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SYMPOSIUM: Oxidative Stress in Neurological Disease

Oxidative Alterations in Alzheimer's Disease

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There is increasing evidence that free radical damage to brain lipids, carbohydrates, proteins, and DNA is involved in neuron death in neurodegenerative disorders. The largest number of studies have been performed in Alzheimer's disease (AD) where there is considerable support for the oxidative stress hypothesis in the pathogenesis of neuron degeneration. In autopsied brain there is an increase in lipid peroxidation, a decline in polyunsaturated fatty acids (PUFA) and an increase in 4-hydroxynonenal (HNE), a neurotoxic aldehyde product of PUFA oxidation. Increased protein oxidation and a marked decline in oxidative-sensitive enzymes, glutamine synthetase and creatinine kinase, are found in the brain in AD. Increased DNA oxidation, especially 8-hydroxy-2'-deoxyguanosine (8-OHdG) is present in the brain in AD. Immunohistochemical studies show the presence of oxidative stress products in neurofibrillary tangles and senile plaques in AD. Markers of lipid peroxidation (HNE, isoprostanes) and DNA (8-OHdG) are increased in CSF in AD. In addition, inflammatory response markers (the complement cascade, cytokines, acute phase reactants and proteases) are present in the brain in AD. These findings, coupled with epidemiologic studies showing that anti-inflammatory agents slow the progression or delay the onset of AD, suggest that inflammation plays a role in AD. Overall these studies indicate that oxidative stress and the inflammatory cascade, working in concert, are important in the pathogenetic cascade of neurodegeneration in AD, suggesting that therapeutic efforts aimed at both of these mechanisms may be beneficial.

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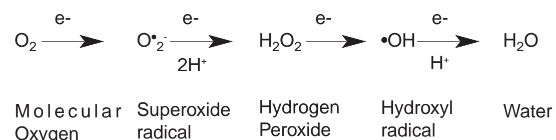
Introduction

A growing body of evidence indicates that increased oxidative stress resulting from free radical damage to cellular function, is associated with the aging process and a number of age-related disorders including atherosclerosis and arthritis. In recent years, considerable data have emerged indicating that free radicals play a significant role in the pathogenesis of neurodegenerative disorders. This evidence has been most clearly demonstrated for Alzheimer's disease (AD), where multiple studies show increased oxidation of brain lipids, carbohydrates, proteins and DNA, and the presence of oxidative stress products in neurofibrillary tangles (NFT) and senile plaques (SP) (Tables 1 and 2). These oxidative modifications not only decrease or eliminate the normal function of these macromolecules, but also may activate an inflammatory process in the brains of AD patients. This report reviews the evidence for oxidative stress in the brain in AD and attempts to show a relationship between oxidative change and inflammatory responses in the pathogenesis of neuron degeneration.

Free radicals are defined as any atom or molecule with one or more unpaired electrons in its outer shell. Numerous radicals exist but some of the most potent are formed in the reduction of molecular oxygen to water as follows:



Reduction of molecular oxygen by one electron yields the superoxide radical ($O^{\bullet}_2{}^-$) which has limited reactivity with some proteins, but is not reactive with lipids or DNA. Under the influence of superoxide dis-

1.	4-Hydroxynonenal (81,83,106)
2.	Protein carbonyls (117)
3.	Nitrotyrosine (37,119)
4.	Advanced glycation end products (133)
5.	Cu/Zn-superoxide dismutase (92)
6.	Hemeoxygenase 1 (115)

Table 1. Oxidative stress products in neurofibrillary tangles in AD.

1.	Advanced glycation end products (114,129)
2.	Cu/Zn-superoxide dismutase (92)
3.	Hemeoxygenase - 1 (115)
4.	Catalase (92)

Table 2. Oxidative stress products in senile plaques in AD.

mutase, hydrogen peroxide (H_2O_2) is formed by addition of an electron and $2H^+$. Although H_2O_2 does not have an unpaired electron and is not a free radical, it is an effective oxidant for many biological molecules. Reduction of H_2O_2 yields the hydroxyl radical (•OH), which is the most reactive oxygen radical and capable of oxidizing lipids, carbohydrates, protein and DNA. These oxygen free radicals plus, H_2O_2, singlet oxygen, and hypochloric acid are spoken of as reactive oxygen species (ROS) (42).

Iron (Fe) and copper (Cu) are important reactive elements that catalyze oxidative reactions and the generation of oxygen radicals. Iron is the most important element in radical generation and may play a role in AD (see below). Iron contains a loosely bound electron and has the ability to exist in more than one valence state. The stable redox state of Fe is Fe^{3+}, but its bivalent form, Fe^{2+}, is capable of transferring one electron and facilitating free radical generation. The reaction of Fe^2 with H_2O_2 produces •OH and is termed the Fenton reaction.

