

Short Communication

Evidence for Neuronal Oxidative Damage in Alzheimer's Disease

Paul F. Good,^{*†} Peter Werner,[‡] Amy Hsu,^{*}
C. Warren Olanow,[‡] and Daniel P. Perl^{*†}

From the Departments of Pathology^{*} and Neurology[†] and
the Arthur M. Fishberg Research Center for Neurobiology,[‡]
Mount Sinai School of Medicine, New York, New York

Oxidative stress has been proposed as a pathogenetic mechanism in Alzheimer's disease. One mechanism of oxidative damage is the nitration of tyrosine residues in proteins, mediated by peroxynitrite breakdown. Peroxynitrite, a reaction product of nitric oxide and superoxide radicals, has been implicated in N-methyl-D-aspartate receptor-mediated excitotoxic damage. Reported evidence of oxidative stress in Alzheimer's disease includes increased iron, alterations in protective enzymes, and markers of oxidative damage to proteins and lipids. In this report, we demonstrate the presence of nitrotyrosine in neurofibrillary tangles of Alzheimer's disease. Nitrotyrosine was not detected in controls lacking neurofibrillary tangles. Immunolabeling was demonstrated to be specific for nitrotyrosine in a series of control experiments. These observations link oxidative stress with a key pathological lesion of Alzheimer's disease, the neurofibrillary tangle, and demonstrate a pathogenetic mechanism in common with the other major neurodegenerative diseases of aging, Parkinson's disease and amyotrophic lateral sclerosis. These findings further implicate nitric oxide expression and excitotoxicity in the pathogenesis of cell death in Alzheimer's disease. (Am J Pathol 1996, 149:21–28)

The major pathological lesions of Alzheimer's disease, neurofibrillary tangles (NFTs) and senile plaques, are composed of abnormally modified pro-

teins, deposited in insoluble aggregates.¹ Because of their insolubility, these lesions have proven difficult to fully characterize and have not completely yielded to biochemical or molecular analysis. However, the major constituents of these lesions have been determined, demonstrating cytoskeletal proteins in NFTs² and the protein fragment β -amyloid³ along with dystrophic neurites in senile plaques. Although the identity of the proteins deposited in these lesions has largely been established, the events leading to their deposition in an insoluble form are not well understood. In Alzheimer's disease, although the relationship is unclear, pathogenetic modifications of normal proteins have been implicated in the transformation of soluble proteins into insoluble forms. Abnormal phosphorylation of the microtubule-associated protein tau,^{4–6} oxidative modification of β -amyloid by free radicals *in vitro*,^{7,8} and advanced glycation end-product post-translational modifications of NFT proteins^{9,10} have been demonstrated in the aggregated proteins of Alzheimer's disease lesions.

Protein nitration by peroxynitrite has been hypothesized to represent a major mechanism of oxidative modification of proteins in association with atherosclerosis,¹¹ inflammation,¹² and ischemia/reperfusion injury.¹³ This aberrant addition of a nitrate group to the ortho position of tyrosine represents one outcome of cellular oxidative stress rendering nitrated proteins dysfunctional¹⁴ and killing neurons in culture¹⁵. Nitrotyrosine formation in proteins has been demonstrated in atherosclerotic lesions¹¹ and has also been proposed for a role in neurodegenerative disease.^{16,17} *In vitro* studies of such a role for protein

Supported by grants AG-5138 and AG-2210 from the National Institutes of Health and the Lowenstein Foundation.

Accepted for publication March 15, 1996.

Address reprint requests to Dr. Paul F. Good, Division of Neuropathology, Box 1134, Department of Pathology, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, New York, 10029.

nitration have identified nitrotyrosine as a product of the peroxynitrite reaction with superoxide dismutase (SOD)¹⁴ or neurofilament protein.¹⁶ Peroxynitrite is produced by a reaction between nitric oxide and superoxide radicals. The nitric oxide radical is generated by the enzyme nitric oxide synthase (NOS), activated by Ca²⁺/calmodulin, secondary to *N*-methyl-D-aspartate (NMDA) receptor-mediated cytosolic calcium influx¹⁸. The potential association of NMDA receptor activity and peroxynitrite production thus represents a link between excitotoxicity and oxidative stress.

