

## Lipoperoxidation Is Selectively Involved in Progressive Supranuclear Palsy

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**Abstract.** Progressive supranuclear palsy (PSP) is a neurodegenerative disorder characterized by extensive neurofibrillary tangle (NFT) formation and neuronal loss in selective neuronal populations. Currently, no clues to the biological events underlying the pathological process have emerged. In Alzheimer disease (AD), which shares with PSP the occurrence of NFTs, advanced glycation end products (AGEs) as well as oxidation adducts have been found to be increased in association with neurofibrillary pathology. The presence and the amount of lipid and protein oxidation markers, as well as of pyrrolidine and pentosidine, 2 major AGEs, was assessed by biochemical, immunochemical, and immunocytochemical analysis in mid-brain tissue from 5 PSP cases, 6 sporadic AD cases, and 6 age-matched control cases. The levels of 4-hydroxynonenal (HNE) and thiobarbituric acid reactive substances (TBARS), 2 major products of lipid peroxidation, were significantly increased by 1.6-fold ( $p < 0.04$ ) and 3.9-fold ( $p < 0.01$ ), respectively, in PSP compared with control tissues, whereas in AD only TBARS were significantly increased. In PSP tissue the intensity of neuronal HNE immunoreactivity was proportional to the extent of abnormal aggregated  $\tau$  protein. The amount of protein oxidation products and AGEs was instead similar in PSP and control tissues. In AD, a higher but not significant level of pyrrolidine and pentosidine was measured, whereas the level of carbonyl groups was doubled. These findings indicate that in PSP, unlike in AD, lipid peroxidation is selectively associated with NFT formation. The intraneuronal accumulation of toxic aldehydes may contribute to hamper  $\tau$  degradation, leading to its aggregation in the PSP specific abnormal filaments.

**Key Words:** Neurofibrillary tangles; Oxidative stress; Progressive Supranuclear Palsy; Tau protein.

### INTRODUCTION

Progressive supranuclear palsy (PSP) is a neurodegenerative disease pathologically characterized by neuronal loss and gliosis in specific subcortical nuclei. Neurons within vulnerable nuclei often form abnormal intracellular inclusions consisting of 12 to 15 straight filaments bundles composed of  $\tau$  protein (1). While the pathogenesis of PSP is unknown, a major focus has been to determine whether  $\tau$  protein fibrillogenesis is a primary cause of the neuronal degeneration or if it is a consequence of other cellular alterations.

In PSP, the  $\tau$  filaments not only differ morphologically from the paired helical filaments of Alzheimer disease (AD) but also occur mainly in subcortical neuronal populations. Additionally, there are distinct differences in  $\tau$  isoform accumulation and the  $\tau$  abnormalities in PSP, unlike in AD, occur in the absence of amyloid- $\beta$  ( $A\beta$ ) deposition (2).

Several studies have suggested that in AD cortical tissue oxidative damage is associated with neurons whether or not they show  $\tau$  abnormalities (3–7). So, while the  $\tau$

protein extracted from AD brain exhibits epitopes of advanced glycated end products (AGEs) (5, 8) and lipid peroxidation products (Smith et al, unpublished observation), the relationship of  $\tau$  abnormalities to oxidative damage is unclear because neurons displaying  $\tau$  abnormalities actually have reduced oxidative damage (7). Conversely,  $A\beta$ , as well as glycated  $\tau$ , induces an oxidative stress in cells in culture (9). Therefore, the relationship of the  $\tau$  abnormalities to oxidative damage in AD is unclear.

To investigate the role of  $\tau$  abnormalities in neuronal oxidative damage, we investigated whether similar oxidative damage found in AD was also found in PSP, a disease in which  $\tau$  accumulation occurs in the absence of  $A\beta$ .

