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PROTEIN OXIDATION IN THE BRAIN IN ALZHEIMER'S DISEASE

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Abstract—In this study we used immunohistochemistry and two-dimensional fingerprinting of oxidatively modified proteins (two-dimensional Oxyblot) together to investigate protein carbonyl formation in the Alzheimer's disease brain. Increased protein oxidation was detected in sections from the hippocampus and parahippocampal gyrus, superior and middle temporal gyri of six Alzheimer's disease and six age-matched control human subjects, but not in the cerebellum. In two brain regions severely affected by Alzheimer's disease pathology, prominent protein carbonyl immunoreactivity was localized in the cytoplasm of neurons without visual pathomorphological changes and degenerating neurons, suggesting that intracellular proteins might be significantly affected by oxidative modifications. Following two-dimensional electrophoresis the positions of some individual proteins were identified using specific antibodies, and immunoblot analysis for protein carbonyls was performed. These studies demonstrated the presence of protein carbonyl immunoreactivity in β-tubulin, β-actin and creatine kinase BB in Alzheimer's disease and control brain extracts. Protein carbonyls were undetectable in spots matching glial fibrillary acidic protein and tau isoforms. Specific protein carbonyl levels in β-actin and creatine kinase BB were significantly higher in Alzheimer's disease than in control brain extract. β-Tubulin did not demonstrate a significant increase in specific protein carbonyl content in Alzheimer's disease brains.

We suggest that oxidative stress-induced injury may involve the selective modification of different intracellular proteins, including key enzymes and structural proteins, which precedes and may lead to the neurofibrillary degeneration of neurons in the Alzheimer's disease brain.

Key words: Alzheimer's disease, protein oxidation, protein carbonyls, immunohistochemistry, 2D electrophoresis, 2D western blotting.

Oxidative modifications of proteins attract considerable interest in aging and age-related neurodegenerative diseases. Alzheimer's disease (AD) is the most prevalent dementing disorder in the elderly, and accumulating evidence points to protein oxidative damage as a major contributor to AD-associated neuron death and decline of cognitive abilities.[9,11,15,40,41] Under oxidative stress conditions, chemical transformations of amino acid residues in proteins can lead to loss of specific protein functions.[10,45] Oxidation often converts proteins to forms that are more susceptible to proteinases, and therefore can "mark" them for degradation by proteolysis.[45] Conversely, oxidative modifications may promote formation of cross-linked protein aggregates, which are resistant to removal by proteinases.[9] Together with possible alterations in the rate of production and removal of oxidized proteins, oxidation-mediated protein aggregation may contribute to the accumulation and damaging actions of oxidized proteins in normal and pathological aging.[9,10] Interactions of proteins with reactive oxygen species play controlling roles in cellular signaling, which affect cell remodeling, growth and death.[10,18,38] Increased reactive oxygen species production and oxidative modification of brain proteins are important in AD pathogenesis,[14,27,28,37] and hence detailed investigation of protein oxidation is necessary to understand how oxidative stress affects cellular functions and eventually leads to neuron death in AD.

Carbonyl formation is an important detectable marker of protein oxidation. Carbonyl derivatives are formed by reactive oxygen species-mediated oxidation of side-chains of some amino acid residues. Carbonyl groups may also be introduced into proteins by glycation and reactions with glycoxidation and lipid peroxidation

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Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; AGE, advanced glycation endproducts; ANOVA, analysis of variance; BSA, bovine serum albumin; CKBB, creatine kinase BB; DNP, dinitrophenyl; DNPH, 2,4-dinitrophenylhydrazine; GFAP, glial fibrillary acidic protein; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphoric acid; HNE, 4-hydroxynonenal; HPG, hippocampus and parahippocampus gyrus; NFT, neurofibrillary tangles; NGS, normal goat serum; PHF, paired helical filaments; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; SMT, superior and middle temporal gyri; TBS, Tris-buffered saline.

products.[9] Protein carbonyls are increased in AD,[1,14,40] and there is a brain regional correspondence between protein carbonyl formation and histopathological markers in AD.[14] However, details of protein carbonyl formation at the cellular level and information about individual protein targets are lacking. Detailed studies of protein oxidative damage have been hampered by lack of appropriate methods to investigate markers of oxidative modifications *in situ* and identify individual oxidized proteins. The recently described methods of 2D fingerprinting of oxidized proteins[33,47] and immunocytochemical detection of protein carbonyls[43] provide the experimental potential to investigate the role of oxidative modifications of brain proteins in the pathogenesis of AD.