In the present study, we have employed an antibody that specifically recognizes nitrotyrosine to investigate the potential role of tyrosine nitration in the production of the insoluble protein aggregates associated with Alzheimer's disease.

Materials and Methods

Five Alzheimer's disease cases (aged 77, 84, 89, 97, and 101 years) and ten controls (aged 2 and 20 days old and 2, 14, 19, 42, 57, 70, 79, and 89 years old) were used in the present study. All specimens were derived from the Mount Sinai Alzheimer's Disease Research Center brain bank or the Mount Sinai Hospital autopsy service. The Alzheimer's disease cases had histories of a chronic, progressive, dementing illness and neuropathological analysis demonstrating sufficient numbers of senile plaques to meet Khachaturian¹⁹ and CERAD²⁰ criteria for Alzheimer's disease. Control cases had no clinical features of neurological disease and on neuropathological examination were free of significant abnormalities. All brain specimens were fixed in 10% neutral buffered formalin for 2 to 4 weeks, blocked, and embedded in paraffin. Sections from the medial temporal lobe including hippocampus and from the orbitofrontal cortex were cut at 5 μ m from paraffin-embedded blocks, deparaffinized, and hydrated through graded alcohols. Sections were then pretreated for 1 hour with 0.1% aqueous saponin (Sigma Chemical Co., St. Louis, MO), microwaved in 0.1 mol/L aqueous sodium citrate, pH 6.5, and finally treated with 3% aqueous hydrogen peroxide to remove endogenous peroxidase activity. Immunocytochemistry was performed using a rabbit polyclonal primary antibody raised against nitrated keyhole limpet hemocyanin¹¹

(Upstate Biologicals, Lake Placid NY; lot 13874) diluted 1:400 in 0.01 mol/L phosphate-buffered normal saline (PBS) with 1% normal goat serum at room temperature overnight. Sections were further processed by the avidin-biotin method (Vector Laboratories, Burlingame, CA) and immunoreactivity was visualized with diaminobenzidine. Finally, sections were briefly counterstained with cresyl violet for Nissl substance.