Nitric oxide (NO) is synthesized in a variety of cells and tissues by the enzymatic oxidation of L-arginine to form citrulline through the action of calcium activated, calmodulin-dependent nitric oxide synthase (71). Nitric oxide is an important mediator of several physiologic processes. Excess NO production is found in excitotoxicity, inflammation and ischemia-reperfusion injury (11). Nitric oxide is a free radical with limited potential reactivity, but in biological systems, it reacts with oxygen, $O_2^{\bullet-}$ and transition metals. Nitric oxide combines rapidly with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$), a powerful oxidant. Peroxynitrite can oxidize carbohydrates, membrane lipids, proteins and DNA. It serves as a nitrating agent, promoting the addition of nitrogroups to aromatic and indolic groups in proteins containing tyrosine, phenylalanine, and tryptophan. In addition, $ONOO^-$ can generate the highly reactive $OH^{\bullet-}$.

To defend against free radicals, living organisms have learned over time to generate antioxidants and repair enzymes to remove and/or repair molecules that are oxidized. A few enzymatic antioxidants are synthesized by cells. These include Cu/Zn- and MN-superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSSG-R), catalase (CAT) and methionine sulfoxide reductase. Other nonenzymatic antioxidants and metal chelators include vitamin E, vitamin C, beta carotene, selenium, ubiquinone, ferritin, ceruloplasmin, and uric acid. Antioxidant defense mechanisms can be upregulated in response to increased free radical or peroxide production (19). Although upregulating antioxidant defense systems may confer protection against free radicals, they are not completely effective in preventing oxidative damage. Also with aging, the efficiency of gene expression may decline or become defective as oxidative damage to the genome increases.

The term oxidative stress is used when free radicals and their products are in excess of the antioxidant defense mechanisms. This may occur as a result of increased radical production or a decrease in antioxidant defenses. If the increased demand on the cell's capacity to detoxify free radicals is not met, alterations occur in cells. Accumulation of oxidized products, such as aldehydes or isoprostanes from lipid peroxidation, protein carbonyls from protein oxidation, and oxidized base adducts from DNA oxidation, serve as markers of excess oxidative stress.

The brain is especially vulnerable to free radical damage because of its high oxygen consumption rate, abundant lipid content, and relative paucity of antioxidant enzymes compared with other organs (26). A role for oxidative stress in the pathogenetic cascade of events in AD and other neurodegenerative disorders is appealing because neurons are post-mitotic cells and gradually accumulate oxidative damage over time, which would account for the late life onset and slowly progressive nature of these disorders (26).

Mitochondrial dysfunction and consequent impairment of ATP production and free radical generation lead to excitotoxicity and neuron degeneration and have been strongly implicated in the pathogenesis of neurodegenerative disorders, especially in AD (7). Mitochondria generate ATP through the reduction of O_2 by the sequential addition of electrons and H^+. The mitochondrial

electron transport system is the major intracellular source of oxygen radicals and H_2O_2. Paradoxically, mitochondria are one of the major targets of ROS. Considerable data show a number of age-related alterations in mitochondria including loss of membrane fluidity, increased proton leakage, and decreased levels of cardiolipin (3). Also there is an increase in $O_2^{\bullet-}$ and H_2O_2 generated in mitochondria with age (120).

It is well recognized that energy metabolism is impaired in the brain in AD (7). Positron emission tomography studies demonstrate a decline in cerebral metabolic rate in parietal and temporal lobes in AD subjects (reviewed in 51). These metabolic defects are present in those at high risk for the disease before symptoms develop (99, 112). Parker *et al*. (93) described a mild generalized reduction of the activity of the electron transport chain complexes (I-IV), but a more marked reduction of cytochrome oxidase (complex IV) in the brains of autopsied AD patients. Several other groups found a reduction of cytochrome oxidase activity in the cerebral cortex of AD subjects (17, 57, 86). Cytochrome oxidase is unique from the other respiratory complexes in that it requires cardiolipin for activity. Oxidative modification of this and other lipids may further damage this complex. Cytochrome oxidase mRNA levels are reduced in hippocampus in AD (109, 110). Parker and Parks (94) purified cytochrome oxidase from AD and control brains and found that control brain cytochrome oxidase had two Km values, but the AD brain had only a single value, suggesting that AD brain cytochrome oxidase may be structurally abnormal. The decrease in cytochrome oxidase function could depress ATP synthesis and divert electrons from the normal pathway into increased $O_2^{\bullet-}$ generation. Partridge *et al*. (95) found •OH in mitochondria after cytochrome oxidase inhibition by sodium azide. Diminished cytochrome oxidase activity could lead to increased ROS generation, oxidative damage to mitochondrial membranes, and increased vulnerability to excitotoxins, and may play a role in the pathogenesis of AD.

Lipid peroxidation

Increased lipid peroxidation has been described in several neurodegenerative diseases including Parkinson's disease and AD, and in ischemic and traumatic brain injury (10, 32, 41). Lipid peroxidation is assessed by measuring a) thiobarbituric acid reactive substances (TBARS), b) alterations in polyunsaturated fatty acids (PUFA), and c) the breakdown products of PUFA, such as aldehydes and isoprostanes.