### MATERIALS AND METHODS

#### Tissue

Five cases fulfilling the clinical and pathological NINCDS criteria for PSP (61–80 years old), 6 cases of sporadic AD (62–78 years old), 6 age-matched controls (53–85 years old), and 2 younger controls (17 and 46 years) were studied. Brains were removed at autopsy and cut along the longitudinal axis; one half of each brain was fixed in 10% formalin and the other stored unfixed at  $-80^{\circ}\text{C}$ . Postmortem intervals were similar in all cases and varied from 4 to 9.5 hours.

#### Immunocytochemistry

Immunocytochemistry was performed on formalin-fixed, paraffin-embedded sections of pons at the level of *nucleus raphe*

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*dorsalis*. Serial sections were processed according to the peroxidase-antiperoxidase method by using an antiserum to  $\tau$  (10) or monoclonal antibody 5E2 to  $\tau$  protein (11) (gift of Kenneth Kosik) (1:500), a rabbit antiserum against a pyrrole adduct of HNE, which recognizes a 2-pentylpyrrole modification of lysine specifically formed by HNE (6) (gift of Lawrence Sayre), antibodies to pyrraline and pentosidine, 2 advanced glycation end products (4), monoclonal antibody against nitrotyrosine (7A2; gift of Joseph Beckman) (1), and a monoclonal antibody to 8-hydroxyguanosine (8OHG) (7). The reaction was developed with 3'-3' diaminobenzidine as co-substrate.

### Biochemical Analysis

For biochemical analysis, frozen samples of periaqueductal gray matter from the brainstem of the above listed cases were homogenized in 10 mM HEPES buffer containing 4.6 mM KCl, 1.1 mM  $\text{KH}_2\text{PO}_4$ , 0.6 mM  $\text{MgSO}_4$ , 137 mM NaCl, 1.1 mM EDTA, pH 7.4, with 2 mM phenylmethylsulfonyl fluoride and protease inhibitors (0.5  $\mu\text{g/ml}$  leupeptin, 0.7  $\mu\text{g/ml}$  pepstatin and 0.5  $\mu\text{g/ml}$  aprotinin), added just before the homogenization. The homogenates were sonicated and divided into 2 aliquots: one was centrifuged at 100,000 g for 5 minutes and used to evaluate protein oxidation and glycation, the other was centrifuged at 1500 g for 15 minutes and used to measure lipid peroxidation products. Supernatants were collected and frozen at  $-80^\circ\text{C}$  until analysis. Protein amount was evaluated by the bicinchoninic acid method (Pierce Chemical Company, Rockford, IL).

Protein oxidation was assessed by the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) (Oxyblot kit, Oncor, Gaithersburg, MD). Brain supernatants (5  $\mu\text{g}$  of protein) were incubated with DNPH for 15 minutes and dot-blotted onto Hybond-C nitrocellulose membrane (Amersham, Buckinghamshire, UK). Membranes were blocked by 5% milk in phosphate buffered saline (pH 7.4) and sequentially incubated with a rabbit antiserum to DNP and horseradish peroxidase-conjugated antiserum to rabbit IgG. Reactions were visualized by the enhanced chemiluminescence system (Amersham) on Hyperfilm<sup>®</sup> ECL (Amersham) and quantified by Molecular Analyst software (Biorad, Hercules, CA). The values of brain samples were within the linear range of the plot curve built with a diluted sample of known concentration.

Lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) (12) and 4-hydroxynonenal (HNE) as quantified by HPLC. The TBARS standard was pure malondialdehyde (monosodium trihydrate), obtained from 1,1,3,3-tetra-ethoxy-propane by hydrolysis following the procedure of Nair et al (13). Fifty  $\mu\text{l}$  of supernatant was added to 750  $\mu\text{l}$  of phosphoric acid (0.44 M), 250  $\mu\text{l}$  of TBA (42 mM) and distilled water (HPLC grade) was used to adjust the volume to 1.5 ml. Samples were incubated at  $100^\circ\text{C}$  for 1 h, cooled, and kept in ice until HPLC analysis was performed. Within 10 minutes of injection onto the column, all samples were neutralized with 0.5 ml of methanol-NaOH solution (4.5 ml of NaOH 1 M and 50 ml of methanol HPLC grade) for protein precipitation. Fifty  $\mu\text{l}$  of clear supernatant was injected in a  $3.9 \times 300$  mm  $\text{C}_{18}$   $\mu$  Bondapack (Waters SpA, Milan, Italy). Mobile phase was performed in 50% methanol and 50% 25 mM phosphate buffer (pH 6.5) at a flow rate of 0.8 ml/min. The detection system

