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BIOMARKERS OF OXIDATIVE STRESS IN NEURODEGENERATIVE DISEASES

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OVERVIEW

The involvement of oxidative stress in Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), suggest that free radicals play important roles in the onset and progress of neurodegenerative process. Understanding the molecular and biochemical basis of disease pathogenesis is critical for the development of potential neuroprotective therapies for neurodegenerative diseases (AD, PD, and ALS). In this chapter we discuss current knowledge of oxidative stress in relation to neurodegeneration.

14.1 INTRODUCTION

A large number of diseases have been described that involve oxidative and nitrosative stress.[1,2] Therefore, at present it is important to clarify if oxidative and nitrosative stress are strictly involved in onset and progression of diseases, and if oxidative and nitrosative stress products could be used for the identification and diagnosis of a specific pathological condition. Many products of oxidative and nitrosative stress have been proposed and studied in order to find biomarkers of disease, since a validated biomarker is especially important in the case of neurodegenerative diseases (Table 14.1). In order that an oxidative and nitrosative stress product could be used as a marker of disease, it is fundamental that it be chemically stable, accurately quantified, reflect specific oxidation pathways, and have its concentration in biological samples correlated with the severity of the disease.[3] By general definition (from NIH),[4] a biomarker is an indicator of normal processes, pathogenic processes, or pharmacological responses to a therapeutic intervention that is objectively measured. It is believed that biomarkers have great potential in predicting chances for diseases, early diagnosis, and setting standards for the development of new pharmacological treatments.

Neurodegenerative diseases are a varied group of central nervous system disorders all characterized by the progressive loss of neuronal tissues.[5] Tremendous efforts have been made in the past years to identify neuropathological, biochemical, and genetic biomarkers of neurodegenerative diseases for a diagnosis at earlier stages, which presumably would be more amenable to therapy. At the moment, the only way to do a valid neuropathological diagnosis of AD is a postmortem autopsy.[6] Having an early diagnosis of the disease might help in the early treatments of the disease or to slow down the progression of the disease (Fig. 14.1).

The brain is particularly sensitive to oxidative damage because of its high oxygen consumption, relatively low levels of antioxidant defenses, and a high content of polyunsaturated lipids that are easily oxidized.[7] Free radicals have been directly or indirectly implicated in the pathogenesis of several neurodegenerative disease associated to aging, such as AD, PD, and ALS.[8,9] Whether oxidative and nitrosative stress in these disease is casual or a secondary consequence of other processes remains

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to be determined. However, monitoring the levels of indicators of such damage might be useful both to follow disease progression and to assess the efficacy of antioxidant treatments.[10] Hence, in this chapter, involvement of oxidative stress in neurodegenerative diseases such as AD, PD, and ALS is reviewed.

TABLE 14.1 Summary of Oxidative and Nitrosative Stress Markers in the Central and Peripheral Compartments in Neurodegenerative Diseases

	Brain	Blood
Lipid peroxidation		
AD	HNE, MDA, Acrolein, TBARs, F2-IsoPs, F4-NP	HNE, MDA, TBARs
PD	HNE, MDA, Acrolein, IsoPs, TBARs	HNE, MDA, TBARs, F_2-IsoPs
ALS	HNE, MDA	HNE, MDA, TBARs
Protein oxidation and nitration		
AD	PC, 3NT	
PD	PC, 3NT	
ALS	PC, 3NT	
Carbohydrates oxidation		
AD	AGEs, RAGE	AGEs
PD	AGEs, RAGE	
ALS	AGEs, RAGE	AGE. RAGE
DNA/RNA oxidation		
AD	8-OHG, 8-OHdG, NPrG	8-OHG, 8-OHdG
PD	8-OHG, 8-OHdG	8-OHdG
ALS	8-OHdG	8-OHdG

AD is the most prevalent form of dementia in the elderly population. In the United States, over 5 million people suffer from AD. This disorder is a progressive disease characterized by death of neurons and synapses mainly in cerebral cortex and hippocampus regions, resulting in deterioration of cognitive functions.[11] The main neuropathological hallmarks of AD are extracellular senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs). The major components of the SP are β-amyloid peptides (Aβ), while the NFT are fundamentally constituted by hyperphosphorylated-insoluble forms of the tau protein.[12]

PD is a neurodegenerative disease that affects more than 1% of all people over the age of 55. Pathological hallmarks include degeneration of dopaminergic neurons between the substantia nigra (SN) and the striatum that causes the characteristic clinical signs (slowed movements, rigidity, tremors).[13,14] Another key neuropathological mark of PD is the formation of Lewy bodies (LBs), which are cytoplasmic inclusions, composed of α-synuclein protein in the dopaminergic neurons of substantia nigra and other brain regions (cortex and magnocellular basal forebrain nuclei).[15] In a small number of families, PD is inherited in a Mendelian autosomal dominant or autosomal recessive way,[16] while AD is inherited in an autosomally dominant manner.