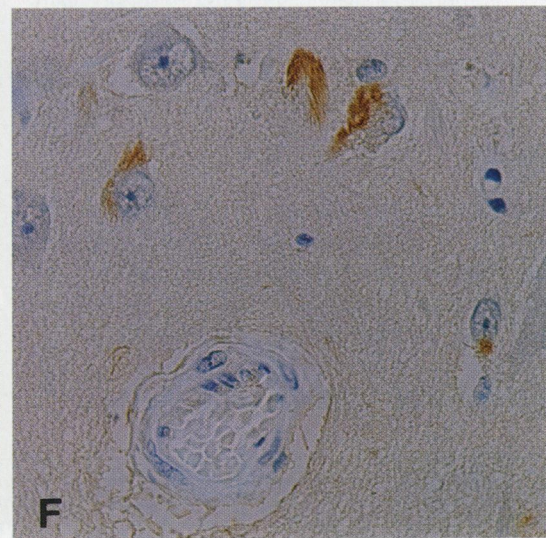
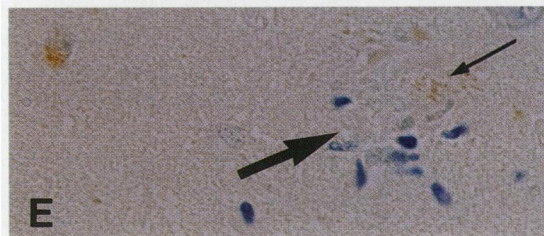
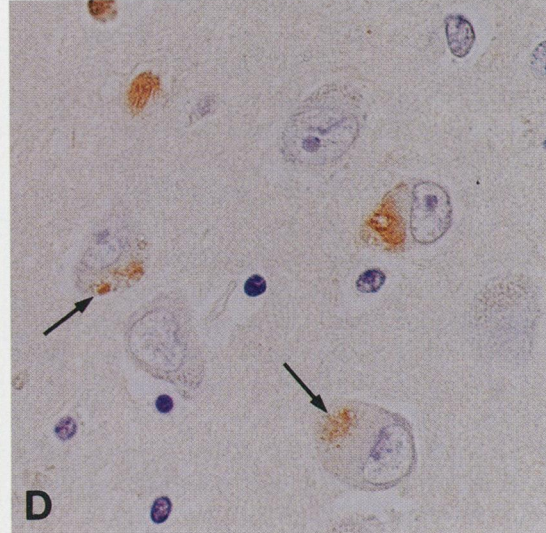
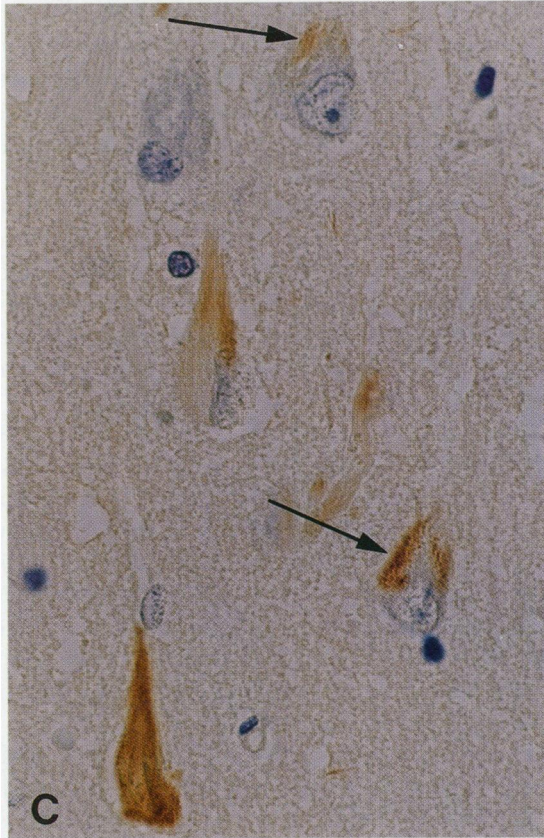
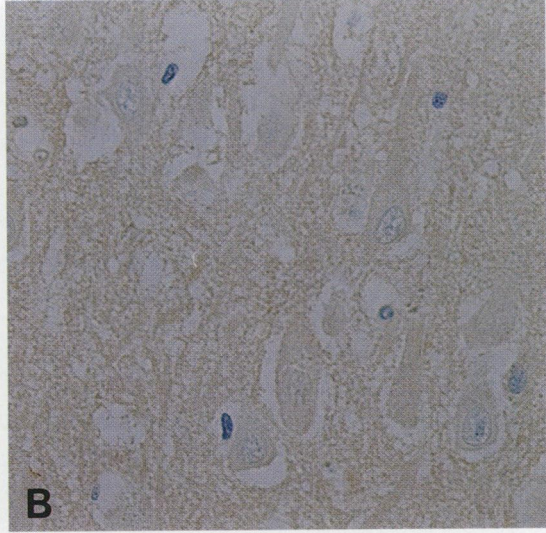
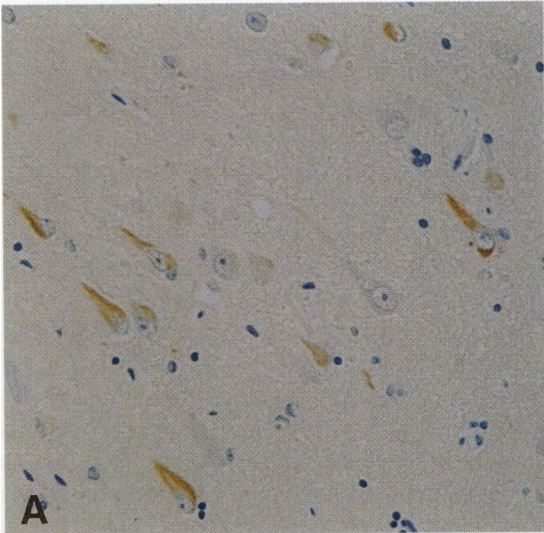
We have performed three series of control studies. First, sections were incubated overnight with the omission of primary antibody and processed as above. Second, antibody prepared at working dilution was preabsorbed with 1 mmol/L nitrotyrosine (Aldrich Chemical, Milwaukee, WI) before overnight incubation of sections and processing as for experimental sections. Third, sections were reduced by reaction with sodium hydrosulfite (Sigma). Aqueous Tris base (1 mol/L) was exhaustively bubbled with argon gas to remove dissolved oxygen, and sodium hydrosulfite was added to a concentration of 1 mol/L reaching a final pH of 9.2. Sections were immediately immersed in this solution for 10 minutes in a sealed coplin jar and washed in PBS. These sections were then incubated overnight in primary antibody and further processed as for the experimental sections.

