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METAL IONS AND OXIDATIVE STRESS IN NEUROLOGICAL DISEASES

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Metal ions and oxidative protein modification in neurological disease

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Summary. - This review highlights the role of oxidative stress and imbalances in metal ion homeostasis in the neurodegenerative diseases Alzheimer's disease and Parkinson's disease and in the progressive demyelinating disease multiple sclerosis. The chemistry and biochemistry of oxidative stress-induced protein damage are first described, followed by the evidence for a pathological role of oxidative stress in these disease states. It is tempting to speculate that free radical oxygen chemistry contributes to pathogenesis in all these conditions, though it is as yet undetermined what types of oxidative changes occur early in the disease, and what types are secondary manifestations of neuronal degeneration.

Key words: neurodegeneration, Alzheimer's disease, Parkinson's disease, multiple sclerosis, metals, oxidative stress.

Riassunto (*Ioni metallici e ossidazione proteica nella patologia neurologica*). - Questa rassegna evidenzia il ruolo dello stress ossidativo e l'alterazione dell'omeostasi di ioni metallici nelle malattie neurodegenerative e da demielinizzazione progressiva (Alzheimer, Parkinson e sclerosi multipla). Vengono descritte in primo luogo la chimica e la biochimica del danno proteico indotto da stress ossidativo, seguite dall'evidenza del ruolo patologico dello stress ossidativo in queste malattie. Si tenta di valutare il contributo dei radicali liberi dell'ossigeno alla patogenesi di tali disordini, sebbene non sia ancora chiaro quali tipi di cambiamenti ossidativi avvengano nei primi stadi e quali costituiscano manifestazioni secondarie della degenerazione neuronale.

Parole chiave: neurodegenerazione, malattia di Alzheimer, morbo di Parkinson, sclerosi multipla, metalli, stress ossidativo.

Introduction

Recent advances in molecular genetics have furthered knowledge of the hereditary basis of familial forms of Alzheimer's disease (AD) and Parkinson's disease (PD). The spontaneously occurring forms of these age-related neurodegenerative diseases, as well as the progressive autoimmune demyelinating disease multiple sclerosis (MS), are of unknown origin, but are thought to reflect a complex combination of hereditary, environmental, and lifestyle factors. In AD and PD, there is compelling evidence for a role of a metabolic imbalance and resulting oxidative stress that is superimposed on hereditary factors, and likely play a larger role in the spontaneously occurring forms of these two diseases [1, 2]. The link between oxidative stress and neuronal death is complex, but there is support for a contributory role of oxidative stress-induced neurotoxicity in both diseases [3]. The fluctuating aspect of MS between periods of exacerbation and remission would suggest that this disease has little in common with age-related neurodegenerative diseases. However, there is growing awareness that the progression of MS is associated with Wallerian degeneration ("dying back" of axons from terminals), and oxidative stress may play a pathological role here as well.

The condition of oxidative stress in each of these disease states is accompanied to a varying degree by a dyshomeostasis of metal ions, including the redox-active transition metals iron and copper as well as redox inactive metal ions such as zinc. The central nervous system is particularly vulnerable to oxidative stress on account of the high rate of dioxygen utilization, the relatively poor concentrations of antioxidants and related enzymes, and the high content of polyunsaturated lipids, the most vulnerable biomacromolecule to oxidation. There is also an accumulation of iron in the brain as a function of age, which can be a potent catalyst for oxidative species formation.

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A characteristic hallmark of AD and PD is the deposition of abnormal forms of specific proteins in the brain, which are immunoreactive to antibodies recognizing protein side-chains modified either directly by reactive oxygen or nitrogen species, or by products of lipid peroxidation or glycoxidation. Although the source(s) of oxidative damage are not entirely clear, this damage may reflect the findings of increased localization of redox-active transition metals in the brain regions most affected. This review describes evidence that suggests a role of oxidative stress and/or metal imbalances in these neurodegenerative conditions as well as in MS.

Definition of oxidative stress

Oxidative stress is defined as the imbalance between biochemical processes leading to production of reactive oxygen species (ROS) and those responsible for the removal of ROS, the so-called cellular antioxidant cascade. Tissues that become subject to oxidative stress witness steady state levels of ROS-mediated damage to all biomacromolecules (polynucleotides, proteins, lipids, and sugars) that can lead to a critical failure of biological functions and ultimately cell death. Several neurodegenerative disorders are associated with oxidative stress that is manifested by lipid peroxidation, protein oxidation and other markers.

Mitochondria are essential organelles for neuronal function because the high-energy requirement makes them highly dependent on aerobic oxidative phosphorylation. However, oxidative phosphorylation is a major source of ROS. All aerobic organisms produce at least minimal levels of ROS, mostly arising from the side-production of superoxide by reaction of molecular oxygen with sites in the electron-transport chain where reducing equivalents accumulate. Superoxide, which is also generated from the respiratory burst of neutrophils, can be subsequently transformed to the other classical ROS species H_2O_2 and hydroxyl radical. ROS can also arise from mutationally-altered or damaged metalloenzymes involved in oxidative metabolism. Reactive species generated by mitochondria have several cellular targets including mitochondrial components themselves (lipids, proteins and DNA). The lack of histones in mitochondrial DNA (mtDNA) and diminished capacity for DNA repair render mitochondria an easy target to oxidative stress events. Mitochondrial dysfunction and free radical-induced oxidative damage have been implicated in the pathogenesis of PD and AD, as well as other neurodegenerative disorders.

Usually considered as the chief instigator of oxidative stress damage, the hydroxyl radical reacts non-discriminately with all biomacromolecules at diffusion-controlled rates, i.e., within nm distances from its site of generation. Hydroxyl radical can be produced by gamma radiation, but is most commonly generated physiologically by the Fenton reaction between reduced transition metals (usually iron(II) or copper(I)) and H_2O_2. Re-reduction of the resulting oxidized transition metal ions (iron(III) or copper(II)) can be effected by cellular reductants such as vitamin C or thiols. In contrast to hydroxyl radical, superoxide radical is chemically unreactive, except at lower pH, where it exists as the hydroperoxy radical. However, superoxide can serve as the reductant of oxidized metal ions for the production of hydroxyl radical from H_2O_2, the so-called Haber-Weiss reaction. Under normal conditions, damage by ROS is kept in check by an efficient antioxidant cascade, including both enzymatic and non-enzymatic entities. Important in the former regard are cytosolic copper-zinc superoxide dismutase (CuZnSOD) and mitochondrial manganese superoxide dismutase (MnSOD), which convert superoxide to O_2 and H_2O_2. The latter, also the normal by-product of oxygen reduction by oxidases such as monoamine oxidase, is removed by catalase and peroxidases, which have ubiquitous tissue distribution, but can result in greater oxidative stress susceptibility in tissues lacking sufficient activity of these enzymes.

In recent years, the upsurge in research on nitric oxide (NO) as a common second messenger in neurotransmission and signaling has resulted in recognition of its enzymatic release from activated microglia (macrophages in the central nervous system), along with superoxide. Accumulating levels of diffusible NO and superoxide give rise to peroxynitrite. Peroxynitrite and related reactive nitrogen species (RNS) are capable of both oxidation chemistry and nitration of the aromatic side-chains of tyrosine and tryptophan [4], resulting in a condition known as “nitrosative stress”. There seems to be growing acceptance that ROS- and RNS-mediated damage seen in the central nervous system may reflect underlying neuroinflammatory processes [5].

