Preface

As the average life expectancy of many populations throughout the world increases, so to does the incidence of such age-related neurodegenerative disorders as Alzheimer's, Parkinson's, and Huntington's diseases. Rapid advances in our understanding of the molecular genetics and environmental factors that either cause or increase risk for age-related neurodegenerative disorders have been made in the past decade. The ability to evaluate, at the cellular and molecular level, abnormalities in postmortem brain tissue from patients, when taken together with the development of valuable animal and cell-culture models of neurodegenerative disorders has allowed the identification of sequences of events within neurons that result in their demise in specific neurodegenerative disorders. Though the genetic and environmental factors that promote neurodegeneration may differ among disorders, shared biochemical cascades that will ultimately lead to the death of neurons have been identified. These cascades involve oxyradical production, aberrant regulation of cellular ion homeostasis and activation of a stereotyped sequence of events involving mitochondrial dysfunction and activation of specific proteases.

Pathogenesis of Neurodegenerative Disorders provides a timely compilation of articles that encompasses fundamental mechanisms involved in neurodegenerative disorders. In addition, mechanisms that may prevent age-related neurodegenerative disorders are presented. Each chapter is written by an expert in the particular neurodegenerative disorder or mechanism or neuronal death discussed. Chapters that consider the role of oxidative stress as a central feature of all neurodegenerative disorders and the fundamental mechanisms of neuronal apoptosis and excitotoxicity, two forms of cell death central to many different neurodegenerative disorders, open this volume. Subsequent chapters focus on specific neurodegenerative disorders. Each chapter presents information on genetic and environmental factors that may contribute to these disorders and cell death cascades involved in these disorders are detailed. Chapters focus on Parkinson's disease, trinucleotide repeat disorders (including Huntington's disease), Alzheimer's disease and Down's syndrome (two disorders that appear to involve shared mechanisms), amyotrophic lateral sclerosis, ischemic stroke, spinal cord injury, and Duchenne muscular dystrophy.

Pathogenesis of Neurodegenerative Disorders will provide a valuable working reference for graduate students and postdocs beginning their careers in this field. In addition, because each chapter presents the most up-to-date specific information in the field, this book is valuable for senior scientists in allowing them to integrate information on cellular and molecular mechanisms across the wide field of neurodegenerative disorders.

Mark P. Mattson

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Oxidative Alterations in Neurodegenerative Diseases

William R. Markesbery, Thomas J. Montine, and Mark A. Lovell

INTRODUCTION

Age-related neurodegenerative diseases represent a major medical problem for modern society. As longevity of our population increases, these disorders could reach epidemic proportions. The key to understanding age-related neurodegenerative disorders is to determine why neurons degenerate and die in specific brain regions in different disorders. Major research is underway to understand the etiology and pathogenesis of these disorders to facilitate rational development of effective therapies. Numerous partially overlapping hypotheses about the pathogenesis of neurodegenerative diseases include genetic defects, altered membrane metabolism, trace element neurotoxicity, excitotoxicity, reduced energy metabolism, and free-radical-mediated damage. Accumulating evidence indicates that increased free-radical-mediated damage to cellular function contributes to the aging process and age-related neurodegenerative disorders. Indeed, increased free-radical-mediated damage relates closely to the reduced energy metabolism, trace element toxicity, and excitotoxicity hypotheses in neurodegeneration.

Free-radical-mediated damage occurs when free radicals and their products are in excess of antioxidant defense mechanisms, a condition often referred to as oxidative stress. Considerable recent data indicate that oxidative stress may play a role in Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and Pick's disease. These diseases share late-life onset and clinical symptoms that relate to region-specific neuron loss in the central nervous system (CNS). Although free-radical damage to neurons may not be the primary event initiating these diseases, it appears that free-radical damage is involved in the pathogenetic cascade of these disorders. The brain is especially vulnerable to free radical damage because of its abundant lipid content, high oxygen consumption rate, and endogenous neurochemical reactions of dopamine oxidation and glutamate excitotoxicity.

A free radical is defined as any atom or molecule with an unpaired electron in its outer shell. Multiple radicals exist, but the most common are formed from the reduction of molecular oxygen to water, and are typically referred to as reactive oxygen species (ROS):

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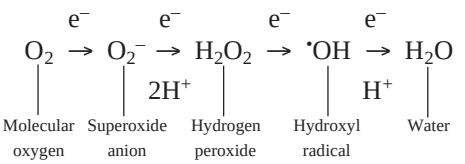

Single-electron reduction of molecular oxygen forms superoxide anion (O_2^-), a free radical that participates in cellular signaling but does not appear to be reactive with DNA or lipids. A second single-electron reduction converts O_2^- to hydrogen peroxide (H_2O_2), a reaction catalyzed by several forms of superoxide dismutase (SOD). Hydrogen peroxide does not have an unpaired electron and is not a free radical; however, it is an effective oxidant for many biological molecules. The hydroxyl radical ($^{\bullet}OH$) is formed from O_2^- and H_2O_2 by the Haber–Weiss reaction:

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

$^{\bullet}OH$ also can be formed by the Fenton reaction, in which H_2O_2 in the presence of the ferrous ion (Fe^{2+}) forms $^{\bullet}OH$:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

$^{\bullet}OH$ is the most reactive oxygen radical and capable of oxidizing lipids, carbohydrates, proteins, and DNA. Thus, the toxicity of O_2^- and H_2O_2 is primarily due to their conversion to $^{\bullet}OH$.

Nitric oxide ($NO^{\bullet}$) contains an unpaired electron and is a free radical, which has several physiologic functions including vasodilation. It is synthesized by the enzymatic oxidation of L-arginine to form citrulline through the action of calcium-activated, calmodulin-dependent nitric oxide synthase (NOS). Nitric oxide is produced in excitotoxicity, inflammation, and ischemia-reperfusion injury, and can react with O_2^- to produce peroxynitrite ($ONOO^-$):

$$NO^{\bullet} + O_2^- \rightarrow ONOO^-$$

Peroxynitrite is a powerful oxidant capable of damaging lipids, proteins, and DNA. It also can form $^{\bullet}OH$ and the nitrogen dioxide radical ($NO_2^{\bullet}$) as follows:

$$ONOO^- + H^+ \rightarrow {}^{\bullet}OH + NO_2^{\bullet}$$

Antioxidants are defined as substances that, when present at low concentrations compared with those of an oxidative substrate, significantly delay or inhibit oxidation of that substrate (Halliwell and Gutteridge, 1989). To defend against free radicals and maintain homeostasis, organisms have developed extensive antioxidant systems and repair enzymes to remove and repair oxidized molecules. Antioxidants have multiple mechanisms of action including preventing initiation of oxidation by radical scavenging, binding or removing catalyzing metal ions, limiting the propagation of the oxidative reaction, and decomposing peroxide (Halliwell and Gutteridge, 1989). Some antioxidants are shown in Table 1. Important enzymatic antioxidants present in the brain include copper–zinc (Cu/Zn)-SOD, manganese (Mn)-SOD, glutathione peroxidase (GSH-Px), and glutathione reductase (GSSG-R). The brain also contains a small amount of catalase (CAT). Numerous other nonenzymatic antioxidants and metal chelators are present in the brain.

Table 1
Antioxidants

Enzymatic	Nonenzymatic
Copper–zinc superoxide dismutase	Ascorbic acid
Manganese superoxide dismutase	Ceruloplasmin
Catalase	Uric acid
Glutathione peroxidase	Bilirubin
Glutathione reductase	Melatonin
Alpha and gamma tocopherol	Glutathione
	Methionine

Antioxidant-defense mechanisms can be upregulated in response to increased free-radical production (Cohen and Werner, 1994). Although upregulation of antioxidant defenses may confer protection, they are not completely effective in preventing oxidative damage, especially with aging of the organism.

SOURCES OF FREE RADICALS

Numerous sources of free radicals are present in the brain but the most common is from oxidative phosphorylation of adenosine 5'-diphosphate (ADP) to adenosine triphosphate (ATP) via the electron transport chain in the inner membranes of mitochondria. ATP is generated through the reduction of molecular oxygen to water by the sequential addition of four electrons and four H^+. Leakage of electrons along the electron transport chain causes O_2^- to form with the potential of forming OH^- via the Fenton reaction. Neurons are highly dependent on oxidative phosphorylation to generate ATP, and because the brain consumes larger amounts of oxygen than other organ, it is more vulnerable to oxidative stress. More active neurons or specific neuron compartments that contain mitochondria, such as synapses, may be particularly vulnerable to oxidative stress through this mechanism.