Results

Sections from the young control cases (<2 years old) demonstrated minimal diffuse neuropil immunoreactivity, and in the specimens from the youngest individuals, immunoreactivity was virtually absent. Sections from older individuals (>50 years old) demonstrated diffuse immunoreactivity throughout the neuropil with no specific neuronal or glial structures immunolabeled for nitrotyrosine (Figure 1B). One control case, an 89-year-old patient with no clinical history of dementia, displayed occasional immunolabeled NFTs in the hippocampus and entorhinal cortex.

Immunolabeling in all five cases of Alzheimer's disease presented a consistent picture. In these cases, there was prominent labeling of NFTs in neurons of hippocampal field CA1, subiculum, and entorhinal cortex of medial temporal lobe sections (Figure 1, A and C-F). Field CA4 also contained moderate numbers of immunolabeled tangles, al-

Figure 1. Nitrotyrosine immunoreactivity in the hippocampal formation of Alzheimer's disease. **A:** Field CA1 of a 77-year-old Alzheimer's disease patient. **B:** Field CA1 of a 53-year-old non-Alzheimer control patient. **C and D:** NFTs of CA1 are labeled (**C, arrow**) in the absence of immunoreactivity in the adjacent cytoplasm, whereas other neurons demonstrate isolated granular immunoreactivity (**D, arrow**). **E:** Plaques are identifiable as a collection of small cells surrounding an unlabeled core (**thick arrow**). Note labeled neurites (**thin arrows**). **F:** Parenchymal blood vessels are unlabeled. Original magnifications. $\times 50$ (**A**); $\times 100$ (**B, C, E, and F**); $\times 150$ (**D**).



though rare labeled tangles were found in neurons of field CA3 and the granule cells of the dentate gyrus. Within neurons bearing immunolabeled tangles, cytoplasm free from NFTs was also free of immunolabeling (Figure 1C). Although quantitative analysis of anti-nitrotyrosine immunoreactivity in the hippocampus of Alzheimer's disease was not performed, it appeared that all NFTs within intact neurons were immunolabeled. Unlabeled or very faintly labeled NFTs were present, but these tangles were either unenclosed by a plasma membrane or were the only remaining structure within an apparently intact neuronal membrane. The faintly labeled NFTs appeared to be in a transitional state to unlabeled extracellular ghost tangles. Within the neuropil there were sparse immunolabeled neurites, which were often seen in association with plaque-like structures (Figure 1E). Senile plaque cores were not immunolabeled by the anti-nitrotyrosine antisera (Figure 1E). Among the large numbers of immunolabeled NFT-containing neurons of CA1 and subiculum were immunolabeled neurons in which a clearly defined NFT could not be observed but instead contained granular immunoreactivity (Figure 1D). Within the parenchyma of the hippocampus, immunolabeling of small blood vessels for nitrotyrosine was not seen (Figure 1F). Sections from the orbitofrontal cortex, chosen for a high density of plaques seen in thioflavin-S-stained sections, presented a similar picture, with widespread immunolabeling of NFTs and no immunolabeled senile plaque cores.