Metal ions and oxidative stress

Copper, manganese, iron, and other trace redox-active transition metals are essential in most biological reactions, e.g., in the synthesis of DNA, RNA, and proteins, and as cofactors of numerous enzymes, particularly those involved in respiration. Thus their deficiency can lead to disturbances in central nervous system and other organ function. However, accumulation of redox-active transition metals in tissues in excess of the capacity of the cellular complement of metalloproteins (catalytic, transport,

storage) can be cytotoxic as a consequence of their participation in an array of cellular disturbances characterized by oxidative stress and increased free radical production [6, 7]. Only trace levels of such circulating excess of redox-active transition metal ions are required for ROS generation, generally reflecting reaction of the reduced metal oxidation states with dioxygen or hydrogen peroxide (Fenton reaction). Although the metal ions are thereby converted to their oxidized forms, these can be re-reduced by superoxide ion or cellular reducing agents such as ascorbate.

Common to both AD and PD is a dyshomeostasis of both redox-active and redox-inactive metal ions. In general, the loss of homeostasis of iron and copper in the brain is accompanied by severe neurological consequences. A role of oxidative stress in AD and PD is consistent with the finding that the areas of the brain affected by these diseases contain abnormally high levels of redox-active metals, particularly iron. However, it is not known whether the metal excesses are a cause of oxidative stress and neurodegeneration or a by-product of the neuronal cell loss. Alterations in the levels of "anti-oxidant" metalloenzymes likely also contribute to altered redox homeostasis in neurodegenerative diseases [8], though it is unclear whether this reflects altered enzyme activities or, indirectly, a disturbance in transition metal homeostasis.

Metal ions that become separated from specific storage and transport proteins still readily coordinate adventitiously to proteins, nucleic acids, and circulating amino acids, so that the concentrations of "free" aqueous metal ions are very low. This does not mean, however, that only the "free" forms are active in ROS generation. As long as the coordination sphere of the metal ion is not saturated (allowing for interaction with O_2 or H_2O_2) and supports the cycling between the oxidized and reduced states of the metal at biologically accessible potentials, the coordinated metal may serve as a catalytic center for generating ROS. For example, it is well known that the iron-EDTA complex is more active than free iron in mediating H_2O_2-dependent damage to DNA *in vitro* [9]. Because the capacity for ROS generation depends heavily on metal coordination environments, redox *inactive* metal ions such as zinc may be pathogenic by virtue of their ability to displace redox-active metal ions from sites where redox activity of the latter is held in check. Transition metals, along with redox-inactive metal ions, may additionally contribute to neurodegeneration through their deleterious effects on protein and peptide structure, such as a pathological aggregation phenomenon. In these cases, transition metals can sometimes exert dual neurotoxic properties.

More than any other transition metal, free iron has been implicated in ROS generation *in vivo* in tissues suffering oxidative stress. Abnormally high levels of iron have been demonstrated in a number of neurodegenerative disorders (vide infra). Importantly, experimental findings of increased *total* iron do not necessarily implicate increased oxidative stress if there are concomitant increases in proteins that store iron in redox inert forms. For example, ferritin (which may be upregulated in PD and AD) contains a core of insoluble, unreactive ferrihydrate. However, the entry and release of iron from ferritin occurs via its more coordinatively labile ferrous state, active in Fenton generation of hydroxyl radical. Microglia are the major sites of ferritin bound iron and are thought to be partly responsible for oxidative damage in PD and other neurodegenerative disorders. Microglia stimulated *in vivo* with phorbol ester show increased lipid peroxidation resulting from a superoxide-dependent release of iron from ferritin [10]. Besides superoxide, ferritin iron can be released by 6-hydroxydopamine, a neurotoxin implicated in PD, and by other easily oxidized catechols [11]. These studies suggest that ferritin iron release contributes to free-radical-induced cell damage *in vivo*.

In recent years, it has become evident that the regulation/management of iron at the cellular level, although primarily by the transferrin receptor and ferritin, is also under the control of the lactotransferrin receptor, melanotransferrin, ceruloplasmin, and divalent cation transporter. Thus, disruption in the expression of these latter proteins in the brain can contribute to altered brain iron metabolism in neurodegenerative disorders [12]. Control of the aforementioned proteins and therefore overall regulation of cellular iron metabolism involves the action of two iron regulatory proteins, IRP-1 and IRP-2 [13]. Under conditions of iron starvation, IRPs stabilize the transferrin receptor and inhibit the translation of ferritin mRNAs by binding to "iron responsive elements" (IREs) within their untranslated regions. In iron-replete cells, IRP1 assembles a cubane iron-sulfur cluster, which prevents IRE binding, while IRP-2 undergoes proteasomal degradation. IRP-1, but not IRP-2, is rapidly activated by extracellular H_2O_2, establishing a regulatory connection between the control of iron metabolism and response to oxidative stress.

In addition to oxidative damage to proteins (see next section), oxidative stress conditions and the occurrence of iron- or copper-mediated Fenton chemistry results also in oxidative damage to nucleic acids, in particular RNA. 8-Hydroxyguanosine (8-OHG), a marker of nucleic acid oxidation, is commonly observed in the cytoplasm of the neurons that are particularly vulnerable to degeneration in AD [14, 15]. 8-OHG is likely to form at the site of

hydroxyl radical production, most likely by the reaction of H_2O_2 with reduced copper or iron bound to nucleic acid bases (the oxidized metal ion thereby generated is re-reduced by cellular reductants such as ascorbate or superoxide). RNA oxidation is also seen in vulnerable neurons in Parkinson disease as well as in dementia with Lewy bodies [16], suggesting that it might represent one of the fundamental abnormalities in age-associated neurodegenerative diseases.

Chemistry of oxidative protein modification occurring in oxidative stress

Protein damage that occurs under conditions of oxidative stress may represent (i) direct oxidation of protein side-chains by ROS [17] and/or RNS [18] or (ii) adduction of secondary products of oxidation of sugars, termed glycoxidation, or of polyunsaturated lipids, termed lipoxidation [2]. In addition to traditional ROS and RNS species such as peroxynitrite, oxidative damage to proteins can occur due to alternate oxidants (e.g., HOCl) and circulating oxidized amino acids such as tyrosine radical generated by metalloenzymes such as myeloperoxidase [19]. The accumulation of oxidized protein is a complex function of the rates of ROS formation, antioxidant levels, and the ability to proteolytically eliminate oxidized forms of proteins. At least in the case of AD, proteomics approaches to identify proteins that suffer oxidative modification in the disease are now yielding information on details of the oxidative stress cascade [20].

Direct protein oxidation

ROS-mediated oxidation of protein side-chains has been reviewed [21], and usually results either in introduction of hydroxyl groups or in generation of protein-based carbonyls detectable by 2,4-dinitrophenylhydrazine (DNPH), usually from oxidation of Ser and Thr side-chains or from oxidative deamination of Lys and Arg side-chains. At least some of the carbonyls seen may represent deterioration of side-chain hydroperoxides, since reductive workup reveals hydroxylated Val and Leu [22]. In addition to protein side-chain oxidation by ROS, carbonyl groups can also result from the hydroxyl radical-like reactivity of peroxynitrite [23]. Use of radical initiators accomplishes oxidation of Met and aromatic amino acid side-chains (His,Tyr,Trp), with little carbonyl generation [24]. Met sulfoxide, which represents the most common type of protein oxidation observed in proteomics studies [18], reflects oxidation of Met by H_2O_2, peroxides, or peroxynitrite. In contrast, oxidation of Met by hydroxyl radical and transition metals generates a cation radical which usually decomposes to give alternate products [25]. Since Met sulfoxide can be reduced to Met by methionine sulfoxide reductases [26, 27], it has become clear in recent years that cyclic oxidation/reduction of Met residues might serve as an antioxidant mechanism to scavenge ROS [28]. Although there is substantial discussion of oxidative crosslinking of proteins that accompanies oxidative stress, the only crosslink structure arising *from direct protein oxidation* identified so far is the oxidative coupling of tyrosine (giving, e.g., dityrosine) [29].