Excitotoxicity refers to the process by which glutamate and aspartate cause heightened neuronal excitability leading to toxicity and death through a mechanism that includes free-radical formation. Glutamate is the major excitatory neurotransmitter in the brain and glutamate-receptor-mediated excitotoxicity contributes to neuron damage in numerous pathological entities. This process is characterized by excessive influx of calcium into neurons resulting from activation of glutamate receptors, especially the *N*-methyl-D-aspartate (NMDA) receptor. The increase in calcium causes activation of phospholipase A2, which leads to release of arachidonic acid. The latter generates O_2^- via its metabolism by cyclooxygenases and lipooxygenases to form eicosanoids. In addition, the increase in intracellular calcium activates proteases that catalyze conversion of xanthine dehydrogenase to xanthine oxidase, which in turn catabolizes purine bases to form O_2^-. Thus, diminished energy metabolism can increase intraneuronal calcium, which leads to excitotoxicity, and these converging mechanisms are capable of generating ROS. Both of these mechanisms are thought to occur in neurodegenerative diseases, especially AD (Beal, 1995).

Another source of oxidative stress in the brain is through the enzymatic oxidative deamination of catecholamines by monoamine oxidase (MAO) to yield H_2O_2 (Fig. 1).

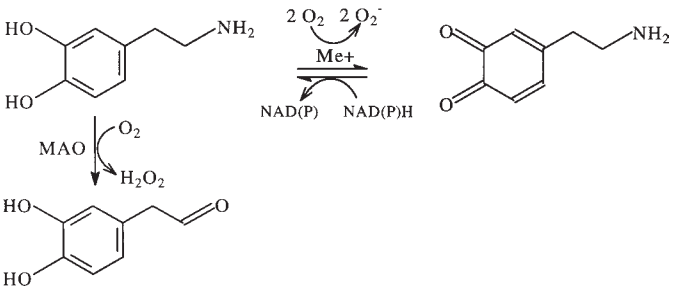

Fig. 1. Dopamine oxidation. Dopamine may be oxidized through both enzyme-dependent and enzyme-independent mechanisms. Enzymatic oxidation of dopamine is catalyzed by monoamine oxidase (MAO) to generate the aldehyde that then is oxidized further to dihydroxyphenylacetic acid. Oxidative deamination by MAO is accompanied by the production of hydrogen peroxide (H_2O_2). Enzyme-independent (autoxidation) of dopamine is catalyzed by paramagnetic metal ions (Me^+) such as iron, copper, or manganese. Autoxidation occurs via sequential one electron oxidation of the catechol nucleus to generate the o-quinone and superoxide anion (O_2^-).

In addition, catecholamines undergo trace-metal-catalyzed autoxidation to generate O_2^- (Cohen and Werner, 1994; Picklo et al., 1999). H_2O_2, generated in catecholaminergic neurons, can be converted by the iron-mediated Fenton chemistry to produce toxic $^{\bullet}OH$, which enhances oxidative neuronal damage and possibly contributes to neurodegeneration in PD and AD (Fu et al., 1998).

Microglial cells, the resident macrophages of brain, are capable of generating free radicals when stimulated. Activated microglia, which are markedly increased in the brain in AD (Carpenter et al., 1993), release O_2^- and H_2O_2 in vivo (Colton et al., 1994). Astrocytes and microglia stimulated with cytokines express NOS and generate NO-derived species including $ONOO^-$ (Goodwin et al., 1995; Ii et al., 1996).

Hensley et al. (1994) demonstrated that aggregated amyloid (Aβ)-peptides are capable of generating free radicals and inducing oxidative events. Dyrks et al. (1992) showed that an in vitro iron-catalyzed oxidation system caused transformation of nonaggregated Aβ-peptides into aggregated forms. Aβ-peptides cause H_2O_2 accumulation in cultured hippocampal neurons (Mattson et al., 1995) and antioxidants are capable of preventing experimental Aβ-peptide-induced neuron death in cultured cells (Goodman and Mattson, 1994; Mattson, 1997). Aggregated Aβ-peptides are capable of generating $NO^{\bullet}$ in cultured neural and microglial cells (Goodwin et al., 1995; Keller et al., 1998), which can produce $ONOO^-$. Thus, it is possible that the increase in aggregated Aβ-peptides in the brain in AD may increase free radical production that could play a role in its neurotoxicity.

TRACE ELEMENTS INVOLVED IN OXIDATION

Iron

An important trace element in oxidative reactions is iron (Fe) because it acts as a catalyst of free-radical generation through the Fenton reaction with formation of $^{\bullet}OH$ (*see* above). Iron, an essential element, is bound to proteins such as hemoglobin and

myoglobin or as a nonheme protein-bound complex such as transferrin, ferritin, and hemosiderin. Following absorption from the gastrointestinal tract, Fe is bound to transferrin, which delivers Fe to tissue where it is stored as ferritin. Brain cells have a high-affinity receptor for transferrin.

An early instrumental neutron activation analysis (INAA) study showed that following growth and development, Fe levels remain relatively stable from age 20 to 80 yr in normal brains, after which there is a small decline (Markesbery et al., 1984). In an INAA study of bulk brain specimens from numerous different regions, we found an elevation of Fe in AD compared with age-matched control subjects (reviewed in Markesbery and Ehmann, 1994). Significant elevations of Fe in AD gray matter were found compared with white matter. In a more recent study of seven brain regions of 58 AD and 21 control subjects, we found significant elevations of Fe in the frontal, temporal, and parietal neocortex, hippocampus, and amygdala, but not in the cerebellum (Cornett et al., 1998). Laser microprobe analyses show a significant elevation of Fe in neurofibrillary tangle (NFT)-bearing neurons in the hippocampus in AD (Good et al., 1992b). Using micro particle-induced X-ray emission (micro-PIXE) analysis, we found a significant elevation of Fe in the cores and rims of senile plaques (SP) in the amygdala of AD subjects (Lovell et al., 1998a). Two other studies (Candy et al., 1986; Edwardson et al., 1991) found elevated Fe in NFT-bearing neurons and SP in AD using a microprobe system.

M. Smith et al. (1997a) showed that redox-active Fe is associated with SP and NFT in AD and catalyzes an H_2O_2-dependent oxidation. Redox-active Fe bound to these pathological lesions of AD suggests the potential for generation of free radicals at the expense of cellular reductants. Iron regulatory protein 2 is associated with NFT, SP, and neuropil threads in AD and co-localizes with redox-active Fe, suggesting impaired Fe homeostasis in AD (Smith et al., 1998a).

Ferritin is present in SP in AD (Grundke-Iqbal et al., 1990) and ferritin from AD patients contains more Fe than brains of age-matched controls (Fleming and Joshi, 1987). An increase in heavy-chain isoferritin (H) to light-chain isoferritin (L) ratio is present in the frontal lobe of AD, but not in PD and the H/L isoferritin ratio is higher in caudate/putamen in PD than AD, indicating regional Fe alterations in both disorders (Connor et al., 1995). Transferrin is present around SP in AD (Connor et al., 1992), but transferrin-receptor density is significantly reduced in the hippocampus and neocortex in AD (Kalaria et al., 1992). The C2 allele of transferrin is significantly elevated in the blood of late-onset AD compared with age-matched controls and is twice as high in AD patients homozygous for apolipoprotein ε4 alleles compared with AD patients with one or no copies of the ε4 allele (Namekata et al., 1997). The Fe-binding protein, P97 or melanotransferrin, is elevated in the serum, cerebrospinal fluid (CSF), and brains of AD patients (Jefferies et al., 1996; Kennard et al., 1996).

Iron interaction with Aβ-peptides is of considerable interest. Iron promotes the aggregation of Aβ-peptides in vitro (Mantyh et al., 1993), and may be capable of modulating amyloid precursor protein (APP) processing (Bodovitz et al., 1995). Low Fe decreases soluble APP production and elevated levels increase soluble APP production. High levels of Fe inhibit the maturation of APP production of downstream catabolites. Iron modulation of APP may be at the level of α-secretase cleavage. Fe and lipid

peroxidation increase the vulnerability of neurons to Aβ-peptide toxicity (Goodman and Mattson, 1994), further supporting a role for Fe in the pathogenesis of AD.