(Waters 470 fluorimeter) was set at 532 nm excitation and 553 nm emission. TBARS were calculated on the basis of malondialdehyde standard calibration curve.

Quantitative determination of free HNE was performed according to Esterbauer (14). Briefly, brain homogenate aliquots were extracted twice with equal volumes of dichloromethane; the extract was collected in a flask containing acetate buffer (0.1 M pH 3); dichloromethane was evaporated under nitrogen stream; the material was applied onto a C18 solid-phase column (Bond-Elut); the nonpolar material removed by a first elution with hexane, and then HNE was eluted with 80% methanol. A volume of the sample was analyzed by HPLC on an S-5 Spherisorb ODS column with 40% acetonitrile as mobile phase and with UV detection at 220 nm.

The amount of AGEs, pyrraline, and pentosidine, was detected by the immunoreactivity of brain proteins with a mouse monoclonal antibody to pyrraline and a rabbit antiserum to pentosidine (gift of Vincent M. Monnier) in a dot-blot assay. Immunoreactions were developed and quantified as described for protein carbonyl groups detection. Statistical difference between control, AD, and PSP cases was performed with a non-parametric test (Kruskal-Wallis) and a post test to check the differences between groups (Dunnett test); a p value lower than 5% was considered significant.

## RESULTS

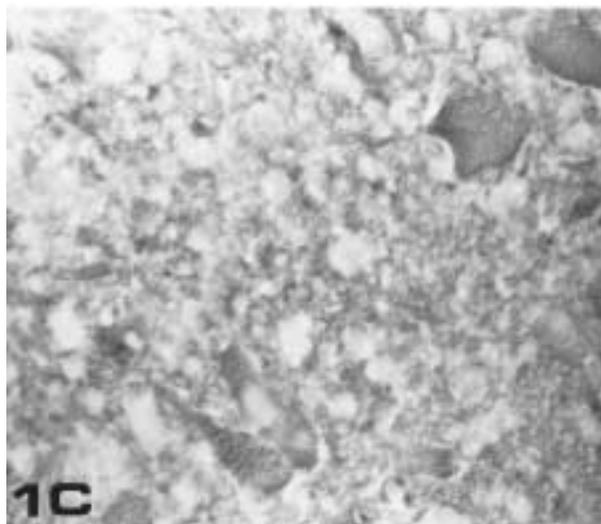
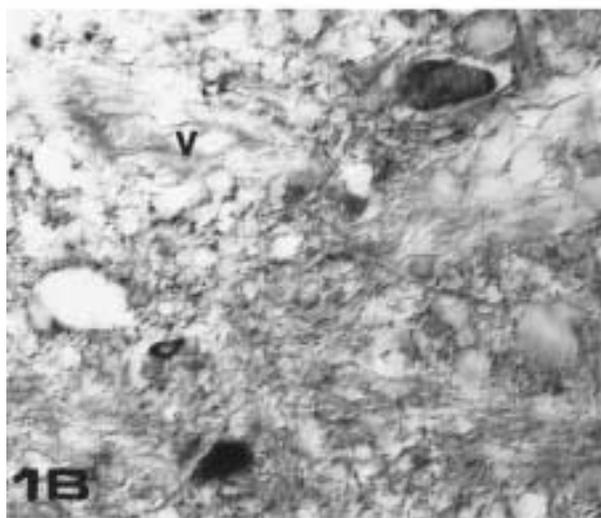
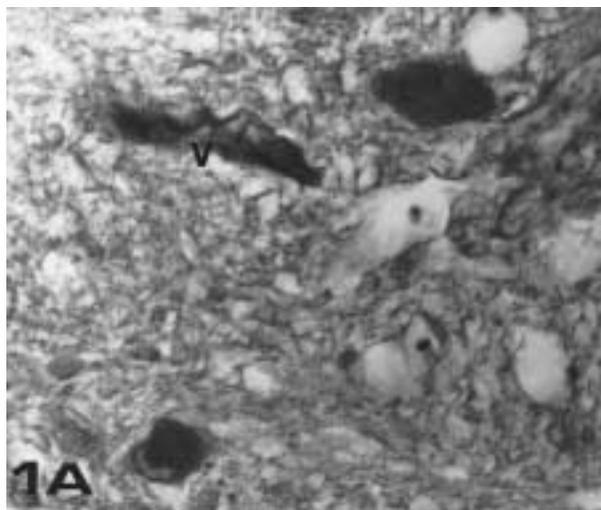
### Immunocytochemistry