The short diffusion distance of the main damaging ROS species, hydroxyl radical, suggests that most metal-catalyzed oxidative damage to proteins occurs via reaction of H_2O_2 (or perhaps O_2) with the sites of metals coordinated adventitiously to the proteins. Examples of such "site-specific" oxidations, which have been modeled using an exogenous reducing agent and O_2 or H_2O_2 as terminal oxidant include (i) conversion of His to 2-oxohistidine [30, 31], (ii) oxidative coupling of Tyr [32], and (iii) oxidation of aromatic amino-acid side chains [33]. Adventitious binding of transition metals to proteins can in many respects mimic redox metalloenzymes, though the rates are expected to be significantly slower. In this regard, although most scientists would consider adventitious protein-bound metals to exert pro-oxidant activity, anti-oxidant effects are thus also possible if these protein-metal systems mimic anti-oxidant metalloenzymes such as SOD or catalase.

Proteins that serve to scavenge adventitious metal ions have been genetically engineered to *abrogate* the redox properties of the metal, such as is the case for the copper-binding site at the amino terminus of serum albumin. Proteins not normally functioning as metal-binding proteins may sometimes act as neuroprotectants by sequestering the metal ions in redox inactive forms. However, adventitiously-bound metals tend to have at least some redox catalytic activity, and it should be possible in some cases for adventitiously-bound metals to possess *greater* redox catalytic activity than the free metal ion. Furthermore, modification of proteins by carbonyl products of glycoxidation and lipoxidation can increase the capacity of the protein to bind copper and iron in a redox-active manner. One readily understood example is the conversion of the lysine e-amino group to e-(carboxymethyl)lysine (CML), which creates the well-known bidentate ligand glycinate [34, 35]. Modification of proteins by 4-hydroxy-2-nonenal (HNE) also increases binding of redox-active copper and iron [36], though this is not yet understood on a structural level. Nonetheless,

since pro-oxidant effects of such bound metals can result in further oxidation of lipids and sugars, these events provide for an autocatalytic mechanism of exacerbated oxidative damage to the proteins and the cell that contain them.

Glycoxidation

The term advanced glycation end-product (AGE) describes either protein damage that results from adduction of reducing sugars and subsequent oxidative evolution, or adduction of more reactive sugar oxidation products, termed glycoxidation. On the basis mostly of the importance of elucidating the structure and presence of AGEs that are implicated in the late-stage pathology of diabetes and diabetic complications, immunochemical tools to detect AGEs became available many years ago. AGEs represent a heterogeneous array of structures that arise mainly from the "Maillard reaction" of reducing sugars with principally the ε-amino groups of lysine residues, though the guanidino group of arginine also becomes heavily modified. This chemistry is related to the "browning" of foods, and complex chemical changes can occur over a long period of time. A significant research effort has been directed at elucidating particularly stable AGE structures, and obtaining antibodies specific for such structures [37].

In recent years, it has become evident that the main epitope recognized by anti-AGE antibodies is CML [38]. How CML arises during the Maillard reaction is not yet entirely clear, though it is presumed that the main pathway involves oxidative cleavage of the so-called Amadori tautomers (Lys ε-$NHCH_2C(=O)R$) of initially formed Schiff bases (Lys ε-N=CHCH(OH)R). Model studies demonstrate that CML can arise from essentially a Cannizzaro reaction of glyoxal with the ε-amino group of lysine, and glyoxal is a known product of glycoxidation as well as lipid peroxidation [39]. Although anti-CML antibody recognition can thus not distinguish between sugar- and lipid-derived modifications of proteins, CML has been popularized as an important marker of oxidative stress.

In addition to CML, condensation of glyoxal with two amine groups leads to a bis-Schiff base that has been implicated as an intermediate to the AGE termed "GOLD" (glyoxal-lysine-dimer), requiring an additional carbon that must arise from cleavage following condensation with a second molecule of glyoxal [40]. The glyoxal bis-Schiff base can also undergo an intramolecular Cannizzaro reaction, leading to a stable glycinamide lysine-lysine crosslink [41, 42]. Our model studies (unpublished) have indicated both GOLD and glycinamide crosslinks appear to form in parallel.

The glycoxidation product methylglyoxal can, in parallel to glyoxal, form N^{ε}-(1-carboxyethyl)lysine (CEL) [43], and the imidazolium compound "MOLD" [44]. Although methylglyoxal is a normal intermediary metabolite in man, arising either enzymatically (e.g., from dihydroxyacetone phosphate (DHAP) by methylglyoxal synthase) or non-enzymatically by elimination of phosphate from glyceraldehyde phosphate or DHAP), its levels in blood are increased in diabetes.

Lipoxidation

Polyunsaturated lipids in lipoproteins and membranes are highly susceptible to oxidative stress damage, and suffer an ensuing radical chain autoxidation process known as lipid peroxidation. Some of the chemically and metabolically stable lipid oxidation products have been used as *in vivo* biomarkers. For example, the levels of isoprostanes, derived from arachidonic acid, and neuroprostanes, derived from docosahexaenoic acid, have been found to be increased in CSF and in diseased regions of autopsy brain tissue in neurodegenerative disease [45-48]. The unsaturated hydroperoxides generated from peroxidation of polyunsaturated lipids can break down, usually in the presence of reduced metals or ascorbate [49], to a host of mono- and bi-functional reactive aldehydes. During the past few decades, there has been an intensive effort directed at ascertaining the nature of protein and DNA modification by these aldehydes, and evidence is accumulating for their being causally involved in many pathophysiological effects associated with oxidative stress in cells and tissues *in vivo* [50].

Initially, most attention focused on malondialdehyde (MDA, 1,3-propanedial), a hydrophilic mediator that serves as the principal aldehyde detected in the TBARS (thiobarbituric acid reactive substances) assay for lipid peroxidation [51]. Although other lipid-derived aldehydes give a positive TBARS signal, MDA partitions so efficiently into the water phase relative to more lipophilic lipoxidation products, that the TBARS measurement constitutes a reasonable definition of released MDA. Although the ability of MDA to modify proteins should be limited by its existence at physiological pH mainly in the form of its "inert" resonance-stabilized enolate anion conjugate base, MDA nonetheless appears capable of forming an array of protein adducts, associated with crosslinking and a characteristic fluorescence (ex/em 390-400/460-470 nm). The most obvious crosslink would be the Lys-Lys bis-Schiff base, which would exist as its resonance-stabilized 1-amino-3-iminopropene tautomer. Although the presence of this crosslink *reversibly formed in solution* is supported by the isolation of the

corresponding propano di-lysine derivative following borohydride reduction [52], the extent to which it contributes to *long-lived* crosslinking of protein by MDA has been questioned [53], and its independent synthesis confirmed its inability to rationalize MDA-derived fluorescence [54]. The 1-amino-3-iminopropene crosslink would have to arise from initial *mono*-Schiff base adducts. Considerable amounts of the latter do form on protein Lys residues, but exist in their resonance-stabilized enamino forms (N^{ε}-(2-propenal)lysine adducts) [55], which would not be expected to react readily with a second amine molecule.

Work by others to determine the nature of the MDA-derived fluorophore in model studies under physiomimetic conditions had led to the isolation of strongly fluorescent 4-methyl-1,4-dihydropyridine-3,5-dicarboxaldehydes [56, 57]. A retro-synthetic analysis of this structure indicates the need for two MDA molecules along either side of the ring, tied together by a C_2 fragment that must result from breakdown of a third MDA molecule [53]. Consistent with this proposal, the same structures are generated in higher yield from a mixture of MDA and acetaldehyde [58]. The mentioned fluorophore would not explain the protein crosslinking potential of MDA, and further studies using a model lysine-containing peptide led to the characterization of another fluorescent MDA adduct, in this case a dihydropyridinyl pyridinium lysine-lysine crosslink derived from four molecules of MDA [59]. The relevance of this type of adduct has been questioned by workers who identified a non-fluorescent imidazole-based Lys-Arg crosslink derived from a single MDA molecule [60].