ALS is an age-dependent motor neuron neurodegenerative disease characterized by neuronal death of the upper and lower motor neurons, skeletal muscle atrophy,

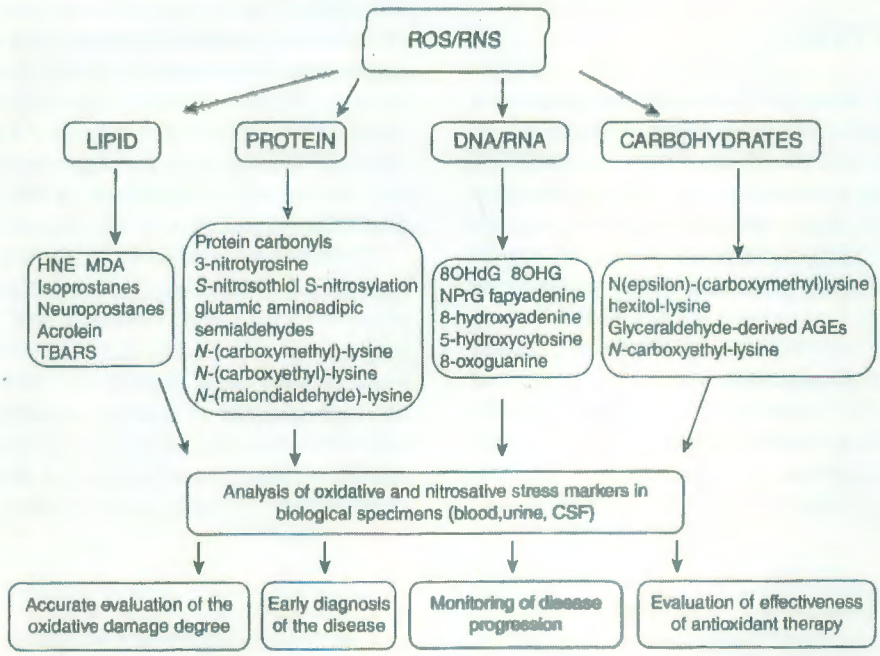

Figure 14.1 Potential use of oxidative and nitrosative markers.

paralysis, and death.[17] The primary goal for scientists with regard to the biomarkers of ALS is to show direct evidence of motor neuronal degeneration within the brain or spinal cord. Approximately 2% of all ALS and 20% of familial cases are associated with mutations in the gene for copper, zinc superoxide dismutase (SOD1).[18]

14.2 BIOMARKERS OF PROTEIN OXIDATION/NITRATION

14.2.1 Protein Carbonyls

Oxidative modification of proteins in most cases is known to affect their function. In this section of protein oxidation, we discuss protein carbonylation and protein nitration that have been used as common makers to study the effect of reactive oxygen species/reactive nitrogen species (ROS/RNS) on proteins. Protein carbonyls are formed by either the direct oxidation of certain amino acid side chains such as Lys, Arg, Pro, Thr, His, and so on, among others, by peptide backbone scission, by Michael addition reactions of His, Lys, and Cys residues with products of lipid peroxidation (e.g., 4-hydroxy-2-nonenal [HNE]), or by glycoxidation reactions with the Lys ε-amino group.[19,20] Protein carbonyls are generally detoxified by enzymes such as aldehyde dehydrogenase (ALDH) or by reduction to their corresponding alcohols by carbonyl reductase (CR).[21] The most commonly used approach for the detection of protein carbonyls is by derivatization of the carbonyl groups with hydrazine compounds such as 2,4-dinitrophenylhydrazine, followed by spectrometry, high-performance liquid chromatography (HPLC), or immunochemical detection.[22–24] In the postmortem frontal- and occipital-pole brain samples from AD, young, and age-matched controls, the levels of protein carbonyls showed an exponential increase with age, at double the rate in the frontal pole compared with the occipital pole.[23] Studies from our laboratory showed an increase of 42 and 37% of protein carbonyls in AD hippocampus and inferior parietal lobule (IPL), respectively, compared to age-matched controls.[25] Furthermore, the levels of protein carbonyls were also found to be increased in the frontal cortex of Swedish APP670/671 FAD mutation.[26] Using immunoprecipitation technique followed by Western blot we found increased oxidation of glutamine synthetase (GS), creatine kinase (CK), and beta actin in AD-affected region, and decreased activities of GS and CK were related to the increased oxidation of these proteins.[25,27]

The levels of CR were found to be increased in brain of AD and Down subjects (trisomy of chromosome 21, which harbor the gene for APP),[21] suggesting that it might be a response to the increased levels of protein carbonyls or decreased clearance of this protein which might have underwent oxidation. Reed et al. showed that CR is HNE-modified in mild cognitive impairment (MPI) brain,[28] which arguably is the earliest form of AD. Studies of animal models of amyloid beta-peptide showed increased levels of protein carbonyls, suggesting that amyloid beta-peptide plays an important role in elevating the protein carbonyls and consequently oxidative stress, cell loss, and AD pathogenesis.[29]

Our laboratory is the first to use redox proteomics techniques to identify carbonylated proteins in the IPL region of AD.[30,31] The redox proteomics approach led to the identification of a number of targets of protein carbonylation in AD brain. The identified proteins perform a wide variety of cellular functions such as energy metabolism, protein degradation, structural, neurotransmission, lipid asymmetry, pH regulation, cell cycle, tau phosphorylation, Abeta production, and mitochondrial function, all of which relate well with the histopathological, biochemical, and clinical presentation of AD.[30–33] For example, energy metabolic alterations in AD brain due to oxidative modification can be correlated well with the positron emission tomography (PET) studies that showed decreased glucose utilization in AD brain.[34] Further, the identification of an oxidatively modified brain protein does not only affect the function of this protein, but it also affects the function of other proteins that interact with it. Studies showed that sometimes a protein could perform multiple functions in a cell. For example, enolase, a protein known to be involved in the glycolytic cycle of glucose metabolism, has been reported to have a number of other nonglycolytic functions such as hypoxic-stress protein,[35] binding to polynucleotides,[36] and *c-Myc* binding and transcription protein,[37] and so on. Hence, oxidation of one protein could dampen a number of cellular functions in neurons and consequently be involved in AD.[38] Redox proteomics approaches also led to identification of peptidylprolyl *cis/trans* isomerase (Pin1) as oxidatively modified protein in AD and also in MCI. Pin1 function is critical for proper protein assembly and folding, intracellular transport, intracellular signaling, transcription, cell cycle progression, and apoptosis. Studies demonstrated that Pin1 has the ability to regulate APP processing, also phosphorylation of tau protein[39,40]; hence the oxidation of this protein could be a potential mechanism in the progression of AD. A recent study from our laboratory on APP(NLh)/APP(NLh) × PS-1(P264L)/PS-1(P264L) human double mutant knockin mice model of AD suggests that amyloid beta-peptide is involved in oxidative modification of this protein.[41] Our laboratory is further exploring the importance of Pin1 and other oxidatively modified proteins in the progression and pathogenesis of AD.