The antiserum against  $\tau$  recognized neurons showing abnormal intracellular fibrils in brainstem of all PSP cases examined (Fig. 1B). Almost all neurons with abnormal  $\tau$  accumulation displayed intense HNE-pyrrole immunoreactivity. In fact, the appearance of the strongest immunolabeling with the antibody to HNE-pyrrole overlapped with that of  $\tau$ , suggesting that the HNE-pyrrole antigen is localized within the  $\tau$  accumulation (Fig. 1A). While neurons not containing  $\tau$  accumulation in the PSP cases showed a higher level of HNE-pyrrole immunoreactivity than in neurons of the same brainstem nuclei of age-matched control cases (Fig. 1C), it was consistently below the level in neurons containing  $\tau$ . In addition, as noted in earlier reports (6), the antibody to HNE-pyrrole recognized the walls of blood vessel in both PSP (Fig. 1A) and control tissues. On the contrary, the immunoreactivity of pyrraline and pentosidine (the 2 AGEs analyzed), as well as nitrotyrosine and 8OHG, did not significantly differ among any of the groups (not shown).

In AD cases, NFTs were detected with anti-tau antiserum in the periaqueductal grey and raphe nuclei. The immunoreactivity of glycation and oxidative markers was elevated in both NFT and non-NFT bearing neurons, but the immunostaining was only slightly higher than that observed in controls (data not shown).

### Biochemistry

When compared with controls, a significant increase of different oxidative markers in PSP and in AD tissue was



observed (Fig. 2). The average TBARS concentration (KW < 0.003) was significantly increased 3.9-fold in PSP ( $1.17 \pm 0.77$  in PSP vs  $0.30 \pm 0.12$  nMol/mg protein in controls;  $p < 0.01$ ) and about 8 times in AD ( $2.36 \pm 0.38$  nMol/mg protein;  $p < 0.001$  vs controls). HNE, similarly (KW < 0.02) increased 1.6-fold ( $48.9 \pm 7.5$  pMol/mg protein vs  $31.83 \pm 8.8$  in controls;  $p = 0.038$  vs controls) in PSP, but only 1.2-fold in AD ( $37.3 \pm 3.7$  pMol/mg protein,  $p$  insignificant). Tissue from AD also showed higher level of free reactive carbonyl groups detected by DNPH on dot-blot compared with controls ( $3.29 \pm 0.57$  vs  $2.09 \pm 0.54$  nMol/mg protein;  $p < 0.01$ ); in contrast, carbonyl groups were similar in PSP and in control groups ( $2.34 \pm 0.96$  nMol/mg). In addition, the immunoreactivities of pyrraline and pentosidine assayed with the same method (dot blot) did not significantly differ in control, AD, and PSP cases (Fig. 2).