In this study, we investigated cellular localization of protein carbonyl immunoreactivity in brain regions differentially affected in AD, and identified several targets of protein carbonyl formation by high-resolution 2D fingerprinting of oxidized proteins.

EXPERIMENTAL PROCEDURES

Tissue samples

For protein carbonyl immunohistochemical analysis, specimens of the hippocampus and parahippocampal gyrus (HPG), superior and middle temporal gyri (SMT), and cerebellum from six AD patients (mean age 80.8 ± 5.0 years, mean *post mortem* interval 3.0 ± 0.3 h) and six age-matched control (mean age 78.8 ± 4.8 years, mean *post mortem* interval 2.8 ± 0.2 h) subjects were fixed in Methacarn (methanol:chloroform:acetic acid, 60:30:10) at 4°C for 24 h. Tissue was dehydrated through ascending ethanol solutions and xylene, and embedded in paraffin.

For 2D fingerprinting of oxidized proteins, specimens from SMT cortex were obtained at autopsy from six AD patients (mean age 80.4 ± 2.7 years, mean *post mortem* interval 3.3 ± 0.97 h) and six age-matched control subjects (81.1 ± 3.6 years, mean *post mortem* interval 3.9 ± 0.5 h). Brain specimens were removed rapidly at autopsy, immediately placed in liquid nitrogen and stored at −70°C.

All AD patients had the clinical diagnoses of probable AD using NINCDS-ADRDA Work Group Criteria.[29] Histopathologic examination of sections from multiple neocortical areas, hippocampus, amygdala and other subcortical, brainstem and cerebellar areas using hematoxylin–eosin and the modified Bielschowsky and Gallyas stains, along with 10D-5 and alpha-synuclein immunohistochemistry, revealed that all AD patients met accepted standard criteria for the histopathologic diagnosis of AD.[32,34] Controls were individuals without a history of dementia, other neurological diseases or systemic diseases affecting the brain. Known causes of death for control subjects were pneumonia, cancer and myocardial infarction. All control subjects were from the University of Kentucky normal volunteer group who underwent annual neuropsychological testing. Neuropathological evaluation of control brains revealed no significant gross alterations and only age-associated microscopic changes.

Immunohistochemical analysis of protein carbonyls

Tissue fixation and immunostaining of brain slides for protein carbonyls were performed as described by Smith *et al.*[43] with some modifications. Six micrometer thick sections were prepared from paraffin-embedded blocks of Methacarn-fixed specimens and placed on Silane-coated glass slides. Paraffin was removed and rehydrated sections were covered with 0.1% solution of 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl and incubated for 1 h to derivatize protein carbonyls. Because derivatization of protein carbonyls was performed in the presence of 2 N HCl, inactivation of endogenous alkaline phosphatase activity in sections was also achieved by this treatment. After incubation was complete, sections were washed three times with Tris-buffered saline (TBS) to remove the unbound DNPH and blocked for 30 min with 10% normal goat serum in TBS (NGS/TBS). After blocking, sections were incubated overnight at 4°C with 1:100 dilution of rabbit polyclonal anti-dinitrophenyl (DNP) antibody (ONCOR, Gaithersburg, MD, USA) in 1% NGS/TBS. Sections were washed three times with TBS and incubated for 40 min with secondary anti-rabbit IgG alkaline phosphatase-conjugated antibody (Sigma, St. Louis, MO, USA) diluted 1:800 in 1% NGS/TBS. After incubation, the secondary antibody solution was removed and sections were again washed three times with TBS. In several preliminary experiments, a rat monoclonal anti-DNP primary antibody (Zymed, San Francisco, CA, USA; 1:1000 dilution in NGS/TBS) and anti-rat IgG alkaline phosphatase-conjugated secondary antibody (Sigma; 1:800 dilution in 1% NGS/TBS) were used to detect protein carbonyls in brain slides. Alkaline phosphatase activity was localized by development for 15 min with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Sigma). Protein carbonyl immunoreactivity was analysed with the 20× or 40× objective of a Nikon microscope. Images were taken by a computer-controlled CCD video camera attached to the microscope. False color images of slides stained for protein carbonyl immunoreactivity were obtained using the MCID/M4 imaging system (Imaging Research, Ontario, Canada).