The levels of protein carbonyls were also found to be elevated in MCI brain.[42,43] Furthermore, redox proteomics studies from our laboratory in MCI brain led to the identification of a number of common targets of protein carbonylation, between AD and MCI, such as enolase, Pin1, and GS, consistent with the hypothesis that oxidative stress is critical to the pathogenesis of AD[33] and might play an important role in the progression of AD.[43,44]

Oxidative stress is elevated in PD brain, and this has been associated with mitochondrial complex I dysfunction.[45] A study using human fetal dopaminergic primary neuronal cultures overexpressing wild-type α-synuclein showed decreased mitochondrial complex I activity and increased ROS production. The increase in the ROS production is related to the metabolism of dopamine by the mitochondrial enzyme monoamine oxidase (MAO) during which molecular oxygen is converted to hydrogen peroxide (H_2O_2), an ROS. This increase in ROS is an essential component of oxidative stress. Postmortem PD brain showed increased levels of protein carbonyls in substantia nigra pars compacta compared to controls and other brain regions.[46] The increase in protein carbonyls is reported in dopaminergic neurons and has been shown to be mostly associated with high-molecular-weight proteins.[46] A recent study in PD subjects showed that increase of protein carbonyls is associated with short telomere length, suggesting that oxidative stress may be involved in the telomere abrasion in PD and consequently in the pathogenesis of PD.[47]

Both hereditary and sporadic PD demonstrate loss of dopaminergic neurons that is accompanied by oxidative stress and preceded by glutathione (GSH) depletion. GSH, the tripeptide γ-glutamyl-cysteine-glycine, is important in maintaining the proper redox balance of the cell, and in the case of neurons, it is also important in the regulation of neuronal excitability and viability. In PD brain the levels of GSH and cysteinyl-glycine (Cys-Gly) were reported to be reduced further, suggesting a role of oxidative stress in pathophysiological mechanisms of PD.[48] An *in vivo* study from our laboratory showed that gamma-glutamylcysteinyl ethyl ester (GCEE), a precursor for GSH synthesis, reduces dopamine-associated striatal neuron loss in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice.[49]

In the hemiparkinsonian animal model, two proteins, that is, α-enolase and β-actin, were identified as being oxidatively modified.[50] Using a redox proteomics approach, we identified carbonic anhydrase (CA-II), alpha-enolase, and lactate dehydrogenase 2 (LDH2)[51] as excessively carbonylated proteins with reduced activity in the brain stem of symptomatic mice with overexpression of an A30P mutation in α-synuclein compared to nontransgenic mice, suggesting that alteration in the cellular process due to oxidative modification of proteins might be important in the pathogenesis of PD.[51]

Oxidative stress is also implicated in the pathogenesis of ALS. Postmortem brain tissue from ALS showed increased oxidative stress.[52] The levels of protein carbonyls have been shown to be elevated in the spinal cord[53] and motor cortex[54] from familial ALS (fALS)[55] and sporadic amyotrophic lateral sclerosis (sALS)[53,56] subjects. Using redox proteomics, our laboratory found SOD1, translationally controlled tumor protein (TCTP), UCH-L1, and αB-crystallin as proteins with elevated carbonyl levels in the spinal cord of G93A-SOD1 transgenic mice compared to wild-type mice.[57] The identification of these proteins suggests the involvement of the protein carbonyl modification and thereby oxidation stress in altering the normal biological functions in the cell, which may be critical in the pathogenesis of ALS.

14.2.2 Protein Nitration

The other marker of protein oxidation, that is, protein nitration, was reported to be increased in AD brain and ventricular cerebrospinal fluid (VF),[58,59] which correlated with increased levels of nitric oxide synthase (NOS) reported in AD brain.[60,61] Furthermore, immunohistochemical studies showed the presence of nitrated tau in pre-tangles, tangles, and tau inclusions in the AD brain, suggesting nitration of tau nitration as an early event in AD pathogenesis.[62,63] Nitration of proteins led to loss of activity of glutamine synthase,[64] ubiquitin,[65] and Mn superoxide dismutase.[66,67]