Altered Fe homeostasis may play a role in dopaminergic neuron loss in PD. Elevated Fe levels were observed in neurons of the substantia nigra and in Lewy bodies using microprobe techniques (Hirsch et al., 1991; Good et al.; 1992a; Jellinger et al., 1992). Aluminum, known to increase lipid peroxidation caused by Fe salts (Gutteridge et al., 1985), is increased in the substantia nigra in PD (Hirsch et al., 1991; Good et al., 1992a). There is an increase in lipid peroxidation in the substantia nigra in PD as noted below. Dexter et al. (1990) showed that ferritin was decreased in the substantia nigra in PD, whereas Riederer et al. (1989) found it increased. Faucheux et al. (1995) observed an increase in lactoferrin receptors in substantia nigra neurons in PD that could be related to the accumulation of Fe within nigral neurons. If free Fe is increased in the substantia nigra in PD, it could enhance free-radical production through catechol autooxidation and Fenton chemistry, and could possibly be important in the pathogenesis of neuron loss.

Copper

Copper is an essential element that plays an important role in many enzymes and modulates numerous regulatory responses in cells. Copper is extremely efficient in generating free radicals owing to its ability to engage in redox reactions. The brain has a high Cu content compared with other organs and its highest level is in gray matter. Copper is bound to numerous enzymes and proteins including Cu/Zn-SOD, cytochrome oxidase, neurocuprein, and ceruloplasmin. Bulk brain studies of Cu show no significant differences in AD and control subjects or a decrease in Cu in AD (Plantin et al., 1987; Tandon et al., 1994; Deibel et al., 1996). Our micro-PIXE study demonstrated a significant increase in Cu in SP in the amygdala in AD (Lovell et al., 1998a). Multhaup et al. (1996) showed that the APP of AD reduces bound Cu^{2+} to Cu^{+}, which leads to disulfide bond formation in the APP. The reduction of Cu involves an electron-transfer reaction that could enhance the formation of $^{\bullet}OH$. The increase in Cu in SP may relate to the finding that soluble Aβ binds one Cu ion, but the aggregated state binds three Cu ions (Atwood et al., 1998).

Zinc

Zinc is an essential element important in numerous brain enzymes and proteins. It is redox inert and not directly involved in free-radical generation. The brain contains three Zn pools: (1) a membrane-bound metallothionein protein, (2) a pool in synaptic vesicles, and (3) a pool of free or loosely bound Zn in cytoplasm (Frederickson et al., 1989). Zinc is maintained within a relatively narrow range in brain and excess levels are neurotoxic (Cuajungco and Lees, 1997). Our INAA study demonstrated that brain Zn remains relatively constant in the brain throughout adult life (Markesbery et al., 1984). Two INAA studies demonstrated elevated Zn levels in frontal, temporal, and parietal lobes, hippocampus, and amygdala in AD (Deibel et al., 1996; Cornett et al., 1998). A micro-PIXE study showed increased Zn in the rims and cores of SP in AD and in AD neuropil compared with control neuropil (Lovell et al., 1998a). The relationship between Zn and Aβ is of considerable interest. Bush et al. (1994a) demonstrated Aβ specifically and saturably binds to Zn. In vitro concentrations of Zn above 300 n*M* rapidly destabilized a human $A\beta_{1-40}$ solution and induced aggregation of Aβ fibrils.

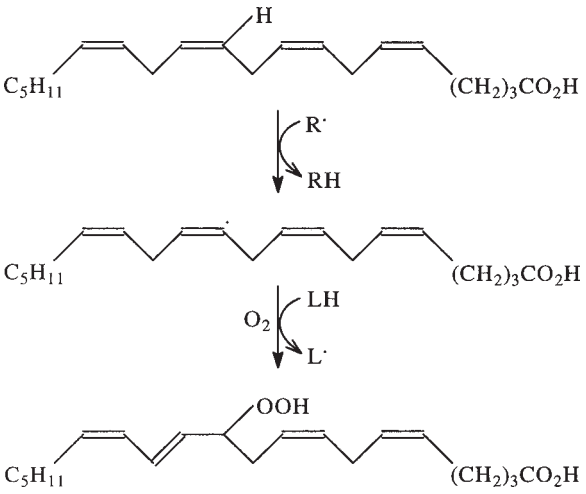

Fig. 2. Lipid peroxidation. Polyunsaturated fatty acids (arachidonic acid shown as an example) begins with hydrogen atom abstraction by a radical (R) to generate a lipid radical that then reacts with O_2 to generate a lipid hydroperoxyl radical (not shown). Propagation of lipid peroxidation occurs when the lipid hydroperoxyl radical abstracts a hydrogen atom from another lipid molecule (LH) to generate a lipid hydroperoxide and another lipid radical (L).

Zinc did not have this effect on rat $A\beta_{1-40}$. The Zn-containing transcription factor NF-κB is one of the regulators of APP synthesis. Zinc binding inhibits the cleavage of APP by α-secretase and inhibits α-secretase cleavage of Aβ (Bush et al., 1994b). Thus, it is possible that elevated Zn may lead to increased levels of transcription factors or influence APP processing (Bush et al., 1994b; Atwood et al., 1999). Hensley et al. (1994) demonstrated aggregation of Aβ has the potential of generating free radicals that can alter membranes and oxidative-sensitive enzymes. This suggests a mechanism by which elevated Zn concentration could contribute to oxidative stress through the accumulation of aggregated Aβ.

Overall, changes in Fe, Cu, and Zn could provide a microenvironment in the brain in which excess generation of free radicals could lead to increased lipid, protein, and DNA oxidation and, in conjunction with multiple other factors, contribute to the pathophysiological cascade of neuron injury in neurodegenerative diseases.

LIPID PEROXIDATION

Lipid peroxidation is one of the major outcomes of free-radical-mediated injury to tissue. Peroxidation of fatty acyl groups, mostly in membrane phospholipids, has three phases: initiation, propagation, and termination. Initiation occurs when a hydrogen atom is abstracted from a fatty acyl chain, leaving a carbon-based radical (Fig. 2). Hydrogen atoms can be abstracted by carbon-, nitrogen-, oxygen-, or sulfur-based radicals. Among the oxygen-based radicals, ·OH is the most reactive at hydrogen atom abstraction. Allylic hydrogens are most labile to abstraction because their carbon–hydrogen bond is made more acidic by the adjacent carbon–carbon double bond. Therefore, polyunsaturated fatty acids are the most vulnerable to lipid peroxidation. The second phase, propagation of lipid peroxidation, begins with reaction of the carbon-

based radical on the fatty acyl chain with molecular oxygen to form a hydroperoxyl radical (Fig. 2). These are extremely reactive species that abstract a second hydrogen atom from nearby fatty acyl chains to generate a lipid hydroperoxide and a new carbon-based radical, thus propagating peroxidation. Finally, termination of lipid peroxidation occurs when two radical species react with each other to form a nonradical product. Thus, lipid peroxidation is a self-propagating process that will proceed until the substrate is consumed or termination occurs. Cellular antioxidant systems may intercede by either preventing initiation of lipid peroxidation (e.g., SOD, CAT, or Fe chelators) or limiting propagation (e.g., ascorbate, α-tocopherol, and reduced glutathione).

There are two broad outcomes to lipid peroxidation, viz., structural damage to membranes and generation of bioactive secondary products. Membrane damage derives from the generation of fragmented fatty acyl chains, lipid–lipid crosslinks, and lipid–protein crosslinks (Farber, 1995). In addition, lipid hydroperoxyl radicals can undergo endocyclization to produce novel fatty acid esters that may disrupt membranes. Two classes of cyclized fatty acids are the isoprostanes and neuroprostanes, derived *in situ* from free-radical-mediated peroxidation of arachidonyl or docosahexadonyl esters, respectively (Morrow and Roberts, 1997; Roberts et al., 1998). In total, these processes combine to produce changes in the biophysical properties of membranes that can have profound effects on the activity of membrane-bound proteins.

