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ELEVATED OXIDATIVE STRESS IN MODELS OF NORMAL BRAIN AGING AND ALZHEIMER'S DISEASE

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Summary

Age-associated neurodegenerative disorders are becoming more prevalent as the mean age of the population increases in the United States over the next few decades. Both normal brain aging and Alzheimer's disease (AD) are associated with oxidative stress. Our laboratory has used a wide variety of physical and biochemical methods to investigate free radical oxidative stress in several models of aging and AD. β-amyloid (Aβ), the peptide that constitutes the central core of senile plaques in AD brain, is associated with free radical oxidative stress and is toxic to neurons. This review summarizes some of our studies in aging and Aβ-associated free radical oxidative stress and on the modulating effects of free radical scavengers on neocortical synaptosomal membrane damage found in aging and Aβ-treated systems.

Key Words: protein oxidation, free radicals, lipid peroxidation, hydroxynonenal, β-amyloid, aging, Alzheimer's disease

Aging and age-related neurodegenerative disorders, especially Alzheimer's disease (AD), Parkinson's disease, and stroke, are major health problems globally. Several hypotheses have been proffered as a molecular basis for the changes which occur in brain due to aging and AD. One of the most compelling is the role of free radical-induced oxidative stress in these disorders (1-5). The dominant risk factor for AD is age; hence, it is critical to learn more of the free radical involvement in aging and age-related neurodegenerative disorders. The continuous generation of reactive oxygen species (ROS), an inescapable cellular sequelae of life in an aerobic environment, causes cumulative damage to key cellular components and eventually leads to age-related pathology. This is the essential basis of the free radical theory of aging (1).

Membrane and cytosolic proteins, along with bilayer lipids, are primary cellular components susceptible to free radical oxidation in brain cells. This review summarizes some of our studies on protein and/or lipid oxidation and their involvement in aging and AD.

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Sources of Free Radicals: Stadtman (3) and Butterfield and Stadtman (6) have reviewed the sources of free radicals for protein oxidation and mechanisms for protein oxidation. Reactive oxygen and reactive nitrogen species, including •OH, $O_2\bullet^-$, $HO_2\bullet$, NO•, among others, as well as several non-radical moieties (O_2, $ONOO^-$, H_2O_2, HOCl, O_3, among others, can be deleterious to cells. These reactive species can be generated *in vivo* from the following reactions:

H_2O_2 + Fe(II) or Cu(I) ----> •OH + OH^- + Fe(III) or Cu(II) (1)

HONOO ----> •OH + $NO_2\bullet$ (2)

Protein-H + O_3 ----> •OH + Protein + O_2 (3)

The H_2O_2, needed to fuel the Fenton reaction, is derived mainly by the dismutation of superoxide anion radical ($O_2\bullet^-$).

$O_2\bullet^-$ + $2H^+$ ----> H_2O_2 + O_2 (4)

$O_2\bullet^-$ for this reaction is formed mainly as a by-product of normal electron transport processes--especially, the transport of electrons to cytochrome C by mitochondria. $O_2\bullet^-$ and H_2O_2 are also formed by mixed-function oxidation systems, by neutrophils and macrophages that undergo an oxidative burst, and are normal products or reactions catalyzed by oxidases (3,6). Peroxynitrite ($ONOO^-$) is produced endogenously by the interaction of NO• with $O_2\bullet^-$, at diffusion-limited reaction rates, to produce a highly toxic species from two relatively benign free radicals. Nitration of tyrosine or methionine by $ONOO^-$, with the latter dependent on the CO_2 partial pressure (7), is one means to induce altered function in proteins and cells. Peroxynitrite-induced nitration of tyrosine residues raises the possibility that nitration could seriously compromise one of the most important mechanisms of metabolic control; to wit, the interconversion of tyrosine residues of regulatory proteins between phosphorylated and non-phosphorylated forms or between nucleotidylated and unmodified forms (3,6). Nitration of tyrosine residues is *irreversible*; hence peroxynitrite effects can put regulatory proteins into a permanently "ON" or "OFF" configuration and thus adversely affect signal transduction pathways of cellular regulation.