Proteomics approach led to the identification of large number of proteins that are excessively nitrated in AD brain.[68,69] These proteins include alpha- and gamma-enolase, lactate dehydrogenase (LDH), neuropolypeptide h3, triose phosphate isomerase (TPI), alpha-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATP synthase alpha-chain, carbonic anhydrase-II, and voltage-dependent anion channel (VDAC).[68,69] The identified nitrated proteins are involved in regulating various cellular functions such as energy metabolism, maintenance of structure, pH regulation, and mitochondrial functions. As stated above, nitration of proteins also leads to loss of functionality,[69] and the identified nitrated proteins also correlated with AD pathology, biochemical changes, and clinical presentation. Guix et al. confirmed our finding of excess nitration of TPI in hippocampus and frontal cortex of AD subjects.[70] However, unlike other proteins, TPI activity was unaffected in AD brain. It is not clear why nitration does not affect the function of this protein, and we speculate that the structure of TPI provides protection against loss of function. Guix et al. suggested a possible link between decreased glucose metabolism, nitrosylation of TPI, and

the formation of Aβ and paired helical filaments.[70] Further, Reyes et al. showed that nitrated tau protein is mostly associated with or in close proximity to amyloid plaques, implying a role of amyloid beta-peptide in inducing nitrosative stress.[71] As stated earlier in the section on protein carbonylation, the nitration of one protein could have implication in various cellular functions. For example, GAPDH is well known for its function in the glycolytic pathway of glucose metabolism; however, this protein also has other functions such as GAPDH can bind to nucleic acid and regulate transcription,[72,73] catalyze microtubule formation and polymerization,[74] bind integral membrane ion pumps associated with Ca^{2+} release,[75] and so on. Furthermore, GAPDH also interacts with a number of small molecules such as tumor necrosis factor (TNF)-alpha, GSH,[76] and so on. Hence, nitration of one protein could have detrimental effect on normal cellular functions. The nitration of proteins like GAPDH and actin also raises a question of these proteins are good to be considered as loading controls in Western blot.

Our laboratory is the first to show increased levels of 3-NT in MCI, arguably the earliest form of AD.[77] Applying proteomics, we identified increased nitration of MDH, α-enolase, multidrug resistant protein-3 (MRP3), glutathione-*S*-transferase Mu (GST M), glucose regulated protein precursor (GRP), aldolase, and 14-3-3 protein gamma, peroxiredoxin 6 (PR VI), DRP-2, fascin 1, and heat shock protein A8 (HSPA8) protein as specifically nitrated in MCI IPL.[78] The reported nitrated proteins are involved in the regulation of a number of important cellular functions, including energy metabolism, structural functions, cellular signaling, and antioxidant. Some of the targets of protein nitration are common between AD and MCI brain, suggesting potential involvement of these pathways in the transition of MCI to AD.[68,69,78] Further studies are required to delineate the role of nitration in the progression of the AD.

Postmortem PD brain also shows increased levels of protein nitration as indexed by increased levels of protein nitration and NOS.[79] Previous studies showed increased nitration of α-synuclein in brain of individuals with synucleinopathy, suggesting a direct link between oxidative and nitrative damage to the onset and progression of neurodegenerative synucleinopathies.[80] A recent study showed that increased nitration of alpha-synuclein could induce dopaminergic neuronal death.[81] Further, an *in vitro* study showed increased nitration of mitochondrial complex I, suggesting the involvement of nitric oxide (NO)-related events in the pathogenesis of PD.[82] Since the mitochondrial electron transport chain is critical for superoxide production, nitration of complex I might lead to increase production or leakage of superoxide consequently leading to increase production of peroxynitrate and enhanced protein nitration in PD. PD pathogenesis appears to be dependent on NO-related events; hence, compounds that prevent nitrosative damage might have therapeutic value in neurological conditions such as PD. Mythri et al. showed that curcumin protects complex I against peroxynitrite-mediated mitochondrial toxicity and oxidative stress.[83]

Spinal cords of sporadic ALS subjects showed increased levels of nitrotyrosine and NOS in the motor neurons, suggesting upregulation of protein nitration ALS.[84] So far, no proteomics studies have been performed to identify the specific target of protein nitration in ALS.

Taken together, studies conducted thus far suggest that oxidation and nitration of proteins are involved in the progression and pathology of AD, PD, and ALS. Further studies are needed to provide potential pathways involved in the progression of these diseases.

14.3 BIOMARKERS OF LIPID PEROXIDATION

Lipid peroxidation is a process resulting from damage to cellular membranes mediated by ROS that generate several relatively stable end products, including aldehydes, such as malondialdehyde (MDA), HNE, acrolein kong,[85] and isoprostanes,[86] which can be measured in plasma or tissues as markers of oxidative stress. MDA, HNE, and acrolein are able to bind DNA and proteins, in particular nucleophilic aminoacidic residues like Cys, His, and Lys generally inducing an alteration of protein conformation and function.[87] Lipid hydroperoxides and aldehydes can also be adsorbed from the diet, and then excreted in urine. For this reason, the measurements of urinary MDA and HNE can be confounded by diet and should not be used as an index of whole-body lipid peroxidation unless diet is controlled.

There are many evidences that lipid peroxidation of polyunsatured fatty acids (PUFA) is involved in the onset and progression of many pathologies such as cardiovascular (atherosclerosis, diabetes), and neurodegenerative diseases.[88,89] For example, in the pathogenesis of AD, lipid peroxidation plays a particular role.[90,91] In fact, a number of studies demonstrated increased levels of lipid peroxidation as indicated by elevated levels of the products of lipid peroxidation such as HNE, acrolein, $F_{(2)}$-isoprostane, $F_{(4)}$-isoprostane, and neuroprostanes in AD brain.[92–94] Further, increased levels of HNE-adducted GSH were found in human postmortem brains from AD patients.[95] Normally in cells, HNE–GSH adducts are eliminated by the systems glutathione transferase (GST) and MRP-1. But in AD brain, this detoxification system was found to be a target of HNE with consequent decreased efficiency to eliminate HNE,