Specificity of primary antibodies was controlled by omitting DNPH treatment and reducing carbonyl groups with sodium borohydride before DNPH treatment. When DNPH treatment was omitted, control slides were treated with 2 N HCl only. Chemical reduction of protein carbonyls was performed using 25 mM solution of sodium borohydride ($NaBH_4$) in 80% methanol for 30 min at room temperature before treatment of control slides with DNPH/2N HCl. Primary antibody was omitted to control the specificity of protein carbonyl immunoreactivity localization.

Brain tissue extract preparation

Brain samples were thawed, minced and suspended in 10 mM HEPES buffer (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$ and proteinase inhibitors: leupeptin (0.5 mg/ml), pepstatin (0.7 mg/ml), type IIS soybean trypsin inhibitor (0.5 mg/ml) and PMSF (40 mg/ml). Homogenates were centrifuged at 16,000 *g* for 10 min to remove debris. Protein concentration was determined by the Pierce BCA method.

Two-dimensional electrophoresis and protein carbonyl immunoblotting

Two-dimensional fingerprinting of brain proteins with carbonyls (2D Oxyblot) was based on Nakamura and Goto[33] and Talent *et al.*[47] Derivatization of brain proteins with DNPH was carried out according to Levine *et al.*[25]

Two-dimensional polyacrylamide gel electrophoresis (PAGE) was carried out in a Multifor II Electrophoresis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Two-dimensional electrophoresis and subsequent immunoblotting for protein carbonyls were performed as described previously.[5] In brief, aliquots of AD and control brain extracts containing 100 μg of DNPH-treated total protein were applied to rehydrated Immobiline DryStrips (Amersham Pharmacia Biotech, pH 3–10, linear gradient) for the first-dimension isoelectric focusing. Until the second dimension, sets of strips containing electrofocused AD and control samples were kept at −70°C. For the second-dimension sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE), pairs of re-equilibrated strips (AD and control) were placed on ExcelGel XL 12–14 gels (Amersham Pharmacia Biotech). After application of prestained molecular weight markers (Bio-Rad, Hercules, CA, USA) and/or standard amount of protein carbonyls (0.4 pmol of protein carbonyl in DNP–BSA band of DNP-protein mixture provided by ONCOR), the electrophoresis was started.

Following electrophoresis, gels were removed from the plastic backing and transferred to nitrocellulose or stained with colloidal