As discussed below, protein-specific spin labeling can be used to monitor oxidative stress and changes in membrane protein conformation. Peroxynitrite added to neocortical synaptosomal membranes causes oxidative stress and alters protein conformation. Both effects are blocked by the antioxidant· glutathione (data not shown), consistent with peroxynitite-induced neurotoxicity.

Reactive carbonyl groups on proteins can be used as a key marker of ROS-mediated protein damage (3,6). Several sensitive methods for the detection and quantitation of protein carbonyl groups have been used in aging research. For example, spectrophotometric analysis of the 2,4-dinitrophenylhydrazone derivatives of protein carbonyls (5,8,9), fluorometric measurements of their fluoresceinamine derivatives (10), fluorescence microscopic image analysis of biotin-conjugate derivatives after treatment with fluorescein-labeled streptavidin (11), radioactivity measurements after reduction to hydroxy derivatives with sodium borotritide (12), or by Western blotting analysis of their 2,4-dinitrophenylhydrazone derivatives using 2,4-DNPH-protein specific antibodies (9), have all been used to show increased protein carbonyls following oxidative stress. EPR spin labeling techniques using protein-specific spin labels also have been used to investigate protein oxidation (13). The protein-specific spin label, 2,2,6,6-tetramethyl-4-maleimido-piperidine-1-oxyl [MAL-6] (14,15), forms covalent bonds with

cysteine SH groups, and in most biological systems is the only source of paramagnetism. A typical EPR spectrum of MAL-6 attached to membrane proteins in cortical synaptosomes is shown in Figure 1.

This spectrum reflects at least two sites of MAL-6 binding to membrane proteins, characterized by the motion of this spin label, i.e., strongly- and weakly-immobilized reaction sites. The relevant EPR parameter to assess structural information is the ratio of the spectral amplitudes of the $M_I = +1$, low-field weakly (W)- to strongly (S)- immobilized MAL-6 reaction sites. This W/S ratio is

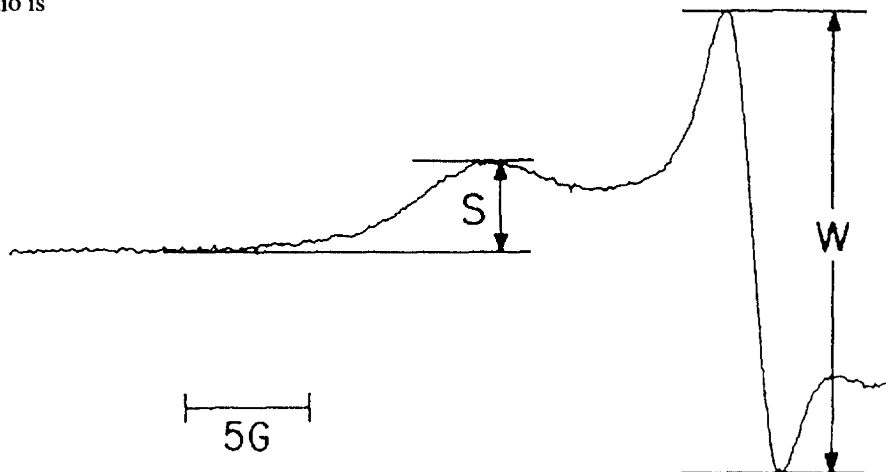

Fig. 1

Typical EPR spectrum of MAL-6 covalently bound to membrane proteins in neocortical synaptosomes. Presented are the low-field resonance lines showing the W and S amplitudes from which is calculated the W/S ratio, a parameter reflective of protein conformation and sensitive to oxidative stress.

highly sensitive to molecular motion, and decreased W/S ratios of MAL-6 are found following slowing of the spin label motion (14,15). For example, conformational alterations in the proteins, decreased segmental motion of spin labeled protein domains, or increased protein-protein interaction lead to decreased W/S ratios. Hydroxyl free radical modification of synaptosomal membrane proteins via Fenton chemistry leads to significantly decreased W/S ratios of MAL-6, implying greater motional resistance of this protein-specific spin label. This is due to increased steric hindrance resulting from increased protein-protein interactions or changes in conformation of membrane proteins (13). Other conditions of oxidative stress also lead to decreased W/S ratio of this spin label covalently attached to cortical synaptosomal membranes (Table I).