Subbarao *et al*. (125) found an increase in TBARS in frontal lobe specimens, but not cerebellum, in AD compared with control subjects. Others described increased TBARS in the temporal lobe but not in other neocortical areas in AD (4, 40, 66, 75). Our study of 13 AD and 10 prospectively evaluated control subjects, all from short postmortem interval autopsies, found elevated TBARS in all AD brain regions compared with controls, except the middle frontal gyrus (60). These elevations were statistically significant in the hippocampus and pyriform cortex, and marginally significant in the amygdala. This study demonstrated that lipid peroxidation is most pronounced in medial temporal lobe structures where histopathologic changes are most pronounced. Although TBARS measure the major lipid peroxidation burden, they also measure a variety of products including non-lipid derived malondialdehyde, C_3-C_{10} aldehydes, and species resulting from chemical interaction among non-lipid molecules during the assay. Thus, it is important to determine other markers of lipid peroxidation to be certain of its presence.

Polyunsaturated fatty acids, which make up the brain's membrane phospholipids, are especially vulnerable to free radical attack because their double bonds allow easy removal of H^+. Because lipid peroxidation is increased in AD, it follows that PUFA would be expected to be diminished, especially arachidonic and docosahexenoic acids, which are especially vulnerable to attack by ROS. *In-vivo* phosphorus nuclear magnetic resonance (PNMR) studies demonstrated that the ratio of glycerophosphorylcholine to glycerophosphorylethanolamine was increased and levels of glycerophosphodiesters and phosphomonoesters were elevated in AD compared with age-matched controls (79, 96). Another PNMR study showed a significant decrease in phosphatidylethanolamine (PE) and the phospholipid precursors–choline and ethanolamine–in AD (90). It was suggested that these alterations were specific for the pathogenesis of AD. Svennerholm and Gottfries (126) showed decreased levels of brain cortical membrane phospholipids in early-onset AD patients but not late-onset AD patients. Our study of membrane phospholipid levels in various brain regions in late-onset AD patients and age-matched controls found that PE and phosphatidylinositol (PI)-derived total fatty acids were significantly decreased in hippocampus of AD subjects (97). In PE-derived fatty acids, stearic, oleic, arachidonic and docosahexenoic acids were significantly decreased, and in PI-derived phospholipids, oleic and arachidonic were significantly decreased. In the inferior parietal lobule, significant decreases were found in total PE-derived fatty acids and in stearic,

oleic, and arachidonic acids. The decreases in PE and PI, which are rich in oxidizable arachidonic and docosahexenoic acids, but no change in the phosphatidylcholine pool, which contains lesser amounts of these fatty acids, suggest that free radicals are responsible for the alteration in membrane phospholipids.

Oxidation of PUFA results in the production of multiple aldehydes with different carbon chain lengths including propanal, butanal, pentanal, hexanal and 4-hydroxynonenal (HNE) (31). Aldehydes have half-lives of minutes to hours and can defuse from their site of origin to more distant sites, much different from the highly reactive free radicals. HNE is a highly reactive α, β-aldehyde responsible for cytotoxicity in conjunction with oxidative stress and is capable of inhibiting DNA, RNA, protein synthesis, and glycolysis, and degrading proteins (31). HNE forms adducts with proteins by covalent bonding to histidine, lysine and cysteine residues through Michael addition or by Schiff base reactions (31, 127). We demonstrated elevations of free HNE in multiple brain regions in AD compared with age-matched controls (69). These elevations were statistically significant in the amygdala, hippocampus, and parahippocampal gyrus, regions showing the most striking histopathologic alterations in AD. We also demonstrated a highly significant elevation of free HNE in ventricular cerebrospinal fluid (CSF) in 19 AD patients compared with 13 control subjects (61).

In immunocytochemical studies, Montine *et al*. (81) demonstrated that HNE pyrrole adducts are present in NFT in AD and are significantly associated with the inheritance of the APOE ϵ4 alleles, a major risk factor for AD (103). Borohydride-reducible HNE adducts were increased in pyramidal neuron cytoplasm in the hippocampus, entorhinal cortex, and temporal neocortex in patients in AD who were homozygous for APOE ϵ4, and pyramidal neurons and astrocytes in AD patients who were homozygous for APOE ϵ3 (83). These studies suggest that APOE, the major lipoprotein trafficking molecule in the brain, might influence HNE accumulation in AD. Sayre *et al* (106) demonstrated HNE-pyrrole immunoreactivity in NFT and in neurons lacking NFT in AD, but not in control brains. They did not find a correlation of HNE-pyrrole immunostaining with any particular APOE allele.

