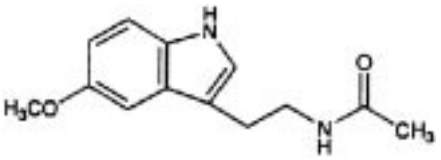

Fig. 9. Melatonin [265].

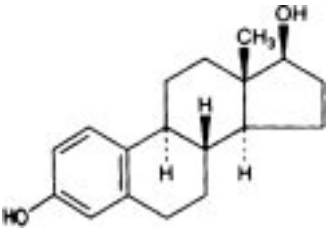

Fig. 10. Estradiol [265].

5. Therapies have been developed to reduce oxidative stress and therefore protect against the development of degenerative diseases such as atherosclerosis, Alzheimer's disease, and Parkinson's disease.

10 PERSPECTIVE

Aging has a strong genetic component, but it can be viewed partly as a degenerative process dictated by free radical reactions, possibly increasing the incidence of several degenerative disease states. The exact interrelationship is unclear, but there is a weighty amount of evidence that free radicals are involved. Free radicals increase with age, alter physiological function, increase disease states and accelerate pathophysiological conditions. This review describes a multitude of studies which clearly show the tremendous power of certain antioxidant interventions and other scientific advances in free radical biology, which will surely continue. However, the difficulties in preventing oxidative damage in vivo partly stem from our lack of understanding which reactive oxidants are active during the aging process and what specific antioxidant therapy provides optimal protection.

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12 ABBREVIATIONS

SOD: superoxide dismutase
Cat: catalase
GPX: glutathione peroxidase
NO·: nitric oxide
$ONOO^-$: peroxynitrite
NO_2^+: nitronium ion
$NO_2^\bullet$: nitrogen dioxide
$Cl\text{-}NO_2$: nitryl chloride

HO·: hydroxyl radical
PBN: *N-tert*-butyl-α-phenylnitrone
GSH: glutathione
NAC: *N*-acetyl-cysteine
DTT: dithiothreitol
MAO-B: monoamine oxidase B
6-OHDA: 6-hydroxydopamine
MPTP: 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine
MPP+: 1-methyl-4-phenyl pyridium
AβPP: β-precursor protein
PMNL: polymorphonuclear leukocytes
LDL: low-density lipoproteins
SAM-P8: senescence-accelerated mice
2-ME: 2-mercaptoethanol
BHT: butyl hydroxytoluene
PDGF: platelet-derived growth factor
HOCl: hypochlorous acid
mtDNA: mitochondrial DNA
$O_2^{\cdot-}$: superoxide
H_2O_2: hydrogen peroxide
GC-MS: gas chromatography and mass spectrometry

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