Widespread Peroxynitrite-Mediated Damage in Alzheimer’s Disease

Mark A. Smith,1 Peggy L. Richey Harris,1 Lawrence M. Sayre,2 Joseph S. Beckman,3 and George Perry1

1Institute of Pathology and 2Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106, and 3Department of Anesthesiology, School of Medicine, University of Alabama, Birmingham, Alabama 35233

Increasing evidence suggests that oxidative damage to proteins and other macromolecules is a salient feature of the pathology of Alzheimer’s disease. Establishing the source of oxidants is key to understanding what role they play in the pathogenesis of Alzheimer’s disease, and one way to examine this issue is to determine which oxidants are involved in damage.

In this study, we examine whether peroxynitrite, a powerful oxidant produced from the reaction of superoxide with nitric oxide, is involved in Alzheimer’s disease. Peroxynitrite is a source of hydroxyl radical-like reactivity, and it directly oxidizes proteins and other macromolecules with resultant carbonyl formation from side-chain and peptide-bond cleavage. Although carbonyl formation is a major oxidative modification induced by peroxynitrite, nitration of tyrosine residues is an indicator of peroxynitrite involvement. In brain tissue from cases of Alzheimer’s disease, we found increased protein nitration in neurons, including but certainly not restricted to those containing neurofibrillary tangles (NFTs). Conversely, nitrotyrosine was undetectable in the cerebral cortex of age-matched control brains. This distribution is essentially identical to that of free carbonyls.

These findings provide strong evidence that peroxynitrite is involved in oxidative damage of Alzheimer’s disease. Moreover, the widespread occurrence of nitrotyrosine in neurons suggests that oxidative damage is not restricted to long-lived polymers such as NFTs, but instead reflects a generalized oxidative stress that is important in disease pathogenesis.

Key words: Alzheimer’s disease; carbonyls; glycation; nitrotyrosine; oxidative stress; protein modification

MATERIALS AND METHODS

Tissue. Hippocampal tissue, including the adjacent entorhinal and neocortex from 16 cases of Alzheimer’s disease (ages 60–91 years; average 79) and five control cases with no clinical or pathological history of neurodegenerative disease (ages 32–82 years; average 55), and cerebellar tissue from three Alzheimer’s disease (ages 69–84) and two control (ages 64 and 74) cases with similar postmortem interval were fixed in methacarn (chloroform/methanol/acetic acid, 60:30:10) at 4°C overnight. The apolipoprotein E genotype of the cases of Alzheimer’s disease was ε4/ε4 (n = 2); ε3/ε4 (n = 3); ε3/ε3 (n = 4); ε2/ε3 (n = 1); unknown (n = 5). After fixation, tissue was dehydrated through ascending ethanol and embedded in paraffin, and 6-μm-thick sections were placed on silane-coated slides (Sigma, St. Louis, MO).

Antibodies and immunocytochemistry. Affinity-purified rabbit antisera (Sigma) was used at a 1:100 dilution in 1% normal goat serum, 50 mM Tris–HCl, pH 7.6 (Beckman et al., 1994). After incubation at 4°C for 16 hr, sections were immunostained with the peroxidase–antiperoxidase method, with 3,3’-diaminobenzidine as cosubstrate (Sternberger, 1986). Adjacent sections were immunostained with antisera to ubiquitin (Manetto et al., 1988) to confirm the identity and location of pathological structures.

Controls consisted of (1) omission of the primary antibody; (2) adsorption of the antibody with 50 μM nitrotyrosine (Gly-Tyr-Ala) peptide at 4°C overnight before application to the section; and (3) chemical reduction of nitrotyrosine by treating sections with 15 mM sodium hydrosulfite in 50 mM carbonate buffer, pH 8.0, for 15 min at room temperature (Cuatrecasas et al., 1968) before immunostaining. These procedures were performed in parallel with the antisera to ubiquitin as controls against artificial inactivation of either primary or secondary antibodies from use of sodium hydrosulfite–reduced sections and against nonspecific adsorption with nitrotyrosine or nitrotyrosine-NHS ester. After they were immunostained, in some cases the sections were stained with Congo red and viewed under cross-polarized light to show NFTs and amyloid-β deposits of senile plaques.