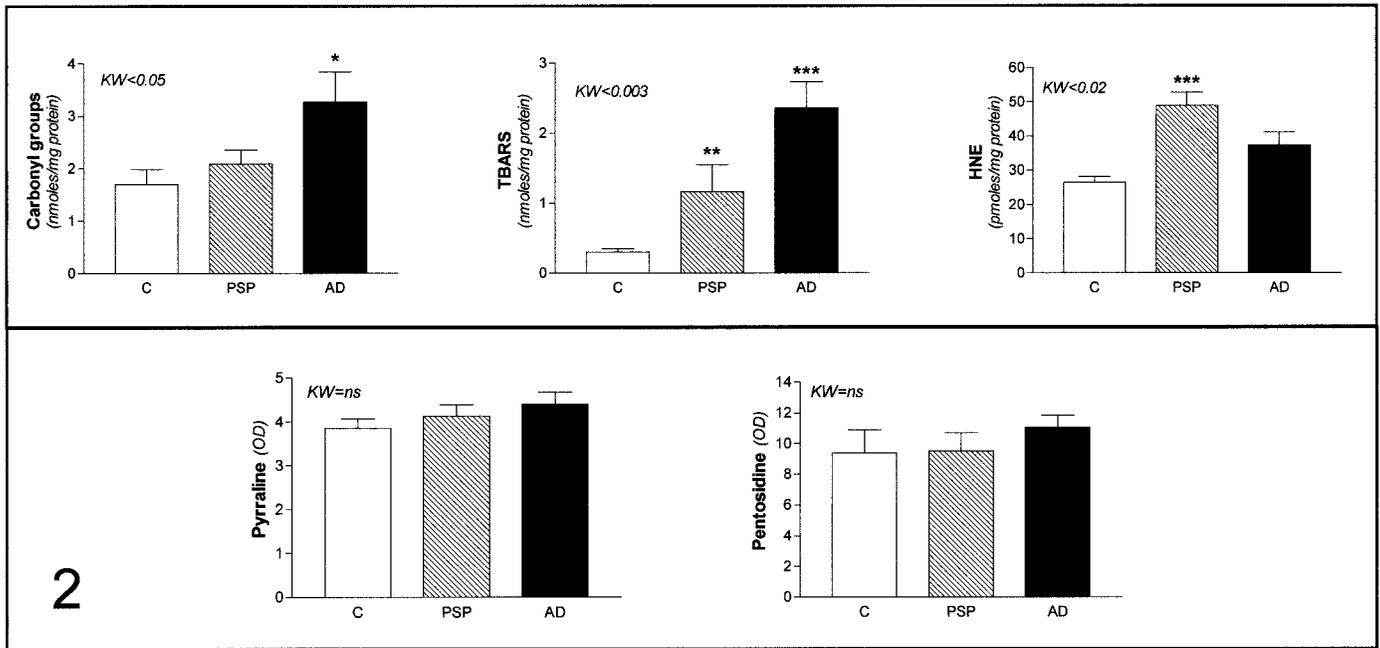
### DISCUSSION

The present study demonstrated that immunocytochemical (HNE-pyrrole) and biochemical (TBARS, free HNE) measure of lipoxidation were increased in the brain areas selectively involved in PSP, while nitrotyrosine (reactive carbonyls) and 8OHG (stable markers of protein and nucleic acid oxidation) did not show any variation between controls and PSP brains. Furthermore, these oxidative changes seem to be related to the formation of NFTs, as indicated by the association of the stable HNE product with intraneuronal  $\tau$ . Our findings differ from those of Montine et al, which reported a negative immunostaining of NFTs in PSP by an antiserum to HNE, unlike the positive reaction displayed by NFTs in AD cortical tissue with the same antibody (15). We do not know the basis of the difference in our findings since essentially the same antigen was used in antibody production in both studies. However, the presence of immunocytochemical and biochemical data supports our results.