Recent studies have suggested that acrolein, another reactive three-carbon aldehyde, is also produced from peroxidation of lipids during oxidative stress. Protein Lys residues appear to constitute the main target of modification by acrolein, and a 3-formyldihydropyridine, constituting condensation of two acrolein molecules with a single lysine, was initially thought to represent the structure of the major adduct based on model studies [61]. Although this adduct was additionally reported to be detected immunochemically in oxidative stress, including AD [62], recent studies determined that the mature adduct present and detected is actually a 3-methylpyridinium structure [63]. In addition to acrolein, lipoxidation-derived α-hydroxyaldehydes have been found to modify protein Lys residues to generate fluorescent 3-hydroxypyridinium adducts [64, 65].

Our lab has focused mainly on the highly reactive bifunctional aldehyde, 4-hydroxy-2-nonenal (HNE), a major product of oxidation of ω-6 polyunsaturated (linoleoyl and arachidonyl) chains that has become the most thoroughly studied biologically-active lipoxidation-derived aldehyde [66]. The propensity of HNE to form Michael adducts with nucleophilic amino acids represents the dominant *initial* reaction pathway of HNE with proteins [51, 67-69]. Unlike simple α,β-unsaturated aldehydes, the Michael adducts in this case are stabilized in the form of cyclic hemiacetals However, although the Michael adducts formed with Cys and His are stable to isolation, Michael adducts to Lys ε-amino groups are formed reversibly [70], and can be isolated only following reductive trapping with $NaBH_4$. Our efforts to characterize an *irreversibly-formed* HNE-lysine adduct led to the discovery of HNE-derived 2-pentylpyrroles [71, 72], that we termed the first example of an "advanced lipoxidation end-product" (ALE).

The bifunctional aspect of HNE allows it in theory to crosslink proteins by Michael addition of Cys, His, or Lys at C3 and Schiff base condensation with Lys at the C1 carbonyl [73, 74]. However, Schiff bases are hydrolytically labile, and our efforts to characterize *stable* adducts that would rationalize the known protein cross-linking activity of HNE [75-77], led to the discovery of a fluorescent four-electron oxidation product, 2-hydroxy-2-pentyl-1,2-dihydropyrrol-3-one iminium, that represents a Lys-Lys crosslink [78-80]. Recent studies in our lab indicate that the fluorescent Lys-Lys crosslink may well constitute the major entity underlying crosslinking of proteins by HNE [81, 82]. Antibodies to the HNE-derived 2-pentylpyrrole or fluorescent crosslink have implicated HNE modification in Alzheimer's disease [77, 83-87], Parkinson's disease [88, 89], ALS [90], systemic amyloidoses [91], and Alexander's disease [92].

In the effort to confirm the structure of the HNE-derived fluorophore by independent synthesis, we found that the 4-keto cousin of HNE, 4-oxo-2-nonenal (ONE), gives rise to the fluorophore in much higher yield [78]. Soon thereafter, ONE was demonstrated to be a direct product of lipid oxidation in its own right [93, 94], suggesting that the fluorescent crosslink may arise more from ONE than from HNE. In addition to the fluorescent Lys-Lys crosslink, ONE forms simple Michael adducts of Cys, His, or Lys as well as pyrrole crosslinks that arise from Paal-Knorr condensation of these initial Michael adducts with protein Lys residues [95]. Recent mass spectroscopic studies have confirmed the formation on proteins of HNE and ONE adducts characterized in model studies [96].

When one examines the bulk of data on the role of AGEs and ALEs in oxidative stress, including the finding that at least some AGE modifications can also result from lipid peroxidation, it seems reasonable to conclude that lipoxidative damage to proteins may be much more prominent than glycoxidative damage in

the complement of neurodegenerative disease conditions. In addition, although MCO events [97] and radical-initiated events [24] can lead to 2,4-dinitrophenylhydrazine (DNPH)-detectable protein-based carbonyls, as mentioned above, the main fraction of DNPH reactivity seen in aged and diseased tissues appears to represent modification of protein side-chains by bifunctional carbonyl-containing products of lipoxidation [98] and/or glycoxidation.

Neuropathology of Alzheimer's disease

AD is characterized pathologically by the presence of two hallmark lesions in the brain, extracellular amyloid plaques, known as senile plaques (SP) and intraneuronal neurofibrillary tangles (NFT), as well as neuropil threads and a selective loss of neurons. SP contain mainly the Aβ peptide, whereas NFT are composed mainly of the microtubule-associated protein (MAP) tau present as paired helical filaments (PHF). Much excitement in recent years has come from the discovery of a multiplicity of mutations in the genes encoding the amyloid-β precursor protein (AβPP) and/or the presenilins, that account for the bulk of the familial cases of AD on the basis of overproduction of AβPP and/or altered AβPP proteolytic processing, both leading to increased Ab. Transgenic mice overexpressing AβPP or other human mutant AD-related proteins, as well as animals expressing more than one of these mutations, exhibit many of the neuropathologic and behavioral features of the human disease, including the development of senile plaques and some neurotoxicity. In addition, NFT can be produced in mice expressing mutant tau protein, and tangle formation is further enhanced in animals that also express mutant AβPP [99]. The animal models of AD have been used to develop and test treatments that reduce brain levels of the Aβ, neuritic plaque load and glial activation, and some have been found to restore learning and memory function [100]. Although the relationship between these genetic models and neuronal loss in AD are tentative, the mechanisms involved may be partially shared with sporadic AD.

There is considerable debate as to whether neuronal loss in AD reflects appearance of either SP or NFT and if so, which one plays a more important role. A majority of workers in the field still favor the amyloid cascade hypothesis, specifying that the onset and progression of AD is initiated by aggregation of Aβ into toxic fibrillar deposits within the extracellular space of the brain, thereby disrupting neuronal and synaptic function and eventually leading to neuronal degeneration and dementia [101]. However, Aβ, formed upon proteolytic processing of AβPP by β- and γ-secretases, has a widespread distribution through the brain and body in healthy individuals throughout life. Moreover, *soluble* Aβ appears to serve a variety of physiological functions, including modulation of synaptic function, facilitation of neuronal growth and survival, protection against oxidative stress, and surveillance against neuroactive compounds, toxins and pathogens [102]. At the other extreme, the mature SP are apparently also non-toxic because neuronal loss in AD does not occur in the vicinity of SP deposition. Also, Aβ deposition is inversely correlated with the levels of 8OHG since this oxidative marker is found physically distant from the Aβ deposits [14]. Thus, recent focus on Aβ toxicity has been on fibrillar polymers that are toxic to cultured neurons, and especially on smaller oligomers [103], particularly those containing more Aβ(1-42) than Aβ(1-40) [104]. The mechanism of neurotoxicity of Aβ(1-42), wherein Met-35 appears to play a critical role, is usually considered to involve induction of oxidative stress and lipid peroxidation [105].

Despite the genetic evidence implicating a pathologic role of Aβ, only tau pathology and NFT have been found to correlate with symptom presentation in patients [106]. In normal adult brain, the microtubule-associated protein tau binds microtubules through its tubulin-binding domains, and this assembly depends partially upon the degree of phosphorylation. By regulating microtubule assembly, τ has a role in modulating the functional organization of the neuron, particularly in axonal morphology, growth, and polarity [107]. Besides the role in microtubule stabilization, tau has other functions such as membrane interactions or anchoring of enzymes [108]. The microtubule-binding domains in tau have been identified, and it is found that phosphorylation of Ser/Thr-Pro moieties within these domains abrogates the association of tau with microtubules [109, 110].