Table I

The W/S ratio of MAL-6 Covalently Bound to Membrane Proteins is Decreased in Oxidative Stress Conditions

Oxidative Stress Condition	Relative Decrease in W/S Ration (% Control)	Reference
Fenton Chemistry, •OH	60-85	[13]
Ischemina/Reperfusion Injury (Stroke)	80-90	[16-19]
Menadione Treatment	80-90	[20]
Lipopolysacchaide	80-90	[21]
Alzheimer's Disease	60-75	[22]

Membrane proteins are not the only target for (ROS) free radical attack. The lipid bilayer of the membrane is also disrupted under conditions of oxidative stress. Free radical-induced, lipid peroxidation leads to lipid peroxides, and, importantly, to the highly reactive alkenal, 4-hydroxy-2-trans-nonenal (HNE). The latter molecule can react by Michael addition with important membrane proteins leading to their dysfunction (23). In our laboratory, brain membrane lipid peroxidation is assessed by three methods. EPR, in conjunction with lipid-specific spin labeling using 5- or 12-doxyl stearate, monitors lipid peroxidation via loss of signal intensity by reaction of the free radical with the unpaired electron on the spin label (24). Free fatty acid release from brain membranes following oxidative stress is a measure of lipid peroxidation (25), and the presence of either free or protein-bound HNE also indicates lipid peroxidation has occurred (26).

Protein Oxidation In Aging: Investigations involving several aging models have reported that intracellular proteins have increased protein carbonyl content, a key marker for protein oxidation, increasing exponentially as a function of age (3,5,6,27-31). Brain protein carbonyl levels have been correlated to deficits in cognitive and motor skills (32). These results are consistent with ROS oxidative stress in aging.

Using MAL-6, the W/S ratio of this protein-specific spin label was significantly decreased in synaptosomal membranes from aged gerbils and rats relative to young animals (33,34), consistent with free radical oxidation of membrane proteins (13,16-22). Hyperoxia has been considered a good model of aging. As in aging, the W/S ratio of MAL-6-labeled synaptosomal membrane proteins from hyperoxia-treated animals was decreased relative to normoxic controls (Table II) (33,34). Increased carbonyl content and decreased activity of the oxidatively-sensitive enzyme glutamine synthetase (GS) were also found (34).

Table II

Oxidative Stress Following Hyperoxia*

System	**W/S Ratio**	**GS Activity (units/mg min)**	**Protein Carbonyl Content (nmol/mg protein)**
Control	7.73 ± 0.20(11)	0.615 ± 0.07(11)	1.85 ± 0.08(6)
Hyperoxia	6.80 ± 0.18(11)[a]	0.440 ± 0.04(11)[c]	2.95 ± 0.19(6)[d]
PBN (20 mg/kg) + Hyperoxia	7.30 ± 0.40(5)[b]	0.560 ± 0.05(6)[b]	(not determined)
Tempol (5-10 mg/kg) + Hyperoxia	7.80 ± 0.17(6)[b]	0.59 ± 0.10(6)[b]	2.02 ± 0.15(6)[e]

* Mean ± SEM (number of samples) are presented
a. P <0.00001 relative to normoxic controls
b. Not significantly different from normoxic controls
c. P <0.000005 relative to normoxic controls
d. P <0.0004 relative to normoixc controls
e. P <0.05 relative to normoxic controls

Unlike younger animals, aged animals were unable to recover from the effects of hyperoxia (38). Others had reported similar age-specific effects for hepatic proteins in rats exposed to hyperoxia (29). If adult animals were dosed with the brain-accessible free radical scavengers,

N-tert-butyl-α-phenylnitrone (PBN) or 4-hydroxy-2,2,6,6-tetramethylpiperdin-1-oxyl (Tempol), (Table II), prior to hyperoxia, all resulting oxidative stress biomarkers were not different than those from normoxic controls (34). Thus, free radical scavengers protect adult animals from oxidative stress.