Control sections incubated without primary antisera and sections incubated with preabsorbed anti-nitrotyrosine antibody (Figure 2, E and F) were devoid of immunoreactivity. Sections reduced with sodium hydrosulfite showed only faintly discernible immunoreactivity within NFTs (Figure 2, C and D).

Discussion

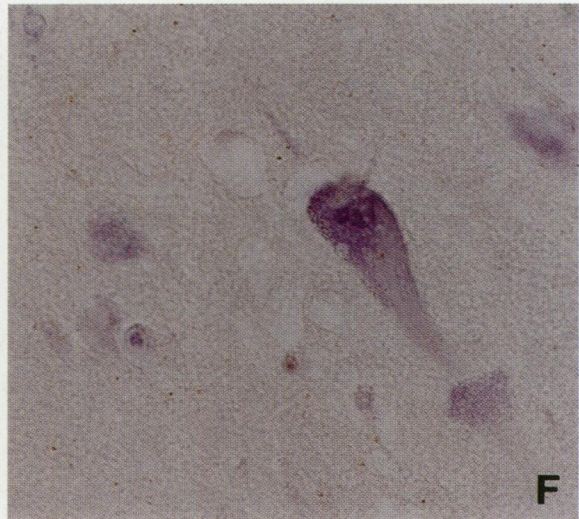
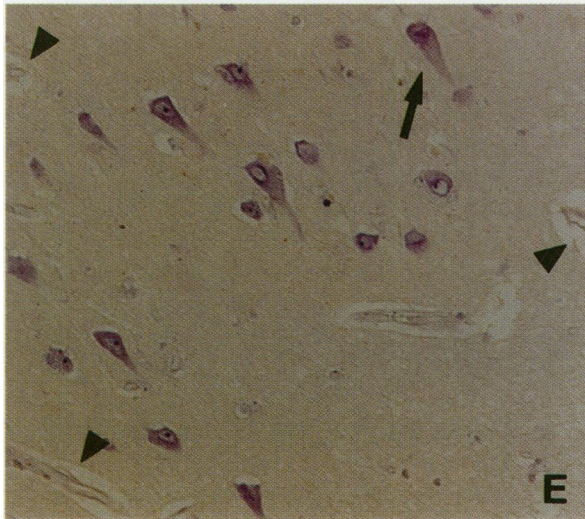
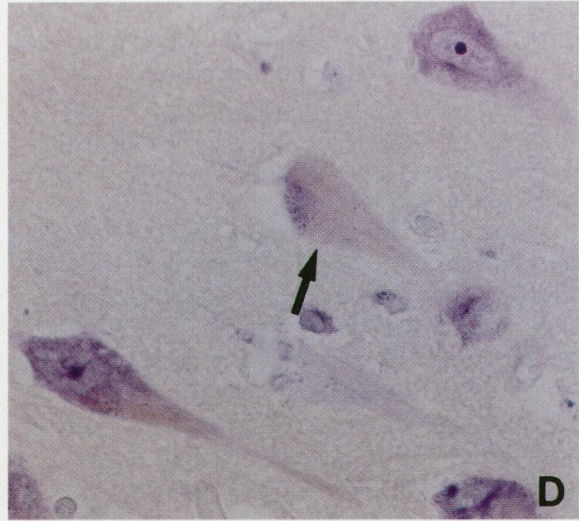
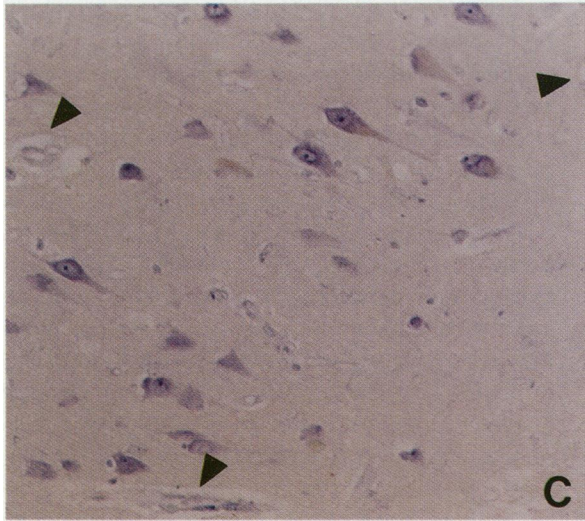
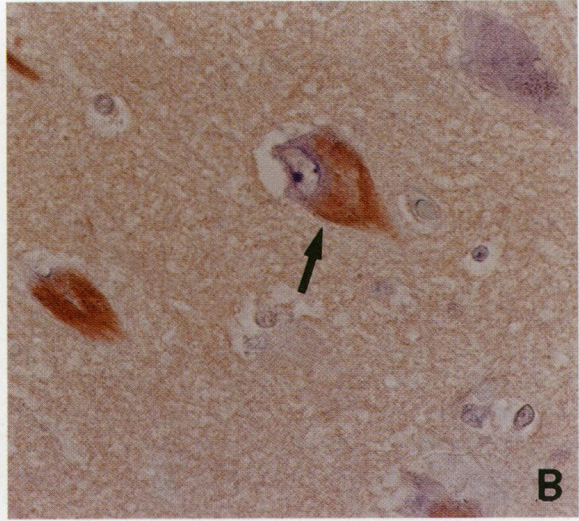
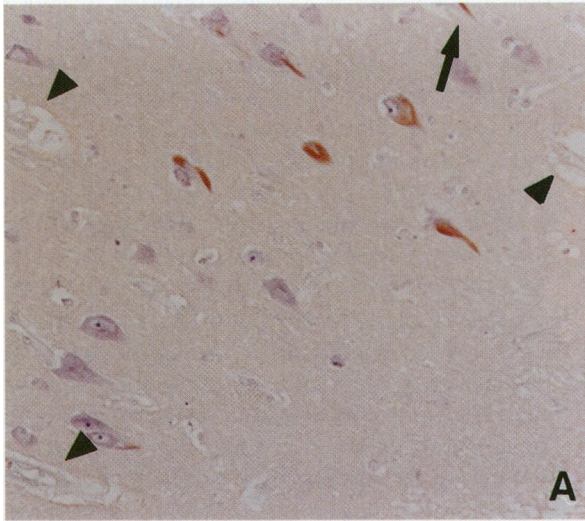
In the present study we have demonstrated the colocalization of nitrated tyrosine residues with the NFTs of Alzheimer's disease. This immunoreactivity was shown to be specific for nitrotyrosine by preabsorption of antisera with the target antigen as well as by abolition of immunoreactivity by reduction of the nitrotyrosine epitope to aminotyrosine by sodium hy-