Fragmentation of lipid hydroperoxides, in addition to producing abnormal fatty acid esters, also liberates a number of diffusible products, some of which are potent electrophiles (Esterbauer et al., 1991; Porter et al., 1995). The most abundant diffusible products of lipid peroxidation are chemically reactive aldehydes such as malondialdehyde, acrolein, 4-hydroxy-2-nonenal (HNE) from ω-6 fatty acyl groups, 4-hydroxy-2-hexenal (HHE) from ω-3 fatty acyl groups, and alkanes (Esterbauer et al., 1991). Alternatively, hydrolysis of abnormal fatty acyl groups generated by lipid peroxidation can liberate abnormal products from damaged lipids. For example, free isoprostanes and neuroprostanes are easily detectable in plasma and CSF (Morrow and Roberts, 1997; Montine et al., 1998b, 1999a, b; Roberts et al., 1998).

Some lipid peroxidation products are thought to contribute to the deleterious effects of lipid peroxidation in tissue. Reactive aldehydes from lipid peroxidation react with a number of cellular nucleophiles, including protein, nucleic acids, and some lipids (Esterbauer et al., 1991). Indeed, many of the cytotoxic effects of lipid peroxidation can be reproduced directly by electrophilic lipid peroxidation products such as HNE (Farber, 1995). These include depletion of glutathione, dysfunction of structural proteins, reduction in enzyme activities, and induction of cell death. Chemically stable products of lipid peroxidation also may contribute to the pathogenesis of lipid peroxidation through receptor-mediated signaling. For example, peroxidation and fragmentation of polyunsaturated fatty acyl groups in phosphatidylcholines can generate platelet-activating-factor analogs that stimulate cellular receptors (McIntyre et al., 1999). Also, at least one isomer of the isoprostanes is a potent vasoconstrictor, likely through a receptor-mediated mechanism (Morrow and Roberts, 1997).

In addition to being potential mediators of tissue damage, products of lipid peroxidation are commonly used to quantify the extent of lipid peroxidation. When considering the quantification of lipid peroxidation, it is necessary to define whether the assay is being applied in vitro or in vivo. Assays such as those for thiobarbituric-

reactive substances (TBARS) or chromatography for specific secondary products are accurate measures of lipid peroxidation in vitro when metabolism of the lipid peroxidation products does not occur. However, in more complicated model systems with metabolic activity and in vivo, extensive metabolism of electrophilic lipid peroxidation products compromises the accuracy of these assays (Gutteridge and Halliwell, 1990; Moore and Roberts, 1998). One solution to the problem of accurately quantifying lipid peroxidation in vivo is to measure one class of isoprostanes, the F_2-isoprostanes. F_2-isoprostanes are chemically stable products of free-radical-mediated damage to arachidonyl esters that are not extensively metabolized *in situ* (Morrow and Roberts, 1997).

Lipid Peroxidation in Neurodegenerative Diseases

Alzheimer's Disease

There is compelling evidence that the magnitude of lipid peroxidation in the brains of AD patients examined postmortem exceeds that in age-matched control individuals. Seminal experiments demonstrated significantly increased TBARS in diseased regions of AD brain obtained postmortem compared with age-matched control individuals (Lovell et al., 1995). Other studies measured free HNE and acrolein in AD brain tissue and showed that both are elevated in diseased regions of AD brain compared with controls (Markesbery and Lovell, 1998; Lovell et al., 2000a).

F_2-isoprostane levels are elevated in the frontal lobe and hippocampus of AD patients compared with controls with short postmortem intervals (Pratico et al., 1998; Montine et al., 1999a). In addition, F_2-isoprostanes are elevated in the cerebral cortex of aged homozygous apolipoprotein E (apoE) gene deficient mice (Montine et al., 1999c; Pratico et al., 1999). A class of free-radical-generated products analogous to the F_2-isoprostanes, but generated from docosahexenoic rather than arachidonic acid, has been described and termed F_4-neuroprostanes (Roberts et al., 1998). Because docosahexenoic acid is more labile to peroxidation than arachidonic acid and docosahexenoic acid is relatively enriched in brain, it was proposed that F_4-neuroprostanes might be more sensitive markers of brain oxidative damage than F_2-isoprostanes. Indeed, F_4-neuroprostanes are significantly more abundant than F_2-isoprostanes in cerebral cortex of aged homozygous apolipoprotein E gene deficient mice (Montine et al., 1999c). One group reported that F_4-neuroprostanes (called F_4-isoprostanes in their publication) are elevated in temporal and occipital lobes, but not parietal lobe of AD patients compared with controls, and that F_4-neuroprostane levels are higher than F_2-isoprostanes in these regions (Nourooz-Zadeh et al., 1999). However, interpretation of data from this study is limited by excessively long postmortem intervals (47 h average in AD patients) (Nourooz-Zadeh et al., 1999).

In contrast to quantification, several groups have studied the localization of lipid peroxidation products in AD brain. These studies used immunochemical detection of protein covalently modified by lipid peroxidation products or displaying protein carbonyls. There is broad agreement among these studies. Consistent with the quantitative studies described above, hippocampus and cerebral cortex from AD patients display protein modifications that are not detectable or are barely detectable in the corresponding brain regions from age-matched control individuals (Sayre et al., 1997; Montine et al., 1997a, b, 1998a; Calingasan et al., 1999; Smith et al., 1998b). Also, in AD patients,

proteins modified by lipid peroxidation products are present in diseased regions of brain but not in uninvolved regions. In diseased regions of AD brain, neuronal cytoplasm and NFT are the major focus of protein modification. Importantly, none of these studies observed modified proteins in or adjacent to neuritic plaques. This stands in sharp contrast to genetically modified mice expressing mutant human APP, where increased HNE-protein adduct immunoreactivity and advanced glycation end product immunoreactivity (*vida infra*) are localized adjacent to or within amyloid deposits (Smith et al., 1998c). One group observed that the tissue distribution of HNE-protein adducts varies with apoE genotype. In these studies, one chemical form of HNE-protein adducts, the 2-pentylpyrrole adduct, co-localized with NFT and was significantly associated with homozygosity for the ε4 allele of apoE (Montine et al., 1997 a, b). Michael adducts, the most abundant chemical form of HNE-protein adducts, were observed in pyramidal neuron and astrocyte cytoplasm of AD patients with an ε3 allele of apoE, but only in pyramidal neuron cytoplasm of AD patients homozygous for ε4 allele of apoE (Montine et al., 1998a). These authors suggested that the tissue distribution, but not the apparent quantity, of HNE-protein adducts in AD is influenced by apoE genotype, perhaps related to the essential role of apolipoprotein E in CNS lipid trafficking. Recent findings suggest that apoE4 promotes, whereas apoE3 and apoE2 suppress, oxidative damage to neurons. The three apoE isoforms differ in that E2 contains two cysteine residues, E3 contains two cysteines, and E4 lacks the cysteines. Apolipoprotein E2, and to a lesser extent E3, can protect neurons against oxidative insults that induce lipid peroxidation, whereas E4 is ineffective (Pedersen et al., 2000). The neuroprotective effect of E2 is correlated with an increased HNE-binding capacity of the protein. Thus, apolipoprotein E genotype appears to affect risk for AD by modifying the antioxidant capacity (specifically, the ability to bind HNE) of the protein.

More recently, CSF has been investigated as a source of CNS tissue, for the assessment of lipid peroxidation, in AD brain. One study measured free HNE in CSF obtained from the lateral ventricles postmortem and showed that its concentration is significantly elevated in AD patients compared with age-matched controls (Lovell et al., 1997). Two studies determined the concentration of F_2-isoprostanes, and in one case F_4-neuroprostanes, in CSF obtained from the lateral ventricles postmortem and showed significant elevations in AD patients compared with age-matched controls (Montine et al., 1999a, b). Importantly, CSF F_2-isoprostane concentrations in AD patients are significantly correlated with decreasing brain weight, degree of cerebral cortical atrophy, and increasing Braak stage, but not with apoE genotype or the tissue density of neuritic plaques or NFT (Montine et al., 1999b). It is noteworthy that all of the postmortem CSF studies in AD patients used material from individuals with very short (average 2–3 h) postmortem intervals.

The aforementioned studies of brain tissue and CSF were performed using material collected postmortem. AD patients undergoing postmortem examination typically have advanced disease and an average duration of dementia of 8–12 yr. Therefore, a serious limitation to analysis of tissue obtained postmortem is that the increased brain lipid peroxidation in AD patients might be a consequence of late-stage disease. Obviously, a late-stage consequence of AD would be a less attractive therapeutic target than a process contributing to disease progression at an earlier stage. A recent study of probable AD patients early in the course of dementia showed that F_2-isoprostanes are signifi-

cantly elevated in CSF obtained from the lumbar cistern compared with age-matched hospitalized patients without neurological disease (Montine et al., 1999a). The average duration of dementia in these probable AD patients was less than 2 yr. In combination with the postmortem studies, this recent study with probable AD patients demonstrates that brain lipid peroxidation is elevated both early and late in the course of AD, and provides a rationale for slowing the progression of AD by suppressing brain lipid peroxidation (Sano et al., 1997).