RESULTS

In all 16 cases of Alzheimer’s disease, all three antibodies to nitrotyrosine recognized neuronal cytoplasm and nuclei in hip-
pocampal sections (Fig. 1A,C), whereas, conversely, in the five control cases no specific structures were stained (Fig. 1B,D). In sections taken from cases of Alzheimer’s disease that were counterstained with Congo red, although the most intensely nitrotyrosine-positive neurons often contained NFTs (arrows) than in those lacking NFTs, which were also intensely stained (arrowheads). In contrast, amyloid-β deposits (❉) and surrounding dystrophic neurites of senile plaques as well as extracellular-NFTs (unmarked) were unstained. The location of NFTs and amyloid-β deposits was determined by Congo red counterstaining. In contrast, in the cerebellum, nitrotyrosine immunoreactivity was present at the same level in cases of Alzheimer’s disease (E) as it was in controls (F). Scale bars: A, B, 500 μm; C, D, 100 μm; E, F, 50 μm.

This distribution is essentially identical to our findings of increased free carbonyls in Alzheimer’s disease that were also localized to neuronal cell bodies and nuclei of both neurons and glia but, as here, was restricted to regions of Alzheimer’s disease pathology (Smith et al., 1996a). Although differences were noted in the intensity of immunoreactivity between cases of Alzheimer’s disease, we found no correlations to apolipoprotein E genotype, age, or postmortem interval for those cases where this information was available. Statistically, for the
21 cases analyzed, neuronal nitrotyrosine immunoreactivity showed complete sensitivity for detecting Alzheimer’s disease and complete specificity in not detecting any of the control cases. Additional studies will be required to evaluate whether the specificity is unique to Alzheimer’s disease or whether neuronal nitrotyrosine immunoreactivity is also increased in other neurological diseases.

To understand whether increased nitrotyrosine represents a global alteration of the oxidative stress in Alzheimer’s disease, we studied the cerebellum. In Alzheimer’s disease, the cerebellum is spared from neuronal degeneration. Pathology in the cerebellum is restricted to diffuse amyloid-β deposits. Cerebellum from controls and Alzheimer’s disease (Fig. 1E,F) showed nitrotyrosine immunoreactivity in Purkinje cells, with no apparent difference between controls and the cases of Alzheimer’s disease. This finding supports the idea that peroxynitrite-dependent damage is restricted to regions of Alzheimer’s disease pathology, although the basal level of protein nitration is dependent on the physiology of the neurons involved.

Nitrotyrosine immunoreactivity is specific, because (1) all three antibodies recognized similar structures, with 7A2 showing the strongest immunoreactivity; (2) no immunostaining was noted with omission of the primary antibody; (3) preabsorption of the antibodies with nitrated BSA or nitrated Gly-Tyr-Ala blocked recognition (Fig. 2); and (4) chemical reduction of nitrotyrosine with sodium hydrosulfite abolished immunoreactivity (Fig. 3). Importantly, there was no diminution of labeling with ubiquitin antisera after hydrosulfite reduction of the section, indicating that chemical reduction did not interfere with the peroxidase–antiperoxidase reaction or antibody recognition of other antigens (results not shown) (Kooy et al., 1995). Moreover, absorption of ubiquitin antisera with nitrated BSA

Figure 2. Nitrotyrosine immunoreactivity (A) is completely blocked by adsorption with nitrated Gly-Tyr-Ala (B). Adjacent serial section with a blood vessel is indicated by ♠ in each section. Scale bar, 100 μm.

Figure 3. Nitrotyrosine immunoreactivity (A) is completely abolished by chemical reduction of nitrotyrosine with sodium hydrosulfite (B). Adjacent serial section with a blood vessel is indicated by ♠ in each section. Scale bar, 100 μm.
or nitrated Gly-Tyr-Ala had no effect on consequent immunoreactivity (results not shown).