dro-sulfite. In addition, others have shown that anti-nitrotyrosine immunoreactivity is not abolished by preabsorption of antisera with either aminotyrosine or phosphotyrosine.¹² The appearance of granular immunoreactivity in the apparent absence of NFTs indicates that nitration of tyrosine residues may precede the formation of the morphological tangle, although a definitive answer awaits additional studies. In control specimens free of neuronal pathology, there was no evidence of anti-nitrotyrosine immunoreactivity other than a diffuse background labeling that appeared to increase with increasing age of the patient. Interestingly, the case of an 89-year-old patient with no history of cognitive impairment demonstrated immunolabeled NFTs within the hippocampal formation. A number of reports²¹⁻²³ have demonstrated that the hippocampus and entorhinal cortex are the first areas affected by neurofibrillary degeneration in otherwise normal individuals and that neurofibrillary degeneration in these areas is associated with the first signs of cognitive decline, again indicating that tyrosine nitration is likely to be an early event in NFT formation.

Neuronal damage by oxidative stress has long been hypothesized as a critical mechanism of cellular damage in normal aging²⁴ as well as in neurodegenerative diseases.^{25,26} Considerable evidence has suggested the presence of oxidative stress or damage in Alzheimer's disease including increased protein oxidation²⁷ and carbonyl formation,⁹ increased lipid peroxidation,²⁸ decreased Cu/Zn-SOD mRNA,²⁹ co-localization of Cu/Zn-SOD, Mn-SOD, and catalase with NFTs³⁰ and the *in vitro* induction of β -amyloid aggregation by an iron H_2O_2 free-radical-generating system⁸. In the present study, the oxidizing agent most likely implicated in the formation of nitrated tyrosine residues is peroxynitrite. The peroxynitrite anion ($ONOO^-$) is a potent oxidizer formed at a near diffusion limited rate by the reaction of nitric oxide (NO^\cdot) with superoxide ($O_2^{\cdot-}$)¹⁴.

Nitric oxide was identified³¹ as a signaling molecule in the brain by demonstration that endothelium-derived relaxing factor, earlier shown to be NO^\cdot , was released upon activation of the NMDA class of glutamate receptors, resulting in an increase in cyclic GMP. Recent studies directed at determining the role of NO^\cdot in synaptic transmission have shown that

Figure 2. Controls for nitrotyrosine immunoreactivity (low and high magnification pairs from serial sections). **A and B:** Anti-nitrotyrosine-immunolabeled section. **C and D:** Section reduced with sodium hydrosulfite before anti-nitrotyrosine immunolabeling. **E and F:** Section incubated with anti-nitrotyrosine antisera, preabsorbed with nitrotyrosine. Note three vessels (**arrowheads**) at edges of low power micrographs (**A, C, and E**). Immunolabeled neuron indicated in **B (arrow)** is also present in adjacent section reduced by sodium hydrosulfite (**D, arrow**). Note that immunoreactivity is almost entirely abolished with only faint reaction product visible within the NFTs. Neuron indicated in **A (arrow)** is present in adjacent section incubated with preabsorbed antisera (**E, arrow**). Higher magnification (**F**) of neuron in **E (arrow)** demonstrates the absence of immunolabeling in the NFT. Original magnifications, $\times 50$ (**A, C, and E**); $\times 150$ (**B, D, and F**).



activation of NMDA receptors with ensuing calcium influx and binding of NOS to calmodulin, stimulates neuronal NOS, oxidizing L-arginine to L-citrulline and liberating NO[•] (for review see Refs. 18 and 32). O₂^{-•} is normally produced as an intermediate oxygen radical in the series of reactions that reduce molecular oxygen to water in the mitochondrial electron transport chain. O₂^{-•} radicals that leak from the electron transport chain are normally detoxified by SOD. The direct stimulation of NMDA receptors has also been demonstrated¹⁵ to generate O₂^{-•}. O₂^{-•}, in the presence of NO[•], would favor the formation of peroxynitrite as NO[•] effectively outcompetes SOD for O₂^{-•} and rapidly forms ONOO⁻. Thus, increased glutamatergic stimulation of NMDA receptors would increase the probability of ONOO⁻ formation. The part that NMDA receptors play in events leading to NO[•] formation is particularly intriguing because hippocampal NMDA receptors are thought to play a key role in synaptic plasticity, learning, and memory. NMDA receptors are found throughout the cortex, but they are particularly dense in the hippocampus^{33,34}, the cortical area that is among the most vulnerable to NFT formation.