HNE is toxic to neurons and astrocytes in P19 neuroglial cultures and caused crosslinking of tau into high molecular weight species that are conjugated with ubiquitin (80). Mark *et al*. (67) demonstrated that HNE caused a time- and concentration-dependent decrease in rat hippocampal neurons in cultures by impairing Na^+K^+-ATPase activity and disrupting calcium homeostasis which led to neuron death. Exposure of cultured rat hippocampal neurons to Aβ induced a significant increase in free and protein-bound HNE (67). HNE caused impaired glucose transport in cultured rat hippocampal neurons and impaired glutamate transport in rat neocortical synaptosomes (54, 68). HNE also impaired coupling of metabotropic acetylcholine and glutamate receptor G_{q11} in cortical neuron cultures (9). HNE is capable of inducing apoptosis in PC12 cells and cultured rat hippocampal neurons suggesting that it is a mediator of oxidative stress-induced apoptosis (59). HNE administered into the basal forebrain of rats, damaged cholinergic neurons, diminished ChAT activity, and impaired visuospatial memory (13). Taken together, these studies suggest that in addition to direct ROS damage to phospholipid membranes, there is an indirect mechanism involving HNE, which also may be involved in neuron death. These studies demonstrate that HNE may be an important molecule in the pathogenetic cascade of neuron degeneration in AD.

Glutathione transferases are a group of enzymes that function to inactivate the toxic products of oxygen metabolism including 4-hydroxyalkenals, such as HNE (27). Recently, we described decreased glutathione transferase activity in eight brain regions in AD including statistically significant reductions in the amygdala, hippocampus, and inferior parietal lobule compared with normal age-matched controls (63). In addition, a statistically significant decrease in glutathione transferase activity was found in postmortem ventricular CSF in AD compared with age-matched controls. This study suggests a loss of protection against HNE in AD that could be important in the pathogenesis of neuron degeneration.

Isoprostanes are prostaglandin-like compounds that are formed nonenzymatically by free radical-induced oxidation of arachidonic acid (84). Because they contain F-type prostane rings, they are referred to as F_2-isoprostanes (F_2-IsoP). Considerable evidence exists to show that F_2-IsoP concentration is a reproducible quantitative index of lipid peroxidation *in vivo* (84). Oxidation of docosahexenoic acid leads to the formation of F_2-IsoP-like compounds which have been called F4-neuroprostanes (F_4-NP) (101). *In vitro* oxidation of docosahexenoic acid yielded higher levels of F_4-NP than F_2-IsoP by 3.4 fold (101). Concentrations of F_2-IsoP in the CSF of AD patients were significantly elevated (72 ± pg/ml) compared with controls (46 ± 4 pg/ml) ($p < 0.05$) (82). Linear regression analysis showed a signif-

icant correlation between F_2-IsoP levels and brain weight in AD. Study of F_4-NP in CSF of a small number of AD and control patients yielded a significantly higher level in AD (110 ± 12 pg/ml), compared with controls (64 ± 8 pg/ml) (101).

These studies indicate that a potential quantifiable marker of brain lipid peroxidation may be present in CSF in AD (Table 3). One or more of these potential biomarkers could be of considerable value in a) quantifying the response to therapeutic interventions to decrease lipid peroxidation, and b) aiding in the diagnosis of AD. Of these, F_2-IsoP and F_4-NP may be the most valuable. Multiple further studies of subjects in various clinical stages of AD and in other slowly progressive neurodegenerative diseases are required to define the value and specificity of lipid peroxidation breakdown products in CSF in AD. It is likely that products of lipid peroxidation are present in CSF in other chronic progressive neurodegenerative disorders such as motor neuron disease, Parkinson's disease, Huntington's disease, corticobasal degeneration and others with focal degenerative changes. However, it may be that the presence of more widespread alterations in AD increases the overall burden of lipid peroxidation and resultant breakdown products.

1. Increased free 4-hydroxynonenal
2. Increased glutathione transferases
3. Increased isoprostanes
4. Increased 8-hydroxy-2-deoxyguanosine (OHdG) in intact DNA and decreased free •OHdG

Table 3. Oxidative stress products in CSF in AD.

Protein oxidation

Hydrazide reactive protein carbonyl (protein carbonyl) analysis is used as a general assay of oxidative damage to proteins (122). Smith *et al*. (113) demonstrated a significant increase in protein carbonyls in the frontal and occipital poles in normal aged subjects compared with young controls. This study also demonstrated that protein oxidation was significantly increased in the frontal pole (where numerous degenerative changes are found) of AD patients compared with normal aged control subjects, although no differences were found in the occipital pole (where few degenerative changes are present). When the level of protein carbonyl was compared to the age of the control subjects, it was found that the frontal pole accumulated carbonyl-bearing protein at twice the rate of the occipital pole. Hensley *et al*. (45) showed that protein carbonyl content was increased in the AD hippocampus and inferior parietal lobule compared with the cerebellum in AD. Control hippocampus and inferior parietal lobule levels were similar to control cerebellum levels. Others have shown a significant elevation of protein-carbonyl levels in the parietal lobe and a trend for elevations in the frontal, temporal and occipital lobes and hippocampus in AD (65). Smith *et al* (117), using immunocytochemical techniques with *in situ* 2, 4 dinitrophenylhydrazine (DNP) labeling linked to an antibody system against DNP, demonstrated the presence of protein carbonyls in NFT and glia but not in nonNFT-bearing neurons in AD. These changes were not found in control brains. In addition increased oxidation of synaptosomes in AD hippocampus and parietal lobe was shown using electron paramagnetic resonance (EPR) (44, 45).