In AD, tau is "hyperphosphorylated", at up to 22 different sites, at the stage where it loses its microtubule-binding and stabilizing function and aggregates into PHF [111]. Tau hyperphosphorylation reflects both an abnormal action of kinases, as well as decreased phosphatase activity [112]. The pool of soluble tau that is incapable of interacting with microtubules becomes prone toward self-aggregation at high concentration. There is evidence also that tau undergoes a hierarchical series of truncations at both N- and C-termini, that affect its conformationally-dependent folding, as it transforms from an unfolded monomer to the structured polymer characteristic of NFT [113].

It seems clear that tau hyperphosphorylation is a critical factor underlying neurofibrillary pathology. Supporting evidence is the appearance of epitopes recognized by anti-PHF antibodies, as a result of kinase

stimulation in neuronal cells, that precedes death of these cells [114]. Not only does hyperphosphorylated tau fail in its normal function in stabilizing microtubules, but it reflects a "gain of toxic function" due to its sequestering normal tau and other MAPs, resulting in the disruption of microtubules [115, 116]. Phosphorylation of tau induces its binding by chaperones such as hsp 27, possibly a cellular survival response aimed at preventing more toxic conformations of the protein [117]. In addition, packaging of dysfunctional tau into PHF of NFT may reflect the futile effort of the cell to reduce the effect of soluble "toxic" tau [116]. A pseudohyperphosphorylated form of tau (substituting Glu for Ser residues in the sequence known to be phosphorylated), expressed in neural differentiated PC12 cells, was shown to exhibit reduced microtubule interaction, to fail to stabilize the microtubule network, to sensitize the cells to other apoptotic stimuli, and, after longer culture, to be cytotoxic [118]. Recent research has focused on evidence that dysfunctional proteasome activity, arising from inhibitory binding of PHF-tau, contributes to the intraneuronal accumulation of oxidatively damaged proteins in the brains of patients with AD, which may be sufficient to induce neuronal degeneration and death [119].

NFT display resistance to proteolysis, and this may reflect in part the presence of transaminase-mediated γ-glutamyl-ε-lysine crosslinks [120] among proteins identified in NFT (hsp27, SN, ubiquitin, parkin) [121]. It has been our contention that the *persistent insolubilization* and *permanency* of NFT aggregates probably represents, at least in part, cementing of the aggregates by processes associated with oxidative stress. In particular, the finding that most of the covalent, including crosslinking, modifications induced by products of oxidative stress (see above) are seen in apparently normal neurons in AD and at pre-NFT PHF-tau stages [86, 122, 123] suggests that these modifications play at least partially a causative rather than by-stander role in the neurofibrillary pathology in AD. We recently showed that seven distinct antibodies raised against NFT that recognize unique epitopes of tau in AD, recognize phosphorylated tau more strongly after treatment with HNE [124]. These findings support the idea that HNE modifications of tau promote and contribute to the generation of the major conformational properties defining NFT.

Metals in Alzheimer's disease

The likely role of an imbalance of redox-active metals in rationalizing the pervasive oxidative stress damage seen in AD brain has been mentioned above. Several studies have indicated imbalances of many trace elements in AD, including Al, Si, Pb, Hg, Zn, Cu, and Fe, with only the latter two being redox active. Micro particle-induced X-ray emission data show that Zn(II), Fe(III), and Cu(II) are significantly elevated in AD neuropil and that these metals are significantly further concentrated within the core and periphery of senile plaques [125]. Over-accumulation of iron in AD has been found in the hippocampus, cerebral cortex and basal nucleus of Meynert, and colocalizes with SP and NFT [126]. These results extend earlier studies reporting increased levels of iron, transferrin, and ferritin in AD. The association of iron with NFT may be, in part, related to iron binding to their primary protein constituent, tau [127]. Dysregulation of iron homeostasis in AD, possibly involving ceruloplasmin [128], is also indicated by the finding that IRP-2 is specifically co-localized in AD with redox-active iron in NFT, SP neurites, and neuropil threads [129]. These results suggest that alterations in IRP-2 may be directly linked to impaired iron homeostasis in AD.

Increased iron in AD may also be explained by data from our laboratory and others indicating that heme oxygenase-1 (HO-1) is induced in AD brains [130, 131]. HO-1 catalyzes the conversion of heme to iron and biliverdin, which, in turn, is reduced to bilirubin, an antioxidant. Since HO-1 is induced in proportion to the level of heme, the induction of HO-1 is suggestive of abnormal turnover of heme in AD. This may be connected to the findings in AD of abnormalities in mitochondria, which contain numerous heme proteins. In turn, the increase in heme induces synthesis of more HO-1 suggesting that mitochondrial turnover promotes oxidative stress via increase of redox-active iron [132].

An assay for *redox-active* iron shows it to be increased in NFT as well as in Aβ deposits [133], and aluminum, which also accumulates in NFT-containing neurons [133], can stimulate iron-induced lipid peroxidation [134] by displacing it from inert redox sites. Also, using an *in situ* iron detection method, we found a marked association of redox-active iron with both NFT and SP in AD [135]. Further studies revealed the presence of non-enzymatic redox activity in both SP and NFT using a more general detection method that responds to redox-active copper as well as iron [36]. These results suggest that metal accumulations are major producers of the ROS responsible not only for the numerous oxidative stress markers that appear on NFT and SP, but also for the more global oxidative stress parameters observed in AD.

Evidence for imbalances of trace metal homeostasis in AD have led over the years to efforts to identify possible interactions of metal ions with both AβPP and Aβ. It has been found that Al, Fe, Zn, and Cu accelerate aggregation of Aβ, with the relative effectiveness being pH-dependent [136,

137]. The finding that chelators can at least partially solubilize β-amyloid deposits from AD [138] suggests a possible physiological role of Cu(II), Zn(II), and/or Fe(II) in Aβ deposition, thereby explaining the enrichment of these metals with SP in AD [139, 140]. Although metal-induced aggregation of Aβ may not reflect redox chemistry, copper and iron bound to soluble or aggregated forms of the peptide do seem to be redox active and thus capable of mediating ROS production [36, 141-143]. At the same time, whereas Aβ complexes of iron and zinc are toxic to cultured neurons, Aβ-copper complexes are not neurotoxic and the neurotoxicity of both iron and copper was reduced by binding to Aβ [140]. A recent study examining the effects of Cu(II), Zn(II), Fe(III), and Al(III) on Aβ(1-42) aggregation that took into account contaminating metals in "Aβ(1-42) only" preparations, revealed a complex interplay between these metals, finding that only the trivalent metal ions induced β-pleated sheet formation [144]. It was concluded that chelation therapy directed at Cu and Zn that does not also chelate Al and Fe, might exacerbate amyloid fibril formation.

There is always the question of whether increased tissue burden of metals reflects at least in part dietary factors. There is mixed evidence for a role of dietary copper in experimental models of AD, one study in transgenic mice suggesting a stabilization of SOD and reduced Aβ production [145], and another study in rabbits showing induction of Aβ plaques and learning deficits [146].

Current research has led to the characterization of AβPP as a copper-binding protein involved in regulating copper uptake and efflux, and copper regulates AβPP expression [147]. Previous work found that binding of AβPP can reduce Cu(II) to Cu(I) concomitant with production of a disulfide linkage [148]. Subsequent exposure to H_2O_2 results in reoxidation of Cu(I) and concomitant site-specific cleavage of AβPP [148, 149]. Redox chemistry associated with Aβ- or AβPP-bound metals could contribute to a perturbation of free radical homeostasis and resulting ROS-mediated neuronal toxicity in AD. An iron responsive element on AβPP points to a role for iron in the metabolism of AβPP and offers another mechanism whereby iron chelators can reduce Abeta peptide burden during Alzheimer's disease [150].