Data obtained from studies of experimental animal and cell-culture models of AD suggest that Aβ-peptide and Fe may be important initiators of lipid peroxidation and neuronal degeneration (Mattson, 1998). Exposure of cultured neurons and synapses to Aβ-peptide results in membrane lipid peroxidation and HNE production. HNE renders neurons vulnerable to apoptosis and excitotoxicity by covalently modifying, and impairing the function of, membrane ion-motive ATPases, glucose transporters, and glutamate transporters (Mark et al., 1995, 1997a, b; Keller et al., 1997). Conversely, HNE enhances calcium influx through membrane glutamate receptor channels and voltage-dependent channels. Aβ-peptide, lipid peroxidation, and HNE can alter neurotransmitter and neurotrophic factor signal transduction pathways involving GTP-binding proteins and transcription factors, which may contribute to cognitive dysfunction in AD (Kelly et al., 1996; Blanc et al., 1997). Recent findings suggest that lipid peroxidation can be suppressed, and AD prevented or delayed, by dietary and behavioral manipulations that either directly suppress lipid peroxidation or activate neurotrophic factor- and stress-responsive signaling pathways (Bruce-Keller et al., 1999; Zhu et al., 1999).

Parkinson's Disease

Idiopathic PD is the second most common neurodegenerative disease. Similar to AD, there is compelling evidence from human postmortem tissue that increased oxidative stress occurs in the midbrain of patients with PD compared with age-matched controls (Coyle and Puttfarcken, 1993; Cohen and Werner, 1994; Jenner and Olanow, 1998). However, the mode of oxidative stress to brain may differ somewhat from AD. For example, depletion of nigral reduced glutathione is proposed to be a relatively early and specific event in PD (Jenner, 1994). Lipid peroxidation appears to be a component of nigral degeneration in PD when examined postmortem. Compared with controls, PD patients have elevated TBARS and lipid hydroperoxides in midbrain, immunochemically detectable HNE-protein adducts in midbrain, and elevated free HNE in CSF (Dexter et al., 1989, 1994; Yoritaka et al., 1996; Shelley, 1998). Interestingly, HNE-protein adducts were present in several midbrain nuclei, and not just in the substantia nigra of PD patients (Yoritaka et al., 1996), a pattern similar to 8-hydroxyguanosine immunoreactivity in midbrain from PD patients (Zhang et al., 1999). Despite the postmortem evidence that associates PD with increased midbrain lipid peroxidation, pharmacologic studies have questioned the significance of lipid peroxidation earlier in the course of PD (Parkinson Study Group, 1989).

In humans and some mammals including mice, exposure to 1-methyl-4-phenyltetrahydropyridine (MPTP) produces selective degeneration of dopaminergic neurons in the CNS. MPTP-induced nigral degeneration in animals has been used widely as a model of PD (Langston, 1994). MPTP-induced dopaminergic degeneration is thought

to involve mitochondrial dysfunction and oxidative damage. Indeed, mice lacking both alleles of the Cu/Zn-SOD1 or the glutathione peroxidase gene (*GPX1*) are significantly more vulnerable to MPTP-induced dopaminergic neurodegeneration than littermate controls (Klivenyi et al., 2000; Zhang et al., 2000). Moreover, the brainstem of mice systemically exposed to MPTP show an acute eightfold increase in HNE concentrations, a 50% reduction in reduced glutathione levels, and a sixfold increase in the concentration of HNE-glutathione adducts within 24 h of exposure (Shelley, 1998).

In summary, these data suggest that some lipid-peroxidation products may participate in the pathogenesis of MPTP-induced dopaminergic neurodegeneration and are elevated in the substantia nigra in late stage PD. The role of nigral lipid peroxidation in earlier stages of PD is not clear.

Amyotrophic Lateral Sclerosis

Similar to AD and PD, ALS has both familial and sporadic forms. Research into the role of oxidative damage in ALS has been fueled by the discovery that mutations in the gene for Cu/Zn-SOD1 are the cause of a subset of familial ALS (Rosen et al., 1993). Indeed, some lines of mice expressing SOD1 with mutations linked to ALS develop a disease phenotype that closely mimics familial ALS and is thought to derive in part from increased free-radical-mediated damage (Dal Canto and Gurney, 1994; Gurney et al., 1994; Tu et al., 1997). Data on postmortem human tissue from patients with ALS suggest that the role of lipid peroxidation in this disease may be complex. Postmortem examination of patients with sporadic ALS showed increased protein carbonyl formation and other signatures of oxidative damage, but not elevated TBARS, in motor cortex and immunoreactivity for malondialdehyde protein adducts in spinal cord (Ferrante et al., 1997). Familial ALS patients, both with and without mutations in SOD1, do not have motor-cortex changes but have spinal-cord changes similar to sporadic ALS patients (Ferrante et al., 1997). Others showed that lumbar spinal cord obtained postmortem from sporadic ALS patients has immunohistochemically detectable HNE-protein adducts in the anterior horn (Pedersen et al., 1998). These investigators also showed by immunoprecipitation that one of the modified proteins is an excitatory amino acid transporter (EAAT2), a molecule whose dysfunction has been implicated in sporadic ALS (Browne and Beal, 1994).

Two studies examined CSF from living ALS patients for evidence of increased lipid peroxidation. One study measured free HNE in CSF obtained from the lumbar cistern of sporadic ALS patients at initial diagnosis and before therapy (Smith et al., 1998). HNE was measured using high-performance liquid chromatography (HPLC) with fluorescence detection following derivatization of CSF, an analytically less rigorous approach than chromatography followed by mass spectrometry, the method used to quantify free HNE in CSF from PD patients. Nevertheless, this study demonstrated a significant elevation in free HNE levels in CSF from sporadic ALS patients compared to patients with several other neurodegenerative diseases, but not when compared to patients with Guillain-Barré syndrome or chronic inflammatory demyelinating polyneuropathy. CSF levels of free HHE were not elevated in the same CSF samples (Smith et al., 1998). HHE is generated by the same chemistry as HNE except ω-3 fatty acids are the substrates for HHE and ω-6 fatty acids are the substrates for HNE. In a separate, smaller study, F_2-isoprostanes in CSF from sporadic ALS patients were not signifi-

cantly different from age-matched controls (Montine et al., 1999a). The average disease duration in this group of ALS patients was approx 2 yr and many of the patients had already initiated therapy. In summary, data from postmortem studies consistently associate ALS with increased lipid peroxidation in spinal cord and suggest a mechanism whereby lipid-peroxidation products could contribute to disease progression. Moreover, a closely related animal model of familial ALS indicates that free-radical-mediated damage may contribute to disease progression. Increased oxidative stress and increased vulnerability to excitotoxicity have been documented in studies of motor neurons from Cu/Zn-SOD mutant ALS mice (Kruman et al., 1999). In addition, Fe and HNE have been shown to impair glucose and glutamate transport in cultured motor neurons, suggesting a role for lipid peroxidation in the deficit in glutamate transport and energy metabolism documented in studies of ALS patients (Pedersen et al., 1999). Finally, some but not all data from CSF support a role for increased lipid peroxidation early in the course of sporadic ALS.

Huntington's Disease

HD is an autosomal dominant disease in which striatal degeneration is proposed to derive significant contributions from impaired energy metabolism, excitotoxicity, and increased oxidative stress (Beal, 1995). Postmortem tissue studies of striatum from HD patients have shown increased indices of a number of oxidative damage markers, including TBARS (Beal, 1995). Moreover, transgenic mouse models of HD have increased levels of free-radical production (Browne et al., 1999). A possible role for lipid peroxidation in HD was recently highlighted by a study showing elevated F_2-isoprostanes in CSF obtained from the lumbar cistern of HD patients compared to patients with multiple system atrophy-parkinsonian type (striatonigral degeneration) or hospitalized controls without neurological disease (Montine et al., 1999b).