Peroxynitrite causes nitration of tyrosine residues yielding nitrotyrosine which is used as an indicator of $ONOO^-$ activity. Several investigators found nitrotyrosine in NFT in the hippocampus in AD (37, 119). In addition, Smith *et al*. (119) found nitrotyrosine immunoreactivity in non-NFT bearing neurons and in nuclei of glia in AD. These studies demonstrate that $ONOO^-$ is likely involved in protein oxidation in AD.

The oxidation of proteins by free radicals may be responsible for damaging enzymes critical to neuron function (122). As demonstrated by Stadtman *et al*. (122), this is a two-step process in which there is oxidation of enzyme amino acids by the free radicals yielding carbonyl derivatives. The second step involves further degradation of the enzyme by proteases to amino acids and peptides. Two enzymes that are especially sensitive to oxidative modification are glutamine synthetase (GS) and creatinine kinase (CK) (122). A significant decline in glia-specific GS activity has been found in the hippocampus and neocortex in AD compared with age-matched controls (45, 113). Decreased levels of GS could result in decreased glutamate turnover causing prolonged NMDA receptor activation and neuron injury in brain areas susceptible to glutamate toxicity. In addition, because glutamate is converted to glutamine, less of the enzyme could alter nitrogen balance, pH and glutathione synthesis in astrocytes. Several studies have demonstrated a significant decline in CK activity in the frontal and temporal lobes in AD (1, 14). One of these studies demonstrated that the decline in CK levels was due to a decrease in brain CK and not ubiquitous mitochondrial CK (1). These studies demonstrate that the increase in protein oxidation in the brain in AD may be at the expense of enzymes critical to neuron energy

metabolism and function.

Yatin *et al*. (136) demonstrated that protein carbonyls develop immediately following oxidative insults in cultured neurons. They showed that ROS generated by Aβ immediately attack amino acid residues causing carbonyl formation. Protein oxidation starts as soon as cells are exposed to Aβ , and protein carbonyl levels are significantly elevated after one hour of Aβ treatment. These changes occur before neuron death and parallel the EPR detection of free radicals. They also demonstrated that Mn-SOD and Cu/Zn-SOD increased rapidly in response to oxidative stress.

DNA oxidation

Oxidation of DNA can cause several damage products including strand breaks, sister chromatid exchange, DNA-DNA and DNA-protein crosslinking, and base modifications (28). Damage to DNA occurs as a consequence of the generation of $O_2^{\bullet-}$, H_2O_2, •OH, $OONO^-$, and singlet oxygen. It has long been suggested that DNA damage accumulating in nondividing mammalian cells, may play a major role in aging changes. The ability to repair DNA damage is a critical factor in the function and longevity of cells and defects in DNA repair may be important in aging and age-associated disorders.

DNA degeneration occurs in apoptotic and necrotic cell death. It has been suggested that neuron death in AD is through an apoptotic mechanism (111, 123), although necrotic cell death also has been proposed (121). A two-fold increase in DNA strand breaks has been described in the brain in AD (85). DNA strand breaks can be detected at the single cell level by terminal deoxynucleotidyl transferase (TdT)-mediated *in situ* end labeling. Su *et al*. (124) showed robust TdT labeling in neurons lacking NFT in the occipital lobe in AD. Nitrotyrosine immunoreactivity was prominent in TdT-labeled neurons. These authors suggested that neurons with DNA damage without NFT may undergo degeneration caused by oxidative mechanisms involving $OONO^-$.

Several biomarkers of oxidative DNA damage have been quantified but the most useful has been the adduct, 8-hydroxy-2'-deoxyguanosine (8-OHdG). Mecocci *et al*. (77) found an increase in 8-OHdG in nuclear and mitochondrial brain fractions in aging. Using HPLC, they demonstrated a 10-fold increase in mitochondrial DNA oxidation compared with nuclear DNA, and a 15-fold increase in DNA oxidation in subjects older than 70 years of age. These same investigators showed a three-fold increase in mitochondrial DNA oxidation in the parietal lobe in AD subjects compared with normal controls (78). Their study demonstrated a small but significant increase in oxidative damage to nuclear DNA and a highly significant increase in mitochondrial DNA oxidation in AD samples compared with age-matched control subjects.