Taken together, the studies by us and others indicating the presence of redox-active iron and copper in AD pathology suggest that these metal accumulations could be major contributors to not only local ROS-mediated damage but also more global oxidative stress parameters tied to neuronal toxicity in AD.

Role of oxidative stress in Alzheimer's disease

Oxidative damage of biomacromolecules [151] has been described in association with neuronal degeneration in AD brains: 1) DNA and RNA oxidation is marked by increased levels of 8-hydroxy-2-deoxyguanosine (8OHdG) and 8-hydroxyguanosine (8OHG) [14, 15] and increased DNA oxidation and decreased repair in CSF [152, 153]; 2) protein oxidation is marked by elevated levels of protein carbonyls and nitrotyrosine [154-156]; 3) lipid peroxidation is marked by higher levels of TBARS and isoprostanes, as well as protein modification by HNE [48, 86, 157]; 4) sugar oxidation is marked by increased protein glycation and glycoxidation [158-164]. Moreover, crosslinking of proteins, such as by bifunctional lipoxidation-derived aldehydes, may lead to the resistance of the lesions to intracellular and extracellular removal even though they are extensively ubiquitinated [165], and this resistance of NFT to proteolysis might play an important role in the progression of AD [166].

Investigations into possible sources of oxidative stress and metal ion excesses that underlie increased ROS generation in AD have focused recently on mitochondria, not only because of their propensity for ROS generation [167], but also because mitochondrial turnover and associated degradation of heme proteins can release iron [168]. Heme oxygenase-1, which is increased in AD [130, 131, 169], catalyzes the conversion of heme to the antioxidant biliverdin, but the other product is iron. There is substantial evidence for a down-regulation of the mitochondrial respiratory chain in AD [170], and evidence for increased turnover of mitochondria is the finding of mtDNA and cytochrome oxidase in the neuronal cytoplasm in AD. Mitichondrial structural abnormalities and mtDNA deletions have also been observed, in each case occurring before neuronal degeneration and amyloid deposition [171]. These findings together suggest that mitochondrial dysfunction, acting in concert with cytoskeletal pathology, serves to increase redox-active metals and initiates a cascade of abnormal events in the early stages of AD pathogenesis.

A state of pervasive oxidative stress underlying the sites of damage in AD is further provided by immunocytochemical evidence for the upregulation of the "antioxidant enzyme" superoxide dismutase [172, 173], glucose-6-phosphate dehydrogenase [174, 175], and increased levels of reduced sulfhydryls [175, 176] in vulnerable neurons. Increased oxidative damage in AD may thus represent a failure of adequate antioxidant defenses [177]. Notwithstanding, there is growing support for the hypothesis that oxidative markers are apparent mainly in initial stages of the disease, and that compensating cellular defense mechanisms eventually result in diminished oxidative damage [178, 179].

In fact, there is now considerable evidence that the deposition of tau in NFT and Aβ in SP may represent responses to the oxidative stress condition, whereby these protein aggregates serve to trap reactive products of oxidative stress. It has been shown that NFT enables neurons to survive decades [180]. Tau and the related neurofilament proteins, especially NFH, appear adapted to oxidative stress due to their high content of lysine [181], suggesting that they can serve as a "buffer" for reactive carbonyl products of lipid and sugar oxidation. Tau also binds iron, and can contribute to iron homeostasis [36]. Also, an antioxidant role for Aβ *in vivo* is supported by the finding that an increase in Aβ deposition in AD cortex is associated with a decrease in neuronal levels of 8OHG, i.e., with decreased oxidative damage [14, 182]. Similar negative correlation between Aβ deposition and oxidative damage is found in patients with Down syndrome [182, 183]. Aβ deposits observed in both studies mainly consist of early diffuse plaques, meaning that these diffuse amyloid plaques may be considered as a compensatory response that reduces oxidative stress [184].

The strong chelating properties of Aβ for zinc, iron, and copper explain the reported enrichment of these metals in amyloid plaques in AD discussed above, but also suggest that one function of Aβ is to sequester these metal ions (though the chelation would have to lower redox activity for this to be protective). Also, Met-35 in Aβ can scavenge peroxides, with the resulting methionine sulfoxide being re-reduced by methionine sulfoxide reductase. Thus, although there is substantial evidence of a pro-oxidant role of especially Aβ(1-42), the *overall* net effect of Aβ on oxidative stress is a complex question. While more studies are required to understand the role of NFT and SP deposition in neuronal and extra-neuronal homeostasis, the results obtained to date clearly implicate a regulated process that, at least through mid-stages of the disease, might have a neuroprotective role.

Role of oxidative stress in the pathogenesis of Parkinson's disease

Parkinson's disease involves the selective destruction of the dopamine-producing cells of the substantia nigra in the midbrain. The cause and pathogenesis of PD remain unknown: mitochondrial dysfunction, oxidative damage, environmental factors, and genetic predisposition might all be involved. Because oxidative stress is intimately linked to other components of the degenerative process, it is difficult to determine whether oxidative stress leads to, or is a consequence of, these events [185]. The principal histopathologic hallmark of PD are Lewy bodies, eosinophilic intraneuronal filamentous inclusions found in magnocellular neurons of brain stem nuclei, predominantly substantia nigra and locus coeruleus. Like PHF, Lewy bodies are insoluble in detergent and are resistant to removal from the extracellular space following neuronal death [186]. Structurally similar Lewy bodies are also found in cortical neurons in PD and diffuse Lewy body disease. The principal protein constituent of Lewy bodies is α-synuclein, originally identified as the precursor of a peptide known as non-Aβ component (NAC) that is tightly associated with amyloid deposits in AD.

There is substantial evidence that mitochondrial complex I inhibition may be the central cause of sporadic PD [187]. Evidence centers on the observed 30-40% decrease in complex I activity in the substantia nigra. The decrease in activity seems to be matched by a decrease in protein content that appears to reflect a mtDNA defect and underproduction of certain complex I subunits [188, 189]. Support for a causative role of the complex I deficiency in PD is that chronic infusion of the complex I inhibitor rotenone in rat brain results in a selective loss of nigral DA neurons and the appearance of cytoplasmic α-synuclein inclusions resembling Lewy bodies, the underlying mechanisms for which appear to involve oxidative stress. Evidence for oxidative stress in PD is the finding of oxidative damage to DNA [190, 191] and protein [192, 193] observed in the nigro-striatal region of PD brain, as well as immunocytochemical evidence for protein nitration [194], glycation [195], and HNE modification [88, 89].

Notwithstanding, with the discovery of missense mutations in two genes, α-synuclein and parkin, which are responsible for rare cases of heritable early-onset PD, there has recently been a major shift in emphasis in PD research [196]. Overexpression of normal α-synuclein only modestly affects cell viability, but most studies show that overexpression of mutant α-synuclein proteins is neurotoxic, most commonly by induction of apoptosis [197, 198]. Although the physiological functioning of α-synuclein remains to be fully defined, recent data are suggestive of a role in regulating membrane stability and neuronal plasticity [199].

Numerous proteins have been shown to associate with soluble α-synuclein [200], and the peptide has been shown to bind to tau and stimulate its phosphorylation [201]. At the same time, when α-synuclein and tau coexist, they tend to form homopolymers rather than heteropolymers. This does not preclude α-synuclein-tau interaction exerting a physiological role, because α-synuclein induces fibrillization of tau, and

coincubation of tau and α-synuclein synergistically promotes fibrillization of both proteins [202]. NFT frequently coexist with Lewy bodies in the same neurons of patients with diffuse Lewy body disease [203]. Moreover, though there is wide recognition of a disease class known as tauopathies (e.g., NFT formation in AD) and another class known as synucleinopathies (e.g., Lewy body formation in PD), emerging evidence indicates that there is frequent overlap of the pathological and clinical features of all these patients [204].