Evidence supportive of the potential use of free radical scavengers in aging and age-associated disorders was gained using the senescence-accelerated mouse (SAM) model of aging. The SAMP8 mouse strain [age-accelerated prone] has decreased life span [12.1 months compared to 18.9 months for SAMR1 (senescence-accelerated-resistant) mice]. Memory loss and learning difficulties are observed in SAMP8 mice relative to the SAMR1 strain (35). Consistent with the free radical theory of aging, chronic PBN treatment of SAMP8 mice led to greatly increased life span of these mice (36). Decreased W/S ratios of MAL-6, increased protein carbonyl content, and decreased GS activity in brain tissue was observed in SAMP8 mice compared to SAMR1 mice (5), which was beneficially modulated by chronic treatment of SAMP8 mice with the free radical scavenger PBN (5). It is interesting to speculate that the increased life span following PBN treatment (36) may be related to relieving the effects of oxidative stress (5). In human accelerated aging, i.e., progeria and Werner's syndrome, increased protein oxidation is also observed (28,29).

Protein And Lipid Oxidation In Alzheimer's Disease: Alzheimer's disease, the principal dementing disorder of the elderly and the fourth leading cause of death in the United States, is characterized clinically by loss of memory, reasoning, and speech and pathologically by the presence of neuritic (senile) plaques (SP), neurofibrillary tangles, and loss of synapses (37). Familial AD is associated with overproduction of the amyloid precursor protein (APP), from which beta-amyloid (Aβ) is derived (38). Aβ is the central constituent of SP, and several lines of circumstantial evidence support a key role for this peptide in the development of AD, including: mutations in the amyloid precursor protein (APP) or the presenilin proteins, both leading to excess Aβ deposition, are associated with early-onset AD; persons with Down's syndrome develop AD eventually, and APP is expressed on chromosome 21; although not a perfect model for AD, APP-overexpressing mice exhibit some characteristics of AD pathology [reviewed in (38)].

Aβ is associated with free radicals in oxygenated buffers as observed by the EPR technique of spin trapping (39-42). In this method a nonparamagnetic nitrone trap such as PBN is reacted with a transient free radical to form a paramagnetic nitroxide, which can then be monitored by EPR (14,43). Highly toxic Aβ yielded three-line EPR spectra with PBN (39-41), a result recently confirmed by others (42). No free radical is formed in the absence of oxygen (39). This Aβ-associated free radical may be peroxyl in nature, and once bound to PBN, is capable of decomposing the spin adduct to yield either a three or four-line EPR spectrum (40,41).

An Aβ-associated free radical model for neuronal death in AD brain has evolved from these observations (4,24,26,39,44,45) (Figure 2). In this model, Aβ-associated free radicals initiate lipid peroxidation and protein oxidation. Reaction of HNE, formed by lipid peroxidation, with numerous proteins can also alter membrane structure and function (23,26). Aβ-associated free radical oxidation and/or modification of key ion-motive ATPases and other transmembrane proteins leads to loss of ion homeostasis, increased intracellular calcium ion concentration, and subsequent cell death (4,44). This model predicts that transmembrane signaling pathways also would be compromised, perhaps leading to hyperexcitability and cell death. The model also is consistent with diffusable reactive free radical reaction products potentially interacting with and disrupting mitochondrial function, cytokine-stimulated transcription factors, or other mechanisms conceivably important in cell death.