Gas chromatography with mass spectroscopy (GC-MS) is a sensitive method used to identify oxidative adducts from DNA bases. Using GC-MS in combined nuclear and mitochondrial DNA specimens, Lyras *et al*. (65) found various bases increased or decreased in different brain regions in AD. The most consistent elevations were in 8-hydroxyadenine, 5-hydroxycytosine, and 8-OHdG in the parietal lobe in AD. We used GC-MS with stable-labeled oxidized base analog standards to study nuclear DNA from four brain regions in AD patients and prospectively evaluated control subjects, all with short postmortem intervals (34). Our studies showed statistically significant elevations of 5-hydroxyuracil, 8-hydroxyadenine, and 8-OHdG in frontal, parietal, and temporal lobes, and 5-hydroxycytosine in the parietal and temporal lobes in AD. The increases in mean 8-OHdG were the largest elevations of all the base adducts analyzed indicating that guanine is the most vulnerable base to oxidation. These studies suggest that the pattern of damage to multiple bases in the brain is due to •OH attack on DNA.

Recently, using GC-MS and stable isotope labeled 8-OHdG as the standard, we studied the levels of 8-OHdG in intact DNA and free 8-OHdG (representing the repair product) in ventricular CSF from AD and control subjects (64). We found significant elevation of 8-OHdG in intact DNA in AD compared with age-matched control subjects. In contrast, levels of free 8-OHdG were significantly decreased in AD samples. This suggests that in AD there may be a double insult of increased oxidative DNA damage and a deficiency of repair mechanisms responsible for removal of oxidized bases. Hermon *et al*. (46) described increased protein levels of two excision repair cross-complementing genes for nucleotide excision repair in AD brain, suggesting ongoing oxidative DNA damage.

Wade *et al*. (130) used immunocytochemistry to show that 8-OHdG was increased in mitochondria in neurons in AD compared with controls. The same investigators demonstrated that the 5Kb deletion, the most common alteration in human mitochondria, was prominent in large hippocampal pyramidal neurons in AD (47). This deletion was present in neurons immunostaining with 8-OHdG. They concluded that oxidative modifications of mitochondrial DNA are an early event in the neuropathologic changes in AD, and that accumu-

lation of deleted mitochondrial DNA may potentiate oxidative damage in vulnerable neurons.

These descriptive studies indicate significant oxidative damage to DNA in the brain in AD. The consequences of the oxidative DNA damage in the pathogenesis of AD will require further study as will the role of nucleotide and base excision-repair pathways in the brain in AD.

Glycooxidation

There are two mechanisms by which glucose can induce changes in proteins (49, 50). Monosaccharides can oxidize when catalyzed by transition metals generating free radicals, H_2O_2, and reactive dicarbonyls (48). Proteins are then damaged by free radicals and by covalent bonding of the carbonyl products to protein components. The other reaction is the nonenzymatic glycation of proteins through the Maillard reaction. Advanced glycation end products (AGE) are post-translational modifications of proteins that are formed when the amino group of proteins, especially N-terminal amino groups and side chains of lysine and arginine, react nonenzymatically with monosaccharides (reviewed in 87). This leads by way of Schiff base products to protein-bound Amadori products. The Amadori products, through oxidation and dehydration including free radical intermediates, form AGE. This reaction, which is catalyzed by transition metals such as Fe and Cu, also yields oxygen free radicals. AGE-modified proteins can also produce free radicals through interaction with microglia (76). Modification of proteins by oxidation, glycooxidation, and products of lipid peroxidation can occur in an additive and synergistic manner (116).

Recent studies indicate a role for AGE in AD (116). AGE have been found in diffuse and neuritic SP in AD (114, 129). AGE-modified Aβ accelerates aggregation of soluble nonfibrillar Aβ *in vitro* suggesting that AGE may enhance SP formation *in vivo* (129). Hyperphosphorylated tau is a major component of NFT. Tau and AGE antigens are co-localized in NFT (114, 133). Glycated tau added to cultured neuroblastoma cells induces lipid peroxidation (133). These studies suggest that AGE may play a role in AD by oxidative modifications of Aβ and tau, the two major proteins involved in this disorder.

Another possible way that AGE may play a role in AD has been described. One of the cell receptors for AGE, termed RAGE, is a member of the immunoglobulin superfamily of cell surface molecules (89,108) that is expressed on endothelium, neurons, smooth muscle cells, and phagocytes (12). RAGE expression is increased in neurons, microglia and blood vessels in AD (134). Yan *et al*. (135) demonstrated that Aβ binds to RAGE and generates oxidant stress, activating nuclear factor -κB(NF-κB), and inducing expression of macrophage-colony stimulating factor. Macrophage-colony stimulating factor enhances proliferation and migration of microglia. This study suggests that a free radical-dependent inflammatory pathway, triggered by interaction of Aβ on RAGE, may be present in AD.

Antioxidant enzymes

The increase in brain oxidative damage described above might be facilitated by a failure of antioxidant defense mechanisms. Antioxidant enzymes that are important in preventing an excessive accumulation of ROS include Cu/Zn- and Mn-SOD, GSH-Px, GSSG-R and CAT.