Our study showed that the markers of nonenzymatic glycation (pentosidine and pyrraline) did not differ between control and PSP cases, suggesting that glycation does not play a role in the pathogenesis of PSP. In the same region of brainstem, all oxidation and glycation markers are more elevated in AD cases compared with control brains, but only protein carbonyls and TBARS

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**Fig. 1.** In PSP, HNE-pyrrole immunoreactivity (A) is increased in neurons of raphe nucleus that are  $\tau$ -reactive (B), while neurons not containing  $\tau$  also demonstrated higher HNE-pyrrole immunoreactivity (not shown) than in age-matched controls (C). HNE-pyrrole immunoreactivity is shown by blood vessels (v) in PSP as well as control cases (not shown), which are not recognized by the antibody to  $\tau$ . (Hematoxylin counterstaining;  $\times 600$ )



**Fig. 2.** Upper panel: level of protein oxidation (Carbonyl groups) and lipoperoxidation (Thiobarbituric acid reactive substance (TBARS) and 4-Hydroxynonenal (HNE)) markers; lower panel: level of glycation (pentosidine and pyrraline) markers in control, AD and PSP brains. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs controls.

are statistically significant. These findings also suggest that in subcortical areas of AD, a general glyco-oxidative stress occurs, proportional to the extent of pathological process, that is much less severe than in cerebral cortex. Accordingly, the immunostaining of all antigens of glycation and oxidation is slightly higher in AD compared with controls.

Our data raise the question of the relationship between oxidative damage, neuronal degeneration, and NFT formation in PSP. Oxidative damage results from an imbalance of intracellular clearance of reactive oxygen. The aging process is a major cause of the alteration of this equilibrium, whereas the central nervous system is particularly vulnerable because of its high metabolic rate, relative low glutathione and catalase (16), abundant lipid content, and inability to replace neurons.

Several studies have shown glyco-oxidative damage in AD cortex. High levels of pentosidine (4), carboxymethyllysine, a well-known AGE (17), TBARS (18) and HNE adducts (6), activation of heme oxygenase-1 (19), a microsomal enzyme that is induced by oxidative stress, nitrotyrosine (3), reactive carbonyls (20), oxidized nuclear and mitochondrial DNA and RNA (7) and upregulation of antioxidant enzymes in neurons bearing NFTs, as well as in neurons lacking  $\tau$  accumulation (21) have all been reported. In this report we show that these markers are increased, to a lesser extent, also in subcortical areas. Disorders such as Parkinson disease, Pick's disease, and corticobasal degeneration, as well as PSP, share with AD the formation of cytoskeleton-derived inclusions in the affected neuronal

populations. In PSP, the abnormal intraneuronal filaments are composed of stable polymers of  $\tau$ , with a prevalence of the longer isoforms that include 4 tandem repeats in the carboxy-terminus of the molecule (22).

In this study, we found that PSP does not display the glycation-related damage or the full spectrum of oxidative abnormalities found in AD, but is instead limited to lipid peroxidation. The reason may be differences in filament morphology or  $\tau$  isoforms involved, although it is more likely that the lack of  $A\beta$  is the key. We base this contention on the finding that global oxidative abnormalities of AD are linked to  $A\beta$  deposition, while  $\tau$  accumulation is a much later event. Indeed, in AD brainstem in which  $A\beta$  deposition occurs, though much less intensely than in cerebral cortex, oxidative damage is not limited to lipid peroxidation (i.e. protein carbonyl groups), but is far less prominent than in cerebral cortex. It is tempting to consider that  $\tau$  aggregation is specifically linked to membrane and lipid peroxidation effects. Therefore, in PSP and perhaps in other degenerative diseases, abnormal  $\tau$  accumulation may be indicative of lipid peroxidation. Finally, our findings suggest that trials with antioxidant compounds are a need in PSP therapeutic strategies.

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