A connection between α-synuclein aggregation and oxidative stress parameters seen in PD is suggested by the finding that amyloid-like aggregates of α-synuclein similar to those seen *in vivo* can be induced by co-incubation with Cu(II) [205], Fe/H_2O_2 [206], or cytochrome *c*/H_2O_2 [207]. Also, studies using anti-nitrotyrosine antibodies have shown the presence of nitrated α-synuclein in human Lewy bodies and other α-synuclein inclusions [208]. Nitrated α-synuclein monomers and dimers but not oligomers were found to accelerate the rate of fibril formation of unmodified α-synuclein when present at low concentrations [209]. This suggests that post-translational modification of α-synuclein could promote the formation of hallmark intracytoplasmic inclusions found in PD and other synucleinopathies.

It seems clear that dysfunction of α-synuclein is a common feature of all forms of PD. However, exacerbating the fact that mutations in or oxidative modifications of α-synuclein can promote aggregation, is the increased evidence for dysfunction of the ubiquitin-proteasome system, which relates to the second set of familial PD genetic defects discovered, namely mutations in two critical enzymes: parkin, a ubiquitin ligase [210], and ubiquitin C-terminal hydrolase L1. Defects of the ubiquitin-proteasome system lead to a condition known as *"proteolytic stress"* [211], where the cell accumulates misfolded or abnormal (e.g., oxidatively modified) proteins that cannot be cleared. The inability of neurons to degrade abnormal protein is now a guiding principle affecting numerous neurodegenerative conditions, and can reflect interference with polyubiquitination [212] as well as inhibition of the proteasomal pathway. It is not surprising that mutant forms of α-synuclein found in familial PD cannot be easily cleared, but proteolytic stress appears also to underlie nigral pathology in *sporadic* forms of PD [213]. Oxidative stress can impair the ubiquitin-proteasome process directly, and products of oxidative damage, such as HNE, can damage the 26S proteasome [185]. Furthermore, proteasome binding to insoluble α-synuclein filaments and soluble α-synuclein oligomers results in marked inhibition of its chymotrypsin-like hydrolytic activity [214]. At the same time, α-synuclein is required for the fibrillar nature of ubiquitinated inclusions induced by proteasomal inhibition in primary neurons [215]. The finding that Lewy bodies in PD are immunoreactive for ubiquitin supports a link between defects in energy metabolism and the disposal of damaged proteins in the development of PD [216].

Metals in Parkinson's disease

Abnormally high levels of iron and oxidative stress have been demonstrated in a number of neurodegenerative disorders characterized by nigral degeneration such as PD, multiple system atrophy, and progressive supranuclear palsy. Selective dopaminergic cell loss in PD is correlated with increased levels of cellular iron. Iron accumulates in astrocytes in the SN pars compacta of old rats [217, 218]. At the same time, there is an increase in the Fe(III)/Fe(II) ratio and a decrease in GSH [219]. One interpretation is that mitochondrial sequestration of redox-active iron in aging nigral astroglia may be one factor predisposing the senescent nervous system to PD. Moreover, there is circumstantial evidence that the intracellular redox imbalance results in aberrant oxidation of dopamine to 6-hydroxydopamine, which in turn can undergo autoxidation to the corresponding quinone concomitant with generation of superoxide. This reaction cascade, either by itself, or as amplified by redox cycling of this quinone leading to further generation of ROS at the expense of cellular reductants, can serve to explain the ultimate demise of these neurons. Studies to clarify the mechanism of dopamine oxidation *in vitro* have demonstrated conversion to 6-hydroxydopamine in the presence of Fe(II) and either H_2O_2 or alkyl peroxides [220]. Since the non-ferritin-bound labile iron pool is presumed responsible for dopaminergic degeneration via Fenton chemistry, efforts have focused on developing brain-permeable iron chelators [221].

An epidemiological study revealed that a dietary iron intake in the highest quartile compared with those in the lowest quartile had an increased risk of PD [222]. Also, there was an apparent joint effect of iron and manganese, with dietary intake above median levels of both together conferring a nearly doubled risk of PD. There is little evidence for a similar effect of dietary iron on AD prevalence. A series of recent studies suggest that iron regulatory proteins (IRPs) coordinate both cellular iron levels and energy metabolism, both of which are disrupted in Parkinson's disease (PD) and may in turn contribute to increased levels of oxidative stress associated with the disease. Iron has also been recently implicated in promotion of

α-synuclein aggregation either directly or via increasing levels of oxidative stress suggesting an important role for it in Lewy body formation [223]. There is substantial support for the hypothesis that oxidative stress, augmented iron deposition, and mitochondrial insufficiency constitute a single neuropathologic "lesion", where any one component in this triad obligates the other two [224].

On the basis of evidence for increased metals seen an PD autopsy tissue, there has been a significant effort to ascertain if there are altered levels of metal ions in bodily fluids of live patients. One study looking at serum levels of 12 elements found significant decreases in Al and S in both early and severe PD compared to controls, Fe and Zn were lower in severe PD, and several other elements (K, Mg, Cu, P) were higher in early and severe PD [225]. In another study, quantification of 8 elements in whole blood, serum, urine, and cerebrospinal fluid (CSF) found a complex interplay between redox-active and redox-inactive elements [226]. Interestingly, the finding that the oxidant status was increased and the anti-oxidant capacity decreased in serum from PD patients, compared to controls, was correlated best with Fe, which along with Zn was increased in blood, whereas serum levels of Cu were lower.

Chronic exposure to Mn results in extrapyramidal syndromes resembling PD, and Mn has therefore been labeled as an environmental toxic factor that induces brain dysfunction. However, Mn(II) itself is inactive in Fenton chemistry, and there is no convincing *in vivo* evidence for a prooxidant role of Mn in the brain. Although Mn was once considered as a possible contributor to idiopathic PD, it is now clear that the PD-like syndrome induced by Mn poisoning may have little connection to nigrostriatal damage occurring in idiopathic PD. Recent data indicates that Mn-induced parkinsonism (termed manganism) can be differentiated from PD because accumulation of Mn and damage occur mainly in the basal ganglia (pallidum and striatum), rather than in the pars compacta of the SN [227, 228].

The role of oxidative stress and metals in multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system that is generally believed to be of autoimmune origin [229, 230]. This disabling disorder is characterized pathologically by selective and coordinated inflammatory destruction of the myelin sheath, with ensuing damage to the underlying axon [231]. Although it is generally accepted that the immune system contributes to tissue damage, the cause of MS is still unclear. However, accumulating data indicate that oxidative stress plays a major role in the pathogenesis of MS.

It is well known that inflammation might raise reactive oxygen and nitrogen species levels leading to oxidative stress. One of the most abundant sources of reactive species, apart from the electron-transport chain of mitochondria, is the respiratory burst system of activated microglia. ROS and RNS generated by macrophages have been implicated as mediators of demyelination and axonal injury in both experimental autoimmune encephalomyelitis (EAE - the generally accepted animal model for the study of MS) and MS [232, 233]. In addition, free radicals can activate certain transcription factors, such as nuclear transcription factor-kappa B, which up-regulates the expression of many genes involved in EAE and MS, such as tumor necrosis factor-α, inducible nitric oxide synthase (iNOS), intracellular adhesion molecule 1 and vascular cell adhesion molecule 1 [234, 235].