We studied a number of antioxidant enzymes in the brains of AD patients and control subjects with short postmortem intervals and found significant elevations of GSH-Px activity in hippocampus, GSSG-R activity in hippocampus and amygdala, and CAT activity in hippocampus and temporal neocortex in AD compared with normal subjects (60). These changes correlated with elevation of lipid peroxidation in the same regions. In that study and subsequent studies, we did not find significant elevations of Cu/Zn- or Mn-SOD activity in AD. In a separate series of AD and control subjects, we studied mRNA expression of oxidative stress-handling genes (2). We found significant elevations of GSH-Px, GSSG-R and CAT mRNA in hippocampus and inferior parietal lobules, but not in cerebellum in AD. An increase in Cu/Zn-SOD mRNA was present in the inferior parietal lobule. Importantly, no deficiencies of antioxidant enzyme expression at the transcription level were observed. The elevations observed in both these studies correlate with increased oxidative damage and may reflect a compensatory rise in antioxidant activity in response to increased ROS generation.

Other studies of antioxidant levels in the brain in AD found variable results. Two studies found no significant difference in GSH-Px in AD and controls (56, 66). Catalase was elevated in the amygdala (4) and in the temporal lobe in AD (66), but in another study it was reduced in several brain regions (39). Several studies reported no significant difference in Cu/Zn- or Mn-SOD activity in the brain in AD compared with controls (39, 53). One study described a significant reduction of SOD in frontal cortex, hippocampus, and cerebellum in AD (100). Other investigators reported a decrease in SOD activity in the caudate nucleus (70), and frontal and tem-

poral lobes in AD (66). In contrast, Kato *et al.* (53) found a significant elevation of Cu/Zn-SOD in AD brains.

Immunohistochemical studies (Table 1 and 2) show an increase in Cu/Zn-SOD and CAT in a subgroup of NFT and SP in the hippocampus in AD (92). Hemeoxygenase -1, an antioxidant, is present in NFT, SP and neuropil threads in AD (115), as well as in neurons and astrocytes in AD (107). Increased expression of hemeoxygenase-1 mRNA and protein content has been described in the frontal, temporal, and occipital lobes in AD (98).

Taken as a whole, studies of antioxidant enzyme activities and expression in the brain in AD do not show a consistent trend. Importantly, they do not demonstrate meaningful deficiencies of any enzyme activity, suggesting that the oxidative stress in the brain is not related to a failure of these defense mechanisms.

Role of iron in oxidative stress

As noted in the Introduction, Fe has a potential major role as a catalyst for free radical generation. It is unique in that it has a loosely bound electron in the Fe^{2+} oxidation state and the ability to exist in two oxidation states. In its bivalent Fe^{2+} form, it is capable of transferring an electron and facilitating free radical generation.

We reported an increase in Fe in the brain in bulk specimens from different brain regions in AD compared with age-matched control subjects in several separate instrumental neutron activation analysis studies (25, 29, 30, 104). Our initial study of separated cerebral gray and white matter showed a statistically significant elevation of Fe in AD gray matter compared with control subjects (30). In our recent study of 58 AD and 21 prospectively evaluated normal control subjects, Fe was significantly elevated in the frontal, temporal, and parietal neocortex, and the hippocampus and amygdala (25).

Good *et al.* (36), using laser microprobe analysis demonstrated a significant elevation of Fe in NFT-bearing neurons in the hippocampus in AD. Using microparticle-induced x-ray emission, we found a significant elevation of Fe in the cores and rims of SP in the amygdala of AD subjects compared with neuropil of AD and control subjects (62).

Several studies found increased Fe and ferritin in SP in AD (22, 24) and ferritin immunoreactivity in microglia in SP in AD (38). One study showed that ferritin from the brains of AD patients contained more Fe than brains of age-matched controls (33). The frequency of the C_2 allele of transferrin was significantly higher in late-onset AD patients than age-matched controls, and twice as high in AD patients homozygous for apolipoprotein E ϵ4 alleles compared with AD patients with one or no copies of the ϵ4 allele (88). Another study showed that transferrin immunoreactivity is homogeneously present around SP in AD (23). The Fe binding protein, P97 or melanotransferrin, is elevated in the serum, CSF, and brains of AD patients compared with control subjects (52, 55). Iron regulatory protein-2 is present in NFT, SP, and neuropil threads, whereas Fe regulatory protein 1 has similar expression in AD and control brains (16). Smith *et al.* (118) showed that redox-active Fe is associated with SP, NFT and neuropil threads in AD and catalyzes a H_2O_2 dependent oxidation. Overall, these studies indicate an increase in Fe in the brain in AD suggesting an abnormality in Fe metabolism. This increase in Fe may contribute to enhancement of oxidative stress in the disease.