**DISCUSSION**

This study demonstrates that tyrosine nitration is increased in the neuronal cytoplasm as well as in the nuclei of both neurons and glia in regions of Alzheimer’s disease pathology. In stark contrast, nitrotyrosine immunoreactivity is undetectable in the cerebral cortex of controls. These findings implicate oxidants derived from nitric oxide, most likely peroxynitrite, in the pathogenesis of Alzheimer’s disease. Although one of the major oxidative modifications of proteins resulting from peroxynitrite is carbonyl formation from side-chain and peptide-bond cleavage, electrophilic nitration of tyrosine phenols is a signature of peroxynitrite involvement. Significantly, peroxynitrite formation is dependent on nitric oxide as well as superoxide, suggesting that nitric oxide synthase (NOS)-containing neurons or microglia may play a role in oxidative damage. NOS production increases after excitotoxicity, and it is suggested that neurodegeneration in Alzheimer’s disease is brought about by excitotoxicity resulting from overstimulation (Gibson, 1989). NOS-containing neurons are relatively spared in Alzheimer’s disease (Hyman et al., 1992), perhaps suggesting that NOS-containing neurons are better able to deal with oxidative stress than other neurons or, alternatively, that nitric oxide, from NOS-positive neurons, diffuses to other cells and reacts with superoxide to form peroxynitrite distal to the NOS-positive neurons. Activated microglia, present in most senile plaques in Alzheimer’s disease (Cras et al., 1991), can also produce nitric oxide (Goodwin et al., 1995; Nakashima et al., 1995; Paakkari and Lindsberg, 1995), and it is of note that the involvement of nitric oxide produced by microglia may provide an additional link to the lower incidence of Alzheimer’s disease with use of anti-inflammatory agents (Marx, 1996).

The strong association of peroxynitrite-related damage to regions of pathology indicates that the source for superoxide must lie in close proximity, because superoxide either reacts with tissue components (Hausladen and Fridovich, 1994) or is readily dissipated by superoxide dismutase, known to be associated with NFTs (Pappolla et al., 1992). The pathological lesions are a likely source, because glycated proteins (Yim et al., 1995), and particularly glycated τ in NFTs, produce superoxide (Yan et al., 1995). Therefore, this offers the possibility that NFT-containing neurons play a role in the oxidative damage of adjacent neurons. This hypothesis is consistent with the coordinate neuronal degeneration seen in sites of Alzheimer’s disease pathology and its dependence on NFT formation.

Although all of the Alzheimer’s disease cases examined show nitrotyrosine immunoreactivity, there was some case-to-case variation in intensity of staining, suggesting that nitrotyrosine may represent a specific pathological stage, that the epitope is often “masked,” or that the extent of involvement of nitrotyrosine is case-dependent. In this latter regard, we found no relationship between the extent of immunostaining and apolipoprotein E genotype. Significantly, our results differ from a recent report using a different antibody and formalin-fixed sections in which nitrotyrosine is described as being limited to NFTs (Good et al., 1996). By marked contrast, using methacarn-fixed material, which is not carbonyl cross-linked, we found evidence of widespread neuronal oxidative damage in Alzheimer’s disease. We were unable to obtain consistent nitrotyrosine immunostaining using formalin-fixed material, a peculiarity that may be restricted to brain, because nitrotyrosine has been localized in other tissues with these same antibodies after formalin fixation (Beckman et al., 1994). In preliminary immunoblots studies, we were unable to establish whether there is a preferential nitration of specific neuronal proteins (our unpublished data). Nonetheless, by extending beyond the lesions, our findings suggest that oxidative damage in Alzheimer’s disease may result from a chronic abnormality of oxidative balance that affects neurons regardless of whether they themselves contain an NFT.

The distribution of nitrotyrosine presented here is essentially identical to the distribution of free carbonyls (Smith et al., 1996a), although additional studies will be necessary to establish the relative contribution of peroxynitrite or other oxidants to protein oxidation. The distinction between neurons, which show damage throughout the cytoplasm and nucleus, and glia, whose damage is limited to the nucleus, suggests cell type-dependent differences with respect to oxidative damage. An understanding of these distinctions may provide an insight into the neuronal specificity of cell degeneration in Alzheimer’s disease.

**REFERENCES**