and subsequent accumulation of HNE protein adducts in neuronal cells.[96,97] Even the proteosome, involved in the removal of damaged proteins from cells, has been demonstrated to form conjugates with HNE and neuroprostanes in both MCI and AD.[98] In addition, significant increase of free HNE in cerebrospinal fluid,[99] amygdala, hippocampus, and parahippocampal gyrus was detected in AD patients compared to control subjects.[100] Moreover, immunocytochemical studies demonstrated that HNE immunoreactivity is present in NFT, but only in some SPs in AD.[101] In blood, some studies demonstrated that HNE is significantly higher in AD compared to healthy subjects.[102,103] Proteomics studies were able to identify regionally specific HNE modification of proteins, that is, ATP synthase, GS, MnSOD, DRP-2 in AD hippocampus, and alpha-enolase, aconitase, aldolase, peroxiredoxin-6, and alpha-tubulin in AD IPL.[104] Also in hippocampus and IPL from subjects with MCI, many proteins oxidatively modified by HNE were identified by proteomics (carbonyl reductase [NADPH], alpha-enolase, lactate dehydrogenase B, phosphoglycerate kinase, heat shock protein 70, ATP synthase alpha chain, pyruvate kinase, actin, and elongation factor tau).[28] Since most of the proteins that undergo HNE modification have been shown to be dysfunctional, these results in the earliest form of AD suggest that HNE-bound proteins may play a key role in the progression and pathogenesis of AD. In addition, these results suggest that proteomics could be a valid method for identification of new markers of neurodegenerative diseases including AD.

In PD–HNE adducts of brain proteins were found.[105] Data from immunocytochemistry demonstrated that HNE levels were increased in dopaminergic cells in the substantia nigra and cerebrospinal fluid in PD.[106] Incubation of rat striatal synaptosomes with various concentrations of HNE induced a dose-dependent decrease of dopamine uptake and Na^+/K^+ ATPase activity and loss of sulfhydryl (SH) groups.[107] These data suggest that HNE is an important mediator of oxidative stress that alters dopamine uptake, and HNE may contribute to the onset and progression of PD. Although the role of oxidation in substantia nigra is well established, the significance of peripheral oxidative stress in PD is still unclear, and the results are apparently contradictory, most probably due to differences in the methods used to measure systemic oxidative stress. However, some studies reported that the concentration of HNE in the cerebrospinal fluid (CSF) and in plasma of PD patients was significantly higher than in control patients.[108] Furthermore, no significant correlation existed between the duration of symptoms and the age of the Parkinson patients with plasma or CSF concentrations of HNE.[108] There also were not significant differences between plasma and CSF concentrations of HNE between patients with untreated PD and those receiving L-DOPA therapy.[108]

HNE plays a critical role also in the motor neuron degeneration in ALS. By immunohistochemistry, HNE-protein modification was detected in ventral horn motor neurons, and immunoprecipitation analysis revealed that one of the proteins modified by HNE was the astrocytic glutamate transporter EAAT2 causing impairment of glutamate transport and excitotoxic motor neuron degeneration in ALS.[109] HNE was also found elevated in CSF of patients with sALS compared with that of the control subjects and other neurological disease.[110,111] By proteomics analysis we[112] analyzed spinal cord tissue of a model of fALS G93A-SOD1 Tg mice to study the HNE-modified proteins. Three significantly HNE-modified proteins in the spinal cord of G93A-SOD1 Tg mice in comparison to the non-Tg mice were identified: dihydropyrimidinase-related protein 2 (DRP-2; neuronal development and repair), heat-shock protein 70 (Hsp70; stress response), and possibly α-enolase (energy metabolism). Since HNE-bound proteins present structural and function alterations, the data found demonstrate that oxidative stress in the form of lipid peroxidation is implicated as a pivotal event in the motor neuron degeneration processes. Furthermore, many other studies were performed by proteomic analysis on CSF from a consistent group of ALS patients and healthy subjects, which identified some proteins with significantly different levels in ALS CSF compared to CSF from control subjects. Therefore, in ALS like in AD, proteomics analysis distinguished the disease condition from the healthy condition.[113–115] At the serum level, HNE was found significantly elevated in ALS patients compared to controls. These levels were higher even at early stages of the disease, and in the sporadic ALS serum than familial ALS, suggesting that familial and sporadic forms are qualitatively different in regard to oxidative stress.[110] This finding may reflect the presence of different mechanisms in the pathogenesis of either form of ALS. Furthermore, the level of serum HNE in ALS patients was positively correlated with the stage of disease, implicating HNE as a possible early indicator of oxidative stress and marker of the disease. The systemic presence of increased HNE in the serum of ALS patients may reflect either the presence of lipid peroxidation in the CNS with diffusion to the peripheral circulation or the activation of additional pathways originating outside the CNS, leading to the formation of HNE and its related adducts.