An analysis of the blood (plasma, erythrocytes and lymphocytes) of 28 MS patients compared to 30 healthy age-matched controls revealed that MS patients displayed significantly reduced plasma levels of ubiquinone and vitamin E, and depressed erythrocyte glutathione peroxidase [236]. It was concluded that the blood of patients with multiple sclerosis shows signs of significant oxidative stress. This conclusion supports an earlier study that found a significant decrease in glutathione peroxidase activity in the erythrocytes of 24 MS patients, compared to controls [237]. Abnormal catalase activity has been reported also in the granulocytes and erythrocytes of MS patients [238] being decreased in the former and increased in the latter, compared to normal controls. In addition, a 38% increase in lipid peroxidation, significantly elevated levels of oxidized glutathione, and a reduction in the plasma vitamin E:lipid ratio has been reported during the active phase of MS [239]. Examination of CSF showed significantly higher concentrations of isoprostanes [240], an increase MDA and glutathione reductase activity, and a decrease of glutathione peroxidase activity [241]. Moreover, direct examination of MS plaques has revealed increased free radical activity, along with decreased levels of glutathione, α-tocopherol and uric acid [242]. Furthermore, it has been shown that activated mononuclear cells of MS patients produce high amounts of reactive oxygen and nitrogen species and that oxidative damage to DNA, including mitochondrial DNA [243], develops in association with inflammation in chronic active plaques [244].

Because commonly activated immune cells release glutamate, glutamate excitotoxicity is thought to be involved in MS lesion formation and axonal damage. Demyelinating lesions caused by excitotoxins can be similar to those observed in MS, causing histologically similar damage. Excessive release of glutamate from damaged neurons can lead to the over-excitation of surrounding neurons and subsequent apoptotic cell death mediated by several types of glutamate receptors [245]. It was found that oligodendrocytes are highly susceptible to glutamate excitotoxicity mainly via AMPA/kainate receptors [246]. Recent experimental studies showed that the treatment with AMPA/kainate antagonists resulted in substantial amelioration of experimental EAE [247]. The AMPA/kainate antagonists also increased oligodendrocyte survival and reduced dephosphorylation of neurofilament H, an indicator of axonal damage [248, 249]. A recent study showed that riluzole, an antagonist of glutamate neurotransmission, dramatically reduces the clinical severity, inflammation, demyelination and axonal damage of EAE mice [250]. Consistent with this possibility, the neurological deficit resulting from EAE has generally been reduced by trial therapies to diminish the concentration of ROS [251]. There is also evidence in MS of an increase in CSF levels of glutamate in association with the severity and course of the disease [252, 253] and that glutamate production by macrophages may underlie axonal damage and oligodendrocytes death in MS lesions [254].

Several investigators have shown that inhibition of iNOS, as well as the use of anti-sense knockdown of iNOS, suppress EAE in mice and rats [255-258]. Furthermore, it has been shown that inhibition of iNOS, or scavenging nitric oxide or peroxynitrite by uric acid, inhibited neurological deficits in mice with EAE, while withdrawal of iNOS inhibitor resulted in the appearance of neurological signs within 24 hours [259, 260].

Evidence for a pathogenic role of ROS in MS pathology has led to the employment of several antioxidant strategies in an effort to ameliorate EAE. It has been shown that oral administration of the oxidant-scavenger N-acetyl-L-cysteine, which can effectively raise intracellular glutathione levels, inhibited the induction of acute EAE [261]. Additionally, the intraperitoneal administration of catalase before the onset of neurological deficit delayed the onset of EAE and reduced its severity and duration [262]. A salen-manganese complex, Euk-8, that may be regarded as a prototype molecule of a new class of synthetic catalytic scavengers with combined superoxide dismutase and catalase activity, was also shown to be effective in EAE mice [263]. It was also shown that α-lipoic acid inhibits the symptoms in EAE mice [264]. However, other workers have suggested that α-lipoic acid can effectively interfere with the autoimmune reaction associated with EAE through non-antioxidant mechanisms [265]. Recently, bilirubin has been found to prevent both acute and chronic EAE in mice [266]. More significantly, bilirubin suppressed ongoing clinical EAE and halted EAE progression when given after disease onset.

Following the encouraging findings in the EAE models, many researchers have suggested that dietary antioxidant intake may help to stop or delay disease evolution. An older study showed that supplementation of the diet of MS patients with antioxidants (selenium, vitamin C and vitamin E) could increase and normalize the glutathione peroxidase activity and the cellular content of linoleic acid in erythrocytes and hematogenous cells within 3 weeks [267]. Also, in a large study of 144 MS patients, adherence to a low-fat diet enriched with vitamin A was shown to delay the progression of symptoms and to lower death rates [268]. Indeed, high dose antioxidant supplementation is now recommended for such patients and has been shown to help normalize the otherwise low glutathione peroxidase activity in MS [269]. It has been shown that low serum uric acid levels of MS patients can be raised and maintained by oral administration of its precursor inosine [270]. Despite the encouraging results obtained with antioxidants, more epidemiological and clinical trials should be performed to clarify the real efficacy of antioxidant strategies in the fight against MS progression.

Although there were early conflicting reports of abnormal iron deposition in the brain in MS, a study using an enhanced staining method revealed that in addition to its normal deposition in oligodendrocytes and myelin, iron was detected in reactive microglia, ameboid microglia and macrophages in MS brains [271]. It is thought that the localization of iron deposition in MS indicates potential sites where iron could promote oxidative damage. The concentrations of ferritin, transferrin and iron were measured in the CSF of MS and control patients [272]. Ferritin levels were significantly elevated in the CSF of chronic progressive active MS patients, but not in patients with relapsing-remitting disease, and levels of transferrin and total iron were similar in all groups. However, in brain tissue from MS patients, disruptions were found in the normal pattern of binding distributions for both transferrin and ferritin [273]. In addition, compared to controls, levels of manganese are significantly decreased and levels of copper significantly elevated in the CSF of MS patients, whereas no differences were found for zinc [274]. Evidence for the role of disrupted iron metabolism and iron-mediated oxidative stress in the pathogenesis of MS and EAE has been recently reviewed [275].

Conclusions

Although demyelination in MS is normally considered an inflammatory disease, evidence for a neurodegenerative component in MS brings this disease process into a common discussion of the role of oxidative stress in age-related neurodegenerative disorders such as AD and PD. In AD and PD, the degree of cognitive and/or clinical impairment is correlated to the degeneration and subsequent loss of specific neuronal populations, presumed to correlate in turn with the pathological lesions. The proteins depositing in these lesions are selectively vulnerable to and, at least sometimes participate in, oxidative stress, and it is tempting to speculate that free radical oxygen chemistry and a dyshomeostasis of metal ions plays a pathogenetic role in all these neurodegenerative conditions. However, it is as yet undetermined what types of oxidative changes occur early in the disease, and what types are secondary manifestations of neuronal degeneration, as well as how the effects of oxidative stress are differentially manifested in the specific neuronal populations affected by the individual disease conditions. Thus, since most antioxidants considered for therapeutic intervention also have metal-reducing capacity, devising a successful regimen of antioxidant action to retard the progression of these diseases remains a complicated goal. Nonetheless, at least in the case of AD, recent clinical data indicate that the use of a combination of various antioxidants might indeed be effective in preventing the disease [276].

Work carried out in our lab and elsewhere has focused on oxidative modifications to biomacromolecules that provide a window to view the disease process. Since these oxidative markers show up, at least in the case of AD, are early stages of development of the classical pathological lesions, it is our contention that oxidative stress-induced modifications contribute to disease pathogenesis. The role of metal ions in mediating oxidative stress reflects the complex intertwining between effects of both redox-active and redox-inactive metals in competing with each other for available metal-binding proteins and adventitious protein binding sites that may either enhance or reduce redox activity. Thus, when considering chelation therapy as an adjunct to possible antioxidant intervention in these disease states, it will be important to recognize the dualistic action of metals in catalyzing both prooxidant and antioxidant reactions. Certainly, developing a more complete understanding of the biochemical bases underlying these disease states will continue to lead to improved animal and cellular model systems that can be used to test pharmacological agents as prospective therapies.

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