Tests of this Aβ-associated free radical model for cell death in AD brain were conducted. In ways completely inhibitable by free radical scavengers, Aβ: inactivated oxidatively-sensitive enzymes and led to hydroxylation products with salicylate (39); caused brain membrane lipid peroxidation (24); generated fluorescence in ROS-sensitive dyes in neuronal and glial cultures and cytosols (11,46); inhibited transmembrane ion-motive ATPases and led to increased intracellular calcium ion content (47); impaired uptake of excitotoxic glutamate into astrocytes (46,48), thereby making neurons vulnerable to NMDA-receptor excitotoxicity; and disrupted transmembrane signaling pathways (49). Others confirmed that free radical scavengers block the neurotoxic effects of Aβ (49-57) and that Aβ peptides insert into lipid bilayers (58).

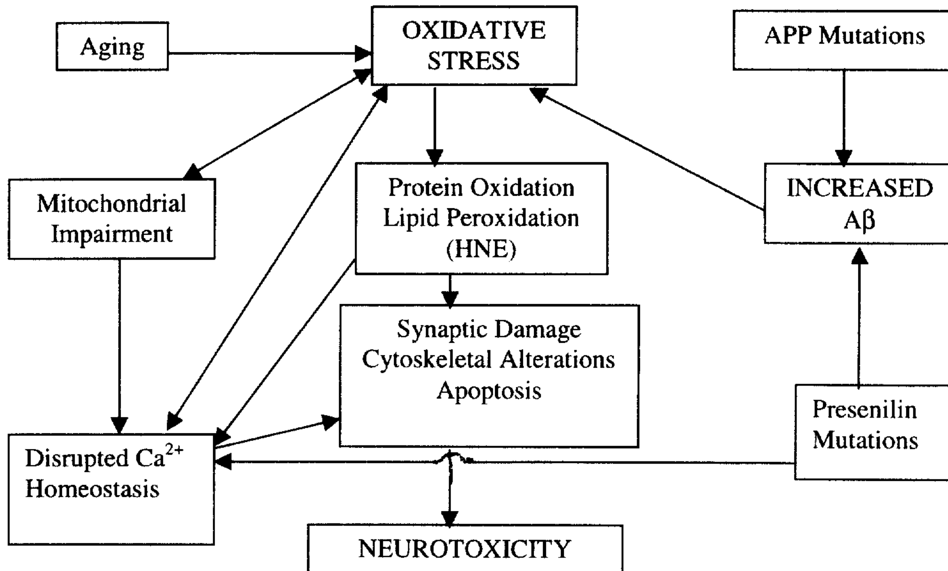

Figure 2. Scheme showing the centrality of oxidative stress to neurotoxicity in AD brain. Aβ-associated free radical oxidative stress leads to protein oxidation, and lipid peroxidation (with subsequent formation of 4-hydroxy-2-trans-nonenal, HNE), which cause damage to synaptic membranes, mitochondria, etc. This damage leads to loss of Ca^{2+} homeostasis, which further causes oxidative stress and eventual neuronal cell death.

The Aβ-associated free radical model of AD neurotoxicity predicts that protein oxidation should occur in brain membranes upon exposure to Aβ-associated free radicals. A histofluorescence method for protein carbonyl measurement following protein oxidation showed that Aβ added to cultured hippocampal neuronal (11) or astrocytic (46) cells led to oxidized proteins. Free radical scavengers abrogated these effects, and Aβ(40-1), the non-toxic reverse sequence of Aβ(1-40), did not lead to increased fluorescence. That Aβ caused protein oxidation in isolated gerbil cortical synaptosomes was confirmed by a decreased W/S ratio of MAL-6 which could be blocked by the free radical scavenger, vitamin E (4). As noted above, it is possible to modify proteins following Michael addition of HNE, which itself is formed from Aβ-associated free radical oxidation of bilayer lipids (59). Mattson and co-workers have shown that HNE, like Aβ, is able to inhibit ion motive ATPases, glutamate and glucose transporters, and G-protein-mediated signaling pathways (59-63), and this alkenal induces apoptosis (64). We wondered if these HNE-induced effects might be due to altered conformations of membrane proteins. To test this idea, we used EPR and the W/S ratio of MAL-6 to show that this parameter was significantly decreased in cortical synaptosomal membranes to which HNE was added (26), consistent with this notion. As predicted, Aβ, too, elicited a decrease W/S ratio of MAL-6 spin labeled cortical synaptosomal membranes (4).