Another main product of lipid peroxidation, MDA, that is able to form covalent adducts with lysine residues of proteins, was found higher in plasma and serum from AD patients compared to controls subjects.[116] Other

studies confirmed these results.[117–119] Further, in AD brain, the increased level of MDA is correlated to decreased levels of one of the most important antioxidant enzymes, superoxide dismutase (SOD).[120] Immunohistochemistry analysis has shown that MDA colocalized with NFT and SP.[121]

MDA was found bound to α-synuclein in the substantia nigra and frontal cortex of all dementia with LB dementias (LBDs) and PD cases examined. Furthermore, it was demonstrated that α-synuclein lipoxidation is an early event in LBDs.[122,123] The basal MDA levels were increased in PD substantia nigra and also in CSF compared with other PD brain regions and control tissue.[124,125] All these observations give support not only to the concept that lipoperoxidation precedes α-synuclein aggregation in LBDs, but also the idea that oxidative-altered proteins are present in cerebral cortex in preclinical PD. Like HNE, the MDA and thiobarbituric-acid-reactive substances (TBARS) levels also were found increased in plasma of PD subjects compared to healthy subjects.[126,127] In addition, Navarro et al. found that the levels of TBARS in frontal cortex brain mitochondria were markedly increased in PD subjects compared to controls, suggesting that PD is not only characterized by substantia nigra dysfunction, but also involves the cerebrum, leading to cognitive decline at the early stages of PD.[128] While plasma MDA levels were inversely related to the age of PD patients, there was no significant correlation between plasma MDA levels and duration of the disease.[129]

In ALS, Hall et al.[130] found an extant lipid peroxidative damage in the spinal cords of a murine model of the disease, the TgN-(SOD1-G93A)G1H mice. Lipid peroxidation was investigated in terms of changes in vitamin E and MDA levels measured by HPLC methods and by MDA–protein adduct immunoreactivity. Compared to non-Tg mice, the TgN-(SOD1-G93A)G1H mice showed an accumulation of spinal cord vitamin E and higher levels of MDA over the 30- to 120-day time span. In addition, MDA–protein adduct immunoreactivity was significantly increased in the lumbar spinal cord and in the cervical cord of the same mice. These results clearly demonstrate an early increase of lipid peroxidation in the lumbar spinal cord in the familial ALS transgenic model, which precedes the onset of clinical motor neuron disease. In another study conducted on TgN-(SOD1-G93A)G1H mice, it was observed a significant elevation in MDA in both red and white skeletal muscles was observed.[131] All these data on murine models were confirmed in spinal cord from sporadic ALS and familial ALS subjects where MDA–adduct proteins were found increased in both neurons and endothelial cells when compared to normal controls.[54] In the periphery, MDA and 2-TBARS levels have been found to be significantly higher in the plasma and serum of ALS patients than in either age-matched controls or young adults.[132–134]

Acrolein has been reported to react with DNA bases like guanine, leading to increased formation of acrolein-deoxyguanosine in AD brain.[135] In PD, α-synuclein is modified by acrolein (ACR) since histopathological observations in dopaminergic neurons from PD brains showed the colocalization of α-synuclein and acrolein.[136] Acrolein-adduct proteins, however, were not detectable in the spinal cord of sALS or fALS patients.[137]

A longitudinal study showed that levels of CFS $F_{(2)}$-IsoPs in AD patients were significantly increased during the follow-up period, and also significantly declined in patients accepting antioxidant treatment.[138] Furthermore, other studies demonstrated higher levels of the isoprostane, 8,12-iso-iPF (2alpha)-VI in CSF in AD[139,140] and MCI,[141] suggesting that this lipid peroxidation product could be another marker to identify AD at early stages. MCI brains showed increased levels of protein-bound HNE, TBARs, MDA, $F_{(2)}$-IsoPs, and $F_{(4)}$-NP.[42,142] The significance of $F_{(2)}$-IsoPs in AD and MCI plasma is still controversial, because Pratico et al. found high levels of $F_{(2)}$-IsoPs in plasma, CSF, and urine of MCI patients,[141] and the same research group showed similar results in AD patients.[143] However, in a 2007 study, plasma $F_{(2)}$-IsoPs levels were not increased in AD or MCI, and most probably, this result was affected by the high percentage of antioxidant used in MCI and AD patients studied.[92] Another research has reported that plasma and urine $F_{(2)}$-IsoPs levels did not accurately reflect CNS levels in AD patients.[144] At the present time, more work will be needed to support the validity of $F_{(2)}$-IsoPs as a plasma biomarker for AD. Like other markers of lipid peroxidation, the isofurans (IsoFs) levels are also significantly high in PD substantia nigra compared to other regions of the PD brain and compared to control and also to AD.[138] On the contrary $F_{(2)}$-isoprostanes ($F_{(2)}$-IsoPs) levels do not change in substantia nigra of PD compared to control individuals.[145] In plasma, several studies found that the $F_{(2)}$-IsoPs levels were unchanged in PD patients compared to control subjects.[92,146] But a recent study using more accurate methods demonstrated that F_2-IsoPs levels were significantly increased in plasma from PD patients.[147] Further analysis of the results revealed that most of the PD subjects analyzed had early PD, suggesting that peripheral oxidative damage is higher in the early stages of PD.

Consequently, there is much evidence for the elevation of the peripheral lipoperoxidation markers in several neurodegenerative diseases. Although it is too early for any firm conclusions to be drawn, the measurement of lipoperoxidation products, which is a simple

and cheap assay to perform, can and should be incorporated into future clinical trials. Such studies would clarify and likely support the hypothesis that oxidative stress is a key component for the evolution of neurodegenerative disease, and it could be considered as a marker of these pathologies.

14.4 BIOMARKERS OF CARBOHYDRATE OXIDATION

Reducing sugars play a pivotal role in modifying proteins, forming advanced glycation end products (AGEs) in a nonenzymatic reaction named glycation. Some of the biological associations of protein glycation include some diseases such as diabetes mellitus, cardiac dysfunction, neurodegenerative disease.[148,149] For this reason, glycation has an important clinical relevance, since it could be considered a potentially useful biomarker for monitoring several diseases.