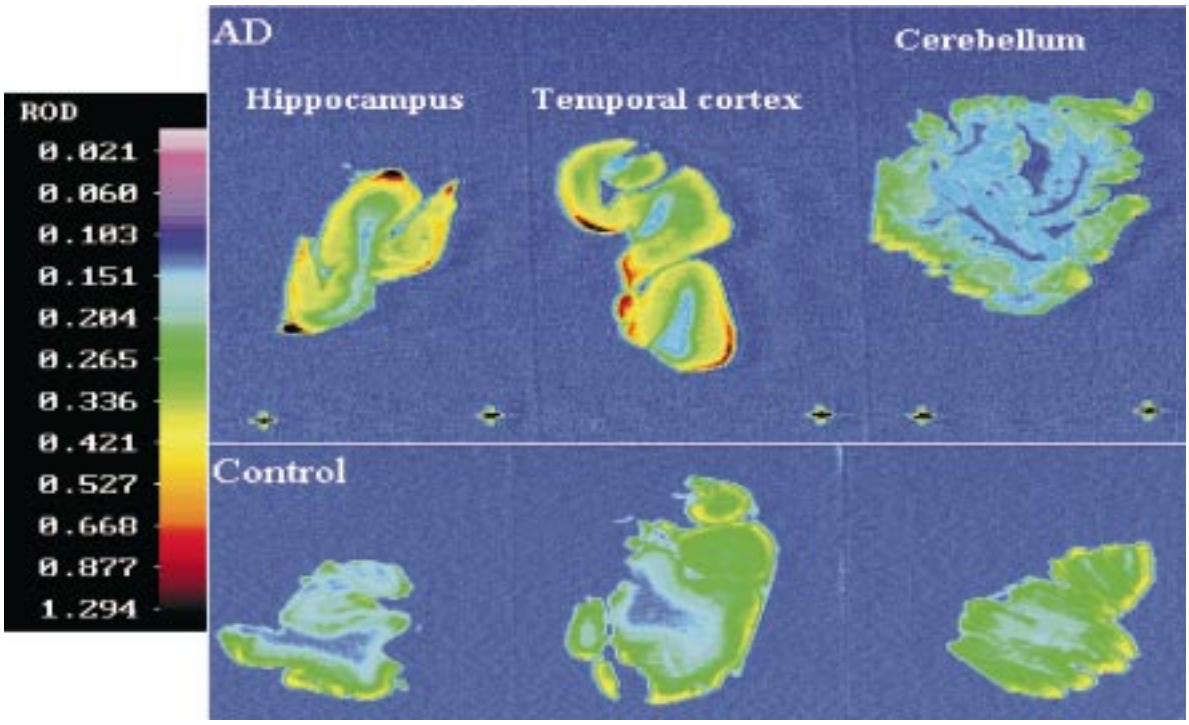

Fig. 1. False-color RGB (red–green–blue) images of 6-μm-thick sections from three different regions of AD and control brains (brain specimens were fixed in Methacarn and embedded in paraffin) stained for protein carbonyl immunoreactivity. Increased protein carbonyl immunoreactivity is present in the hippocampus and superior middle temporal gyrus AD brain sections. Color mapping of the images was performed using MCID/M4 imaging software. The scale on the left represents the relationship between color pattern and optical density. ROD, relative optical density.

Coomassie Blue (Sigma). Immunodetection of oxidized proteins on 2D blots was performed using polyclonal rabbit anti-DNP antibody (ONCOR)

Localization of β-actin, β-tubulin, creatine kinase BB, tau and glial fibrillary acidic protein on two-dimensional brain protein fingerprints and determination of specific carbonyl levels in identified individual proteins

Polypeptide spots corresponding to β-actin, β-tubulin, CK BB, tau and GFAP were identified on 2D protein fingerprints of DNPH-treated AD and control brain tissue extracts using specific antibodies.

Monoclonal anti-β-tubulin, anti-β-actin and anti-glial acidic fibrillary acidic protein (GFAP) antibodies were obtained from Sigma. Monoclonal anti-tau antibodies were purchased from Zymed (San Francisco, CA, USA) or obtained as a gift from Elan Pharmaceuticals (Dublin, Ireland). Anti-tau antibody from Zymed recognized an epitope near C-terminal region (404–441 aa) of human tau[8,20] and reacted with all human tau isoforms. Anti-tau 8011 antibody from Elan Pharmaceutical recognized a phosphorylation-independent epitope of all human tau isoforms.[50] Creatine kinase BB (CKBB) was detected with polyclonal anti-CK BB antibodies described previously.[1,2,4]

Conventional (1D) western blotting was used to verify the specificity of all antibodies before using them for identification of the individual proteins on 2D blots. For 1D western blotting analysis, SDS–PAGE was performed according to method of Laemmli.[21] Immunodetection of β-actin and CKBB on 1D and 2D blots was performed as described by Aksenova *et al*.[2] Working dilutions of specific antibodies for the detection of β-tubulin and GFAP on 1D or 2D blots were 1:300 and 1:200. Working dilutions of anti-tau antibodies were 1:1000 (Zymed) or 1:5000 (Elan Pharmaceuticals). Anti-mouse IgG AP-conjugated secondary antibodies were diluted 1:15,000.

Two-dimensional blots and gels were digitized and quantified using MCID/M4 imaging software (Imaging Research). Computer-assisted alignment of images of 2D blots immunostained for β-actin, β-tubulin, CKBB, tau and GFAP with images of 2D gels and Oxyblots was performed using "Shadow Alignment" option of the MCID/M4 imaging software package.

Protein carbonyl levels in all identified individual proteins were assessed by comparing the intensities of anti-DNP staining of the subsequent spots on 2D Oxyblots with an internal protein carbonyl standard. Specific protein carbonyl levels were determined in proteins that matched anti-DNP-positive spots on 2D Oxyblots as the ratio of anti-DNP immunostain to the content of matching protein. The content of matching proteins was determined by western blotting. Results of western blotting analyses were verified by quantitation of the subsequent polypeptide spots on 2D gels stained with colloidal Coomassie blue (Sigma).

Statistical analysis

Statistical comparisons of specific carbonyl levels in brain proteins, which matched with anti-DNP positive spots on 2D Oxyblots from AD and age-matched control subjects, were made using analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. Significant differences were set at $P < 0.05$.