PROTEIN OXIDATION

Free radicals can attack amino acids and lead to damage and inactivation of enzymes or receptors as well as cause protein–protein crosslinking. Oxidative modifications of protein residues in neurons are mediated by a variety of systems including lipid peroxidation, as noted above. Oxidation of amino acid residues in protein can have deleterious effects on functional properties of cell homeostatic balance. Oxidative damage to enzymes critical to brain function can cause neuron degeneration and result in accelerated brain aging (Stadtman, 1992). Stadtman described a two-step process in which the first step is oxidation of enzyme amino acids by free radicals yielding carbonyl derivatives. The second step involves further degradation of the enzyme by proteases to amino acids and peptides.

Protein Oxidation in Neurodegenerative Disease

Alzheimer's Disease

Protein carbonyl formation, a measure of oxidative damage to proteins can be quantified by derivatization with 2,4-dinitrophenylhydrazide (DNP). An early study demonstrated that there is increased brain protein carbonyls in aging and AD (Smith et al., 1991). Subsequently, other studies demonstrated an increase in protein carbonyls in the brain in AD (Hensley et al., 1995; Gabbita et al., 1999), and in Pick's disease and

dementia with Lewy bodies (Aksenova et al., 1999). Smith et al. (1996), using immunocytochemical techniques with *in situ* DNP labeling linked to an antibody system against DNP, demonstrated the presence of protein carbonyls in NFT and glia, but not in NFT-free neurons in AD. These changes were not found in control brains and are similar to what has been observed for protein adducts from lipid-peroxidation products.

The enzymes glutamine synthetase (GS) and creatine kinase (CK) are especially sensitive to oxidative modification (Stadtman, 1992). Two studies reported a significant decline in glia-specific GS activity in the hippocampus and neocortex in AD compared with age-matched controls (Smith et al., 1991; Hensley et al., 1995). Decreased levels of GS could result in diminished 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, loss of the enzyme could alter nitrogen balance, pH, and glutathione synthesis in astrocytes.

Creatine kinase BB is a member of the CK gene family and the predominant cytosolic CK isoform in the brain. Creatine kinases are a family of enzymes that catalyze a reversible transfer of a phosphoryl group between ATP and creatine. Cells respond to external challenges by mobilizing the creatine phosphate (CrP)/CK system (Struzynska et al., 1997) and increasing CK BB expression (Aksenov et al., 1998). Activation of the CrP/CK system and changes in CK expression may be an early indicator of oxidative and bioenergetic stress. Hensley et al. (1995) demonstrated that decreased CK BB activity in brain correlates well with the presence of NFT in severely affected regions. Aksenov et al. (1997) and Aksenova et al. (1999) demonstrated that CK BB is decreased in several neurodegenerative diseases including AD, Pick's disease, and dementia with Lewy bodies, but is decreased most in AD. These studies demonstrated that the CK BB decline is not a result of gene expression, which suggests that posttranslational oxidative modification of the enzyme contributes to the loss of CK BB activity (Aksenov et al., 1997, 2000a). A study using two-dimensional fingerprinting of oxidatively modified protein demonstrated decreased specific activity of CK BB that correlated with an increase in protein carbonyl content in the enzyme (Aksenov et al., 2000a). This suggests that introduction of carbonyl groups into amino acid residues in the active site of CK BB by oxidation or reaction with reactive aldehydes participates in the inactivation of the enzyme. A subsequent two-dimensional fingerprinting study demonstrated significantly elevated protein carbonyls in β-actin and CK BB in AD, but insignificant elevations of β-tubulin and undetectable protein carbonyl immunoreactivity in tau isoforms and glial fibrillary acidic proteins (Aksenov et al., 2000b). David et al. (1998) described a correlation between reduced CK activity and decreased ATP binding to CK BB in the brain in AD. The ATP-binding domain of CK BB contains arginine, histidine, and lysine, amino acid residues that may be converted into carbonyl derivatives by free radicals and modified by lipid peroxidation products. Oxidative inactivation of CK BB may involve the modification of these amino acid residues in the ATP-binding site of CK BB.

Methionine, cysteine, and tryptophan are also amino acid vulnerable to oxidation. The principal product of methionine oxidation is methionine sulfoxide (MetSO). Several studies have demonstrated that proteins lose their biological activity when specific

methionine residues are oxidized to MetSO (Brot and Weissbach, 1983; Swaim and Pizzo, 1988; Vogt, 1995). Levine et al. (1996) suggested that methionine residues act as a last line of antioxidant defense in proteins. High concentrations of methionine in proteins allow effective scavenging of oxidants to form MetSO before an attack on residues critical to structure or function. The enzyme responsible for reduction of MetSO back to methionine is methionine sulfoxide reductase (MsrA). Most biological systems contain disulfide reductases and MetSO reductases that can convert the oxidized forms of cysteine and methionine residues to their unmodified forms. According to Berlett and Stadtman (1997), methionine and cysteine oxidative modification products are the only residues that can be repaired within protein. Moskovitz et al. (1998) found high levels of MetSO in yeast MsrA mutants when exposed to oxidative stress compared with wild-type strain, which indicates MsrA possesses an antioxidant function. This suggests that MsrA could have an important role in providing cells with a defense system against oxidative stress. A recent study showed MsrA activity in the brain of AD patients was diminished in all regions studied and reached statistical significance in the superior and middle temporal gyri, inferior parietal lobule, and hippocampus (Gabbita et al., 1999). Messenger RNA analysis suggested that the loss in enzyme activity may be the result of a posttranslational modification of MsrA or defective translation resulting in inferior processing of the mRNA. This study suggests that a decline in MsrA activity could reduce the antioxidant defenses and increase the oxidation of critical proteins in neurons in the brain in AD.

Peroxynitrite causes nitration of tyrosine residues that yield nitrotyrosine, which is used as an indicator of $ONOO^-$ activity. Investigators using immunohistochemistry found nitrotyrosine present in NFT in the hippocampus in AD (Good et al., 1996; Smith et al., 1997b). Hensley et al. (1998) demonstrated significant elevations of protein-bound 3-nitrotyrosine and 3,3'-dityrosine in the hippocampus, neocortex, and ventricular CSF of AD subjects compared with normal control subjects. These studies indicate that nitric oxide and its redox congeners, especially $ONOO^-$, are likely involved in protein oxidation in AD.

Parkinson's Disease

Alam et al. (1997b) described a generalized increase in protein carbonyls in the brain in PD, but not in brain of patients with incidental Lewy body disease (putative presymptomatic PD), suggesting that oxidative protein damage occurs late in PD or that L-Dopa treatment contributes to protein oxidation. Good et al. (1998) demonstrated the presence of nitrotyrosine immunoreactivity in Lewy bodies and in amorphous deposits in intact and degenerating neurons of the substantia nigra in autopsied PD patients, indicating that oxidative modification of proteins has occurred in the target cells of this disorder. A relatively selective inhibitor of the neuronal isoform of nitric oxide synthase, 7-nitroindazole, protects against MPTP-induced dopamine depletion in mice (Schulz et al., 1995) and against dopamine depletion and loss of tyrosine hydroxylase-positive neurons in the substantia nigra of MPTP-treated baboons (Hantraye et al., 1996). Knockout mice deficient in the neuronal isoform of NOS are resistant to MPTP neurotoxicity (Przedborski et al., 1996). These studies suggest that $NO^{\bullet}$ may play a role in PD and an experimental model of PD.

GLYCATION

Analogous to protein adduction by lipid-peroxidation products, nonenzyme-catalyzed posttranslational modification of proteins by reducing sugars is associated also with some neurodegenerative diseases. This process, termed glycation, is initiated by reversible Schiff-base formation between a protein-bound amino group and an aldose that undergoes Amadori rearrangement to regenerate carbonyl activity. Subsequent irreversible rearrangements, fragmentations, dehydrations, and condensations yield a complex mixture of protein-bound products termed advanced glycation endproducts (AGEs). The chemical structures of AGEs have been partially characterized; the best studied are pentosidine, pyrraline, and *N*-(carboxymethyl)lysine (CML). Both the reversible adduct formation and subsequent evolution to AGEs are accelerated by oxygen in a process called glycoxidation (Smith et al., 1995).