Inflammatory alterations in AD

Numerous epidemiologic studies indicate that use of nonsteroidal anti-inflammatory drugs (NSAID) or corticosteroids is associated with slowing of the progression or delaying the onset of AD. Meta-analysis of 17 epidemiologic studies from nine countries demonstrated that the use of corticosteroids or NSAID, was associated with a low risk of AD (74). The use of NSAID had a stronger level of significance than use of corticosteroids. A six-month trial on indomethacin showed a slowing of the progression of AD, although the number of patients used in the study was small (102). The major effect of NSAID is through decreasing the activity of cyclooxygenase which catalyzes the first steps in the biosynthesis of prostaglandins from arachidonic acid. Cyclooxygenase-2 (COX-2) is induced by stimuli associated with inflammation, such as the cytokines, interleukin I (IL-1), interleukin 2 (IL-2), and TNF-α (128). Thus, the ability of NSAID to inhibit COX-2 makes them effective as anti-inflammatory agents in a number of different disorders.

Cultured murine and rat astrocytes express COX-2 and it is upregulated by IL-1β and TNF-α (91). Rat microglia stimulated with lipopolysaccharide express COX-2 (6). Expression of COX-2 mRNA is decreased in the brain in AD (18). One explanation for the lower COX-2 activity in the AD brain is that in end-stage disease the loss of neurons and decreased synthesis of COX-2 are greater than the increase of COX-2 in activated glia (128). Another explanation might be that the efficacy of NSAID in AD is through mechanisms that do not involve COX-2.

The above studies suggesting that anti-inflammatory

drugs may play a protective role against AD have stimulated an increased interest in the role of inflammation in the brain in AD. Support for the presence of an inflammatory response in the brain in AD has been gained from numerous autopsy studies. A number of markers of inflammation are present in the brain in AD and some are related to the morphologic changes of AD (reviewed in 73). These include the classic-complement cascade and their regulating cytokines, acute phase reactants, proteases, and protease inhibitors. Immunohistochemical studies have shown the presence of C1q, C4d, C3c, and C3d in amyloid in SP. Elements of the membrane attack complex, C7 and C9, are found in Aβ-deposits in AD, and other components of the membrane-attack complex have been found in neurites in SP and in some NFT. The acute phase proteins, α-1 antichymotrypsin, α-antitrypsin, C-reactive protein, and α-2 macroglobulin are upregulated in the AD brain and found around SP. Mutations in the α-2 macroglobulin gene have been found in AD and appear to be a major risk factor for the disease (8).

Microglia cells are the immunological scavengers of the brain and may be the key factor in the inflammatory response in AD. Activated microglia cells are markedly increased in the brain in AD (15). Microglia cells are capable of releasing a number of interleukins, especially IL-1 and IL-6 and TNF-α (131, 132). Astrocytes also have the ability to produce interleukins, prostaglandins, complements, coagulation factors, proteases, and protease inhibitors (73). The relationship between reactive astrocytes and reactive microglia in the inflammatory cascades may be important in propagating the response.

The relationship between the inflammatory response and free radical generation, although not clearly worked out in AD, is of considerable theoretical and therapeutic interest. Activated microglia demonstrate respiratory burst activity which is derived from the hexose monophosphate shunt (20). Respiratory burst activity can be initiated by fatty acids, high extracellular calcium, eicosanoid derivatives and various synthetic peptides including Aβ (58). Activated microglia are capable of generating $O_2^{\bullet -}$ which could lead to H_2O_2 and •OH formation (5, 20). Activated microglia are also capable of generating NO (21) which, as shown above, can generate the potent radical $OONO^-$. Thus, the activated microglia in AD may be part of a neurotoxic mechanism through generation of free radicals.

Another possible pathway of neurodegeneration in AD is through the ability of IL-1 to cause an upregulation of APP expression (35). This could lead to increased Aβ which has been shown to cause H_2O_2 accumulation in cultured hippocampal neurons (72). Hensley *et al*. (44), using EPR analysis, demonstrated that aggregated Aβ is capable of generating free radicals. Aβ is capable of inactivating GS and CK in cell free incubates and in cell cultures (43). Thus, microglia releasing IL-1 could set in motion a self-propagating series of events that lead to more free radical generation and neuron destruction.

It is most probable that AD is associated with multiple etiologies and pathophysiologic mechanisms. The above review clearly demonstrates that oxidative stress and the inflammatory cascade are, at least, a part of a pathophysiologic mechanism in AD. While they may be thought of as separate events, it appears that they work in concert. Although we do not know the initiating events, these mechanisms appear to be involved in the pathogenesis cascade that leads to specific neuron destruction in AD and possibly other neurodegenerative diseases. Thus, it seems logical to direct therapeutic efforts at oxidative and inflammatory events in the pathway of neuron degeneration in AD. Preliminary evidence that antioxidant therapy (105) and anti-inflammatory therapy (74) slow or delay AD supports this concept. Because some of the risk factors for AD are known, treatment of individuals at high risk long before they develop symptoms, using antioxidants and anti-inflammatory agents, may delay or prevent the onset of AD.

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