A second mechanism responsible for overproduction of NO[•] is the increased synthesis of the inducible form of NOS. Inducible NOS (iNOS) has a sufficiently high affinity for calmodulin such that it binds calmodulin at the low levels of intracellular calcium normally found in neurons. iNOS is therefore always in the activated state, constitutively synthesizing NO[•]. Induction of iNOS is generally thought to occur in macrophages after immunogenic stimulus by lipopolysaccharide or cytokines. The role of iNOS in the production of peroxynitrite in the central nervous system is unknown, although it has been shown that reactive oxygen intermediates such as are generated by glycated tau are able to activate NF-κB,³⁵ a transcription factor for iNOS.³⁶

The mechanism of neurotoxicity of ONOO⁻ is not well understood. Peroxynitrite has been demonstrated to preferentially nitrosylate tyrosine residues¹⁴ resulting in nitrotyrosine immunoreactivity as observed in the present study. Demonstration of nitrotyrosine epitopes co-localized with NFTs raises the question of the nature of the protein bearing the nitrated residues within NFTs. Among molecules identified as nitrosylation targets of ONOO⁻ are the cytosolic and mitochondrial forms of SOD¹⁴ as well as the low molecular weight subunit of the neurofilament triplet.¹⁶ Nitrosylation of proteins has been shown to be catalyzed by two distinct mechanisms. Both superoxide and Fe³⁺-EDTA can catalyze the nitration of tyrosine by peroxynitrite.³⁷ Catalysis by

both mechanisms is certainly possible in light of observations that both SOD³⁰ as well as iron³⁸ have been found in association with NFTs.

Nitrosylated proteins present in intimate relationship with the primary intracellular lesion of Alzheimer's disease appears to represent an abnormal oxidative modification of the cytoskeletal components that constitute the NFT. Although it is not known precisely what the effects nitrosylation of cytoskeletal or other proteins are, such modifications would likely interfere with tertiary structure, rendering them dysfunctional, and may be a key step in the transformation of soluble cytoskeletal components into NFTs. In this context it has been observed that neuronal populations with a high neurofilament content are more vulnerable to NFT formation.³⁹ The presence of nitrosylated neurofilaments represents a possible mechanism by which this vulnerability may be conferred.

Finally, nitrotyrosine immunoreactivity may indicate the presence of a generalized state of oxidative stress. Protonated peroxynitrite forms peroxynitrous acid (HONOO), which spontaneously dismutates into nitrogen dioxide (NO₂[•]) and hydroxyl radicals (•OH),⁴⁰ capable of initiating lipid peroxidation and nucleotide hydroxylation.⁴¹ In addition, it appears that NO[•] can reduce iron from Fe³⁺ to Fe²⁺, liberating it from ferritin,⁴² thus increasing the concentration of iron available to participate in the generation of hydroxyl radicals by way of Fenton chemistry.⁴³ These factors, in combination with those cited above, suggest that oxidative stress is indeed an active process in Alzheimer's disease.

The hypothesis of oxidative stress with potential pathogenetic mechanisms has also been proposed for the other two major neurodegenerative diseases of aging: amyotrophic lateral sclerosis (ALS) and Parkinson's disease. In familial amyotrophic lateral sclerosis, numerous mutations in the gene encoding Cu/Zn-SOD have been found,⁴⁴ resulting in dysfunctional SOD^{45,46} with the prospect of overproduction of superoxide, increased peroxynitrite,¹⁷ and ensuing oxidative stress. The relation between familial and sporadic amyotrophic lateral sclerosis remains to be determined, but the highly similar phenotype of the two forms argues for a similar pathogenetic mechanism. In Parkinson's disease, factors leading to oxidative stress include decreased glutathione,⁴⁷ increased iron,⁴⁸ and decreased mitochondrial complex I activity⁴⁹ in the substantia nigra. In this setting, hydrogen peroxide liberated from the oxidative deamination of dopamine can undergo Fenton chemistry resulting in the increased lipid peroxidation⁵⁰ observed in Parkinson's disease. The results

of the present study raise the possibility that Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease share a common pathogenetic mechanism and represent the spectrum of a single disorder united by oxidative stress.

Acknowledgments

The authors thank Drs. Gerald Cohen, Tom Mittag, and John Morrison for many fruitful discussions of oxidative stress and neurodegeneration.

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