AGEs have been studied most extensively from the perspective of diabetes mellitus and its complications where their accumulation is correlated with the degree of hyperglycemia. However, accumulation of AGEs in tissue is also associated with advancing age and with some disorders that are not characterized by hyperglycemia but are associated with oxidative stress, e.g., uremia and AD. Moreover, molecules other than reducing sugars can lead to AGE formation under conditions of increased oxidative stress. For example, other carbohydrates and even ascorbate can lead to AGE formation (Dunn et al., 1990; Dyer et al., 1991; Grandhee and Monnier, 1991).

Like lipid-peroxidation products, the biological consequences of AGEs may be viewed as deriving either from biochemical reactions or from receptor-mediated processes. AGEs can damage structural proteins and enzymes, thus rendering them dysfunctional. For example, enzymatic activity of human Cu/Zn-SOD is inactivated by glycation (Arai et al., 1987). AGEs can also be redox active, generate oxidative stress themselves, and induce neuronal apoptosis (Yan et al., 1994; Kikuchi et al., 1999). In addition to these deleterious biochemical reactions, AGE-modified proteins may bind to a receptor (RAGE) that exists on several cell types including neurons and glia. One role of RAGE is thought to be incorporation of AGE-modified proteins for degradation. However, binding of ligands, including Aβ-peptide, to RAGE on neurons in culture stimulates production of reduced oxygen species, and binding to RAGE on microglia in culture leads to cellular activation (Du Yan et al., 1997).

Glycoxidation in Neurodegenerative Diseases

Alzheimer's Disease

AGE formation in neurodegenerative diseases has been investigated most extensively in AD. These studies have focused on immunohistochemical localization of AGEs in brain. The results are not as consistent as those for localizing lipid-peroxidation products. Antibodies to pentosidine or pyrraline protein adducts are immunoreactive with neuritic and diffuse SP as well as intracellular and extracellular NFT in the hippocampus of AD patients (Smith et al., 1994). This study also observed immunoreactivity with both antibodies to vessel walls. The same pattern of immunoreactivity is present in hippocampal sections from control individuals as well as patients with diabetes; however, the extent of staining is much less than in AD because SP and NFT densities were much lower (Smith et al., 1994; Sasaki et al., 1998). Using different antibodies to AGEs, others observed a similar pattern of immunoreactivity in AD brain (Dickson et al., 1996; Munch et al., 1998; Sasaki et al., 1998). One group noted

that intracellular NFT are more strongly immunoreactive than extracellular NFT and that granulovacuolar degeneration is immunoreactive for AGEs (Sasaki et al., 1998). Others showed that Hirano bodies are immunoreactive for AGEs (Munch et al., 1998). Diffuse neuron cytoplasmic immunoreactivity was not reported in these studies. Other groups using antibodies directed at CML demonstrated diffuse pyramidal neuron cytoplasmic immunoreactivity that apparently increases with age and is present in hippocampal sections from AD patients (Kimura et al., 1996; Takedo et al., 1996). One of these studies noted that while CML immunoreactivity in hippocampus from AD patients is also present extracellularly, CML immunoreactivity is not co-localized with SP (Takedo et al., 1996).

Other Neurodegenerative Diseases

Investigation of AGEs in neurodegenerative diseases other than AD has been limited. Pentosidine and pyrraline immunoreactivity has been reported in the substantia nigra and locus ceruleus of patients with PD, and in the frontal and temporal cortex, but not hippocampus, of patients with dementia with Lewy bodies (Castellani et al., 1996). Staining was localized to the periphery of some Lewy bodies. Another study detected AGEs on intraneuronal hyaline inclusions of spinal cord from patients with ALS linked to mutations in SOD1 and in mice that express mutant human SOD1 (Shibata et al., 1999). AGE immunoreactivity has been demonstrated in NFT from patients with progressive supranuclear palsy, Guamanian parkinsonism–dementia complex, and Guamanian ALS (Sasaki et al., 1998). Finally, AGE immunoreactivity has been observed with Pick bodies and granulovacuolar degeneration in temporal lobe sections of patients with Pick's disease (Sasaki et al., 1998).

DNA OXIDATION

Accumulated damage to DNA in nondividing mammalian cells may play a role in aging and age-associated diseases. Oxidation of DNA causes strand breaks, sister chromatid exchange, DNA–DNA and DNA–protein crosslinking and base modification. Defects in DNA synthesis can produce mutant genes that lead to altered proteins. These damaged proteins can alter functions and result in functional defects and eventually cell death.

Multiple oxidants of DNA exist including $^{\bullet}OH$, $ONOO^{-}$, and singlet oxygen. Other species, O_2^{-} and H_2O_2 are linked to DNA damage by the Fe-mediated Fenton reaction. Secondary effects of oxidation can lead to DNA damage through aldehyde breakdown products of lipid peroxidation that form aldehyde–DNA adducts (Douki and Ames, 1994; Ames et al., 1995). Multiple oxidative DNA adducts have been identified but the most thoroughly studied is the adduct involving the C-8 hydroxylation of guanine, 8-hydroxy-2-deoxyguanosine (8-OHdG), which has become the most popular method for monitoring DNA oxidation in vivo (Beckman and Ames, 1997).

Cells respond to DNA alterations by repairing the damage and restoring the physical and functional state of the genotype. Three modes of DNA repair are: (1) base excision repair, (2) nucleotide excision repair, and (3) mismatch repair (Friedberg and Wood, 1996). Base excision repair is carried out by a group of enzymes termed DNA glycosylases that recognize the damaged base and cleaves its glycosylic bond. Nucleotide excision repair removes damaged oligonucleotide fragments via large enzyme complexes. Defective DNA repair may be of considerable importance in aging and age-associated neurodegenerative disorders.

DNA Oxidation in Neurodegenerative Disease

Alzheimer's Disease

The study of oxidative DNA damage in the brain is in its infancy and most of the reports are from aging, AD, or PD studies. Mullaart et al. (1990) described a twofold increase in DNA strand breaks in the brain in AD. Mecocci et al. (1993) reported an increase in 8-OHdG in nuclear and mitochondrial brain fractions in aging. Using HPLC, they demonstrated a 10-fold increase in brain mitochondrial DNA oxidation compared with brain nuclear DNA oxidation and a 15-fold increase in brain DNA oxidation in subjects older than 70 yr of age. These same investigators demonstrated a threefold increase in mitochondrial DNA oxidation in the parietal lobe in AD (Mecocci et al., 1994) and a small but significant increase in oxidative damage to brain nuclear DNA.

More recent studies of DNA oxidation have used gas chromatography with mass spectroscopy (GC-MS), a highly sensitive method for identifying oxidative adducts from DNA bases. Lyras et al. (1997), using GC-MS, found various bases increased or decreased in total brain DNA in different brain regions in AD. The most consistent elevations were in 8-OHdG, 8-hydroxyadenine and 5-hydroxycytosine in the parietal lobe in AD. Using GC-MS with stable isotope-labeled oxidized base analogs for standards, we studied nuclear DNA from four brain regions in AD patients and prospectively evaluated control subjects, all with short postmortem intervals (Gabbita et al., 1998). This study demonstrated statistically significant elevations of 8-OHdG, 5-hydroxyuracil, and 8-hydroxyadenine 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. The pattern of damage to multiple bases in the brain suggests that this is due to $^{\bullet}OH$ attack on DNA. Wade et al. (1998) used immunocytochemistry to show that 8-OHdG was increased in mitochondria in neurons in AD compared with controls. The same investigators showed that the 5 kb deletion, the most common alteration in human mitochondria, was prominent in large hippocampal pyramidal neurons in AD (Hirai et al., 1998). This deletion was present in neurons following immunostaining for 8-OHdG. These authors concluded that oxidative modification of mitochondrial DNA is an early event in the neuropathologic changes in AD and accumulation of deleted mitochondrial DNA may potentiate oxidative damage in vulnerable neurons.

Recently, we evaluated the levels of 8-OHdG in intact DNA and free 8-OHdG, which represents the repair product, in ventricular CSF from AD and control subjects (Lovell et al., 1999). A significant elevation of 8-OHdG in intact DNA in AD compared with age-matched control subjects was found. In contrast, levels of free 8-OHdG were significantly decreased in AD samples. This indicates that there may be a double insult of increased oxidative damage and a deficiency of repair mechanisms responsible for removal of oxidized bases in AD. Indeed, fibroblasts and lymphocytes from familial AD patients have a deficiency of DNA repair after exposure to fluorescent light (Parshad et al., 1996). Hermon et al. (1998) described increased protein levels of two excision-repair cross-complementing genes for nucleotide excision repair in the brain of AD patients, suggesting ongoing oxidative DNA damage. We found statistically significant decreases in 8-oxyguanine glycosylase activity, which is responsible for the

excision of 8-oxyguanine, in the nuclear fraction in the hippocampus, superior and middle temporal gyri, and inferior parietal lobule in AD (Lovell et al., 2000b). DNA helicase activity was elevated in all nuclear DNA samples and statistically significantly elevated in the hippocampus and cerebellum. This study demonstrates that the base excision repair capability for 8-oxyguanine is decreased in AD. The increase in DNA helicase activity in some brain regions may interfere with base-excision repair mechanisms.