In AD, glycation is believed to play an important role in NFT formation as well in the development of SPs. Involvement of AGEs in AD was first suggested in several papers published successively during 1994–1995.[150,151] Indeed, immunohistochemical studies showed the existence of AGEs such as pyrraline and pentosidine in SPs and NFTs.[152] Tau glycation enhances the formation of paired helical filaments in AD frontal cortex, reduces its ability to bind microtubules *in vitro*, and increase the fibrillization of tau.[151] Interestingly, glycation agents such as methyglyoxal are able to activate p38 MAP kinase, which is able to phosphorilate tau,[153] an important step in the formation of NFTs.[154] Furthermore, AGE-modified tau leads to an increase in the production and secretion of amyloid beta-peptide, followed by formation of ROS.[150] Glycation by methylglyoxal promotes the formation of β-sheets, oligomers, and protofibrils.[155] Glycation likely causes increased oxidative stress, inflammation, and apoptosis. However, is not clear if glycation is an early- or a late-stage marker for AD. Some data suggest that AGE formation is a late secondary event in AD, since amyloid beta-peptide alone induces free radical generation that can promote cross-linking between peptides and sugars.[156] But glycation of AT8, a known precursor of NFTs, suggests that it could be an early event.[157] Although AGE levels increase with age, in AD, the increase is much greater (37.5% and 72.6%, respectively).[158] In addition, AGEs were found in CSF of AD patients,[159,160] suggesting that this may be explored as a biomarker for AD. Receptor for AGE (RAGE) is normally expressed in a variety of cells, including microglia, neurons, and pericytes,[161] and has been found to be a specific cell surface receptor for amyloid beta-peptide, promoting neuronal cell death and dysfunction. In addition, by immunohistochemistry, it was demonstrated that RAGE levels are increased in microglia from AD brains compared to non-AD brains, especially microglia surrounding neuritic plaques.[162] Moreover, double transgenic mice with neuronal overexpression of neuronal RAGE and mutant amyloid beta-protein precursor (mAβPP) displayed early abnormalities in spatial learning/memory, accompanied by altered activation of markers of synaptic plasticity and exaggerated neuropathological findings.[163] All these observations support the active participation of the AGE-RAGE system in AD. Researchers started to think that serum or CSF AGE could become a promising biomarker for early detection of AD. But the current results available about AGEs levels in blood from AD and non-AD patients are controversial. Many groups found that AGEs and soluble RAGE (sRAGE, a C-terminal truncated isoform of RAGE) levels were lower in blood from AD patients and from MCI compared to healthy control or other forms of dementia,[164–166] but in contrast, some other groups demonstrated that AGEs and sRAGE blood levels did not change at all or increased.[167,168] Unfortunately, the blood circulating levels of AGEs do not reflect completely what happens in the CNS. Perhaps it is just matter of methods used to measure AGEs or maybe such analyses are influenced by other external factors such as food intake

The formation of LBs in PD is still unclear, but it seems that in addition to oxidation and phosphorylation, glycation might constitute another factor affecting the aggregation process. Glycation was first reported in the substantia nigra and locus coeruleus, showing higher immunoreactivity at the periphery of LBs in PD patients.[169] These results suggest that glycation may be involved in the chemical cross-linking and proteolytic resistance of the protein deposits. Further, a study showed that AGEs and α-synuclein are similarly distributed in very early LBs in the human brain in cases with incidental LBs disease, suggesting that most probably AGEs promote formation for LBs.[170] Although glycation was also detected in the cerebral cortex, amygdala and substantia nigra of older control subjects, the number and levels of glycated proteins were significantly higher in PD patients.[123] sRAGE was also highly expressed in cerebral cortex of PD patients when compared to age-matched controls,[123] suggesting a role for AGEs in the disease. One important feature of PD is an acute decrease in the levels of cellular reduced glutathione (GSH) in early stages of the disease, which results in a lower activity of the glyoxalase system, an important catabolic pathway of the most important glycation agent *in vivo*, that is, methylglyoxal.[171] This would cause an increase in AGEs concentration that would increase oxidative stress, which consequently induces AGEs for-

mation. This vicious cycle would contribute to cell damage in dopaminergic neurons and death, that is, this glycation-prone environment promotes development of PD.

Glycation was first detected in both sporadic and familial forms of ALS, in spinal cord, and brain samples.[172] Initially, it was hypothesized that glycation could be involved in the cross-linking of neurofilament protein.[173] Subsequent studies have revealed that AGEs levels in spinal cord were higher in patients carrying SOD1 mutations and in mutant SOD1 transgenic mice compared to control cases.[174] Glycation, although it is a random process, affects superoxide dismutase 1at lysine residues level, causing a decrease of its activity.[175] This could justify the observed oxidative stress in ALS. Surprisingly, levels of soluble RAGE (sRAGE) are significantly lower in the serum of ALS patients.[176] Furthermore sRAGE, lacking the transmembrane-anchoring domain, was found to ameliorate the deleterious effects of RAGE by scavenging its ligands without further activating RAGE mediated-processes.[177] Thus, sRAGE may function as an endogenous protection factor in ALS, indicating that the low sRAGE levels may pose a risk factor in the disease. Moreover, it was demonstrated that the concentration of N-ε-(carboxymethyl)lysine (CML, an AGE derived from the reaction between glyoxal and the side chain of lysine residues) was significantly increased in serum and CSF of ALS patients. This result could be a potential biomarker for diagnosis of ALS, as well as point out the relevance of glycation in ALS.[178]