A prediction of the Aβ-associated free radical model for neuronal death in AD brain is that regions of AD brain rich in Aβ-containing SP should exhibit protein oxidation, while SP-poor regions should be relatively immune from protein oxidation. The hippocampus and inferior parietal regions of AD brain have significant numbers of SP, while cerebellum has essentially no SP (22). Using the University of Kentucky rapid autopsy protocol, synaptosomal membranes were isolated from these three regions from AD and age-matched controls with no neurological involvement (average postmortem interval was about 2-3 hours). Four biomarkers of protein oxidation -decreased W/S ratio of MAL-6 in EPR studies, levels of protein carbonyls, and activities of the oxidatively-sensitive enzymes GS and creatine kinase (CK) - were used to assess protein oxidation (22). All four biomarkers provided evidence of protein oxidation in the hippocampal and inferior parietal regions of AD brain, and with the exception of CK activity decrease, no evidence of protein oxidation in cerebellum. In control brain there were no differences across regions among the cerebellum, hippocampus, and inferior parietal lobule, and all values were consistent with minimal protein oxidation (22). These results support the model of Aβ-associated free radicals and neurotoxicity in AD brain (4,24,39,44,45). The increased protein carbonyl levels in AD brain observed (22) confirm earlier results (31) and suggest that protein oxidation may be of fundamental importance in AD.

Since Aβ forms HNE in neurons (59) [and both cause neuronal cell death], evidence for Aβ-associated free radical induced lipid peroxidation was also sought. Aβ leads to lipid peroxidation in cortical synaptosomes (24,25,65) as assessed by EPR spin labeling methods and by free fatty release. Both measures of oxidative stress were normalized by pretreatment with the free radical scavenger vitamin E (25). In AD brain, lipid peroxidation was found (66) and recently high levels of HNE were observed in ventricular fluid in AD (67).

Oxidative stress could arise in AD brain by other mechanisms in addition to Aβ-associated free radicals. Mitochondrial electron leakiness, trace metal imbalance, advanced glycation end products (AGEs), often associated with cytoskeletal tau protein in AD NFTs, and activation of AGE receptors may lead to elevated oxidative stress in AD [reviewed in (68)].

Given the presence of protein and lipid oxidation products in AD brain and in brain tissue treated with Aβ, abrogation of the latter by free radical scavengers (4,11,25,44,46-57), and the beneficial effects of vitamin E in AD patients (69), the role of oxidative stress in AD needs further investigation. Aβ-associated free radical oxidative stress and elevated oxidative stress in AD brain (4,68), suggest brain-accessible free radical antioxidants as one promising class of compounds for AD therapeutics. Studies along this line are ongoing.

Aging and age-associated neurological disorders will become even more important in the near future as the population increases in median age. It now seems clear that free radical processes are intimately associated with normal aging. Further, aging is the single most important risk factor for Alzheimer's disease. Free radical-associated lipid and protein oxidation in this and other age-related brain disorders appear (16-19,70,71) fundamental to the pathogenesis and etiology, and, hence, treatment of each. Other neurological disorders of the brain are associated with free radical oxidative stress, e.g., Parkinson's disease [reviewed in (70)], amyotrophic lateral sclerosis (71), stroke (16-19), traumatic brain injury (72), etc. Hence, greater understanding of free radical processes and their treatment and/or prevention in AD may provide insight into the basis of and treatment for other neurological disorders of oxidative stress.

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