One of the consequences of DNA strand breaks is the activation of poly (ADP-ribose) polymerase (PARP), a Zn-finger DNA binding protein (Lautier et al. 1990). Over-activation of PARP in response to oxidative damage causes depletion of intracellular NAD^+ resulting in depletion of energy stores and cell death (Zhang et al., 1994; Eliasson et al., 1997). Love et al. (1999) demonstrated enhanced PARP activity in the brain in AD using immunohistochemical staining of frontal and temporal lobes. Su et al. (1996) showed that bcl-2 expression is increased in neurons with DNA damage in AD and that bcl-2 has an antioxidant effect. Deng et al. (1999) demonstrated that bcl-2 in PC12 cells inhibited peroxynitrite-induced cell death and enhanced DNA damage recovery, which suggest neuronal upregulation of bcl-2 may facilitate DNA repair following oxidative stress.

Parkinson's Disease

Sanchez-Ramos et al. (1994) described increased 8-OHdG in the substantia nigra in PD but also observed elevations in the basal ganglia and cerebral cortex. Alam et al. (1997a) described an increase in 8-hydroxyguanine in total cellular DNA in the substantia nigra, frontal pole, and putamen, plus a decrease in fapy guanine in PD. They suggested that the rise in 8-hydroxyguanine could be due to change in the 8-hydroxyguanine/fapy guanine ratio rather than an increase in total oxidative guanine damage. Zhang et al. (1999), using immunohistochemical methods, demonstrated increased 8-OHdG in the cytoplasm of substantia nigra neuron in PD and determined that the oxidative damage targeted RNA and mitochondrial DNA, similar to what has been described by others in the hippocampus of AD patients (Wade, 1998).

Further studies to define the role of oxidative DNA damage and DNA repair in the brain in aging and neurodegenerative diseases could yield important information about the pathogenesis of neuron degeneration.

ANTIOXIDANTS IN NEURODEGENERATIVE DISEASES

As described above, a broad spectrum of enzymatic and nonenzymatic antioxidants provides important protection against oxidative stress (Fig. 1). Most of the research on antioxidants in neurodegenerative diseases has been in AD. Studies of the activity and expression of brain antioxidants in AD have yielded inconsistent results. For example, two reports showed no significant difference in Cu/Zn- or Mn-SOD activity in AD and control brains (Kato et al., 1991; Gsell et al., 1995), whereas two studies found a reduction of SOD activity in several brain regions in AD (Richardson et al., 1993; Marcus et al., 1998). CAT activity was elevated in the amygdala in one report (Gsell et al., 1995), but reduced in several brain regions in another (Marcus et al., 1998). Two studies demonstrated no difference in GSH-Px activity in AD and controls (Kish et al., 1986; Marcus et al., 1998), whereas one study showed a elevation of GSH-Px (Lovell et al., 1995). One possible explanation for the variability in brain antioxidants in AD is that most of

these studies used brains with prolonged postmortem intervals, which can affect enzyme activity. It has been well demonstrated that the overall efficiency of antioxidant defenses is attenuated during aging and, thus, closely age-matched controls are critical in such studies.

In a study using short postmortem interval AD and age-matched, longitudinally evaluated normal control subjects, we found significant elevations of GSH-Px activity in the hippocampus, GSSG-R activity in hippocampus and amygdala, and CAT activity in hippocampus and temporal neocortex in AD (Lovell et al., 1995). These changes correlated with elevations of lipid peroxidation in the same regions. We did not find a significant alteration in Cu/Zn- or Mn-SOD activity in AD. In a separate series of AD and control subjects, we evaluated mRNA expression of oxidative stress handling genes and found significant elevations of GSH-Px, GSSG-R, and CAT mRNA in hippocampus and inferior parietal lobule in AD (Aksenov et al., 1998). It is possible that the elevations observed in these studies represent a compensatory rise in antioxidant activity in response to increased free-radical generation. It should be emphasized that none of our studies defined a deficiency of antioxidant enzyme activity or mRNA expression, which suggests that the increased oxidative stress in AD is not related to failure of these defense mechanisms.

As noted above, MsrA may play a role in antioxidant defenses in proteins by its ability to reverse MetSO back to methionine, which can scavenge oxidants. Our study showed a marked decrease in MsrA in multiple brain regions in AD, which suggests an important defense mechanism from protein oxidation may be impaired (Gabbita et al., 1999).

Thioredoxin (Trx), a ubiquitous protein containing redox-active disulfide/dithiol within its conserved active site (Holmgren, 1985), functions in the reduction of protein disulfides and as an ROS scavenger (Mitsui et al., 1992). Thioredoxin reductase (TR), a selenium-containing enzyme, functions to change oxidized Trx to reduced Trx. Together, Trx and TR operate as a potent NADPH-dependent protein disulfide reductase system that can repair oxidized proteins or maintain levels of reduced Trx, which can interact directly with ROS. We found decreased Trx protein levels in five AD brain regions compared with normal control subjects, with statistically significantly decreases in amygdala and hippocampus (Lovell et al., 2000c). Thioredoxin reductase activity was increased in five AD brain regions and reached statistical significance in amygdala and cerebellum, which suggests a compensatory rise in response to oxidative stress. Neuron culture studies showed that Trx was protective against Aβ-peptide-induced neuron degeneration, which is mediated in part through free-radical mechanisms.

Uric acid acts as a scavenger of $ONOO^-$ in vitro and inhibits nitration of tyrosine in cultured neurons challenged with oxidative stress (Whiteman and Halliwell, 1996; Mattson et al., 1997). Hensley et al. (1998) described decreased uric acid in multiple brain regions and in ventricular CSF in short-postmortem-interval AD patients compared with age-matched control subjects, suggesting that diminished defenses against $ONOO^-$ may be important in AD.

Defenses against the secondary products of oxidation may also play a meaningful role in the brain in neurodegenerative disorders. Glutathione transferases (GST) are a multigene family of enzymes that catalyze the nucleophilic conjugation of glutathione with many electrophilic compounds. These enzymes protect against toxic compounds including aldehydes such as HNE. Exogenous GST protects cultured hippocampal neu-

rons against HNE toxicity (Xie et al., 1998). In a study using short postmortem interval AD and age-matched control brains, we found GST activity and protein levels significantly depleted in multiple brain regions and in ventricular CSF in AD (Lovell et al., 1998b). This indicates that a protective enzyme against aldehydes formed from lipid peroxidation is significantly decreased in AD and may play a role in neuron degeneration in this disorder.

Although the study of antioxidant defenses in the brain is in the early descriptive stage, there are indications that several defense mechanisms in the brain are diminished, at least in late-stage AD subjects compared with age-matched controls. Perhaps, as we learn more about the antioxidant defenses in the brain, it will enhance our understanding of oxidative stress in brain, more clearly define the mechanisms of neurodegeneration, and determine new therapeutic targets.

CONCLUSION

This review demonstrates that, although a number of environmental or genetic factors may cause or trigger neuronal degeneration in neurodegenerative diseases, free radical-mediated damage is at least a part of the pathogenetic cascade of events. Oxidative damage to lipids, proteins, or nucleic acids is found in AD, PD, ALS, HD, and Pick's disease. In addition, there is a decline in some antioxidant systems in AD and ALS.

The presence of free-radical-mediated damage is supported by clinical findings that suggest protective mechanisms against oxidative stress may play a role in slowing, or perhaps, eventually preventing disorders such as AD. It also appears that upregulating the natural defense mechanisms developed by cells against the constant challenge of oxidative stress could be beneficial.

Further research will be aimed at defining specifically how oxidative events are involved in the pathogenetic cascade leading to neuron death, and developing methods to decrease oxidative stress and enhance antioxidant defense mechanisms with the ultimate goal of preventing neurodegenerative disorders.

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