14.5 BIOMARKERS OF NUCLEIC ACID OXIDATION

Among all the free radicals produced during normal metabolism and/or by exogenous sources, the hydroxyl radical ($HO^{\bullet}$) is the most toxic and most highly reactive, and it conceivably could be responsible for the most oxidative damage to biological molecules, including nucleic acids (RNA and DNA). Hydroxyl radical, produced in the vicinity of nucleic acid, can easily modify RNA and DNA because they are reactive and cannot diffuse from their site of formation. More than 20 different types of base damage by hydroxyl radicals have been identified,[179] but guanine is the most reactive of the nucleic acid bases.[180] Therefore, the oxidized bases 8-hydroxyguanosine (8-OHG) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) are the most abundant among the oxidized bases, and they are used as markers of RNA and DNA oxidation, respectively.[181]

There is a considerable amount of evidence supporting early involvement of RNA oxidation in the pathological cascade of neurodegeneration, especially in AD. RNA oxidation has been observed in postmortem brains of cases with early stage AD,[182] a presymptomatic case with familial AD mutation,[183] and Down syndrome cases with early stage AD pathology.[184] One of the markers of RNA oxidation, 8-hydroxyguanosine (8OHG), is inversely correlated to Aβ deposits, NFT, and duration of dementia.[185] Increased level of 8-OHG and 1-*N*2-propanodeoxyguanosine (NPrG) were found not only in MCI, but also in AD brains at latest stages, suggesting that mRNA is highly sensitive to oxidative damage.[186] Ribosomal RNA also was oxidatively modified in AD brain, and oxidation of rRNA by bound redox-active iron suggested its role in impairments in protein synthesis also in MCI.[187] A decreased level of ribosomal RNA and protein synthesis rate were reported in MCI. Furthermore, approximately fivefold increased levels of oxidized RNA in CFS were reported for AD cases than controls.[188]

Elevated RNA oxidation has also been observed in both postmortem substantia nigra tissue and CSF from living PD patients. Studies about the correlation between levels of 8-OHG in the CFS and the duration of the disease suggest that RNA oxidation may occur at the early stage of the disease.[189,190]

The role of mRNA oxidation in ALS was demonstrated for the first time in the transgenic mouse model of ALS TgN-(SOD1-G93A)G1H.[191] RNA oxidation in ALS is an early event, at presymptomatic stage, before the degeneration of motor neurons. Many mRNA species that have been found oxidized in TgN-(SOD1-G93A)G1H mice are related to ALS, included SOD1, dynactin 1, vesicle-associated membrane protein 1 (VAMP), and neurofilament subunit.[191] Moreover, protein levels as a consequence of oxidized mRNA species are significantly decreased.[192]

All the data about RNA oxidation in neurodegenerative diseases suggest that oxidative modification of mRNA causes not only reduction of protein levels, but it also induces translation errors *in vivo* with alteration of protein structure and function.

A substantial body of evidence indicates that oxidative DNA damage is a feature of AD in the brain as well in peripheral tissues.[193,194] Higher concentrations of oxidized pyridines and purines were detected in lymphocytes and leukocytes of AD patients compared to controls,[195–197] and in DNA from ventricular CSF of AD patients.[198] Mecocci et al were the first to demonstrate the mitochondrial DNA oxidation in AD.[199] Later, it was reported that a DNA oxidation was not limited only to the mitochondrial compartment, but also at the nuclear level in both MCI and AD brain.[182,193] No difference in DNA oxidation in the cerebellum was observed in AD, consistent with lack of Aβ pathology and other markers

of oxidative stress in this brain region.[25] Both RNA and DNA oxidation markers were found in MCI and also in AD, suggesting that nucleic acid oxidation may be an early event in the progression of AD.[200] Furthermore, the levels of base excision repair (BER) enzymes that are correlated with the number of NFTs, but not SPs, were found significantly decreased in both MCI and AD.[201]

DNA damage in PD appears to occur as the levels of 8-OHdG are increased in the substantia nigra and some other brain regions.[190,202] However, levels of another product of guanine oxidation, fapyguanine, were decrease in substantia nigra in PD.[202] The oxidative damage to the DNA occurs widely in PD brain, but the substantia nigra is particularly vulnerable. One explanation could be that one of the drugs used for PD treatment, levodopa (L-DOPA), might lead to the formation of ROS and widespread oxidative damage. In fact, it was demonstrated that levodopa induces oxidative stress and degeneration of cultured dopaminergic neurons.[203,204] Moreover, the 8-OHdG levels in serum, CFS, and urine are increased in PD patients compared to healthy people.[189,205,206] Based on this background, 8-OHdG potentially could be a good biomarker for PD.

Also familial and sporadic ALS subjects had an increased level of nuclear 8-OHdG in the motor cortex[54]; it was also 10-fold higher in the spinal cord tissue in ALS than in controls.[207] In plasma, urine, and CSF, levels of 8-OHdG are higher in ALS subjects compared to healthy people.[208–210] In addition, all these data were confirmed in a mouse model of ALS, TgN-(SOD1-G93A).[211]

Oxidative stress is involved in a number of diseases, including neurodegenerative diseases discussed above. However, so far, there are no unique set of markers that can help to differentiate these diseases or to use of the above discussed oxidative stress markers as specific biomarkers of the disease. Studies are ongoing in our laboratory to identify disease-specific biomarkers which can be used in both diagnosis or to monitor the protective efficacy of therapeutic agents. A recent comprehensive review of redox proteomics in neurodegenerative disorders from our laboratory has been accepted for publication.[212]

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