RESULTS

Anti-DNP immunostaining of the AD brain sections showed increased protein carbonyl immunoreactivity in the HPG and SMT compared with the cerebellum. Severely affected brain regions in AD exhibited increased staining intensity compared with controls (Fig. 1).

Intense anti-DNP immunoreactivity localized in neuronal cell bodies was consistently found in HPG and SMT sections from AD patients. In control sections from the same brain regions, neurons with excessively

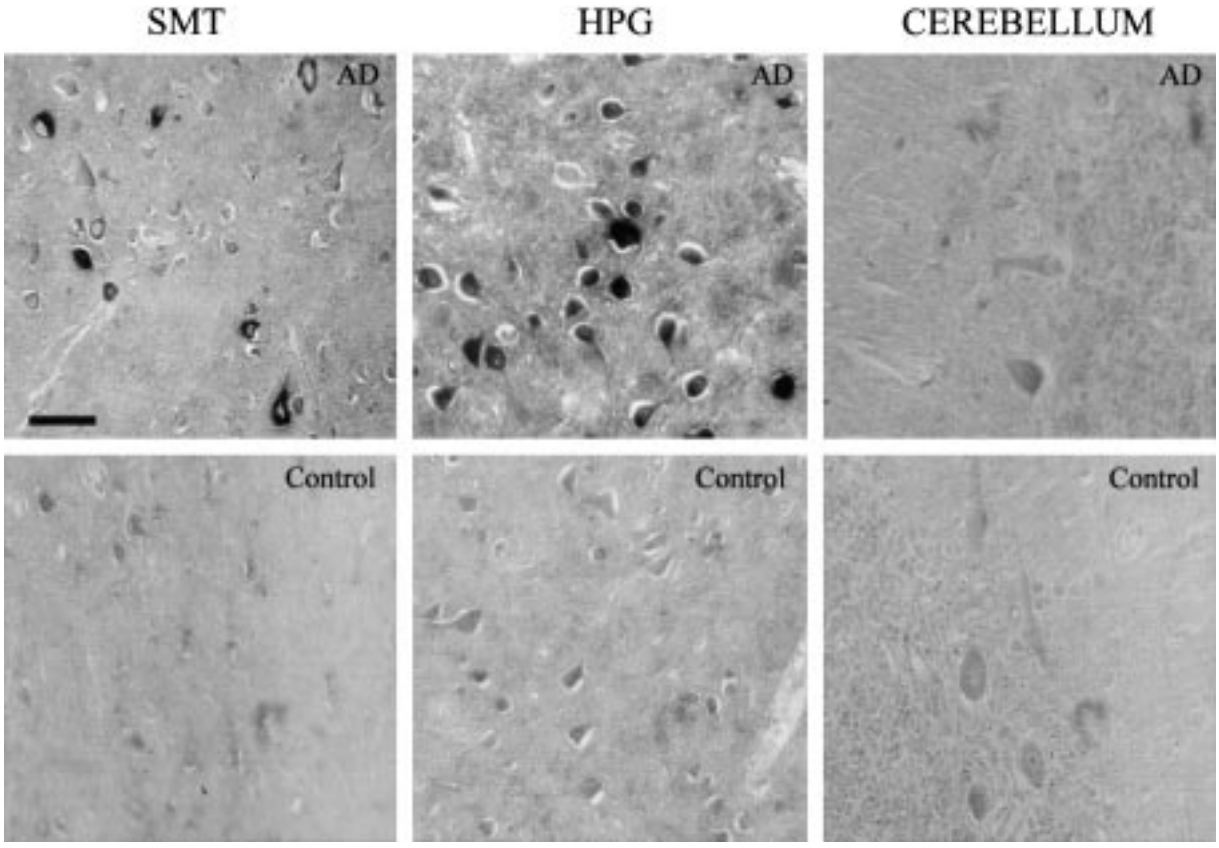

Fig. 2. Representative microscopic images illustrating results of immunohistochemical detection of protein carbonyls in the SMT cortex, HPG and cerebellum of AD and control subjects. All images were captured at the same lighting conditions and magnification. Scale bar = 100 μm.

oxidized proteins in the cytoplasm were rare. In cerebellar sections, faint protein carbonyl immunoreactivity was evenly distributed and no significant differences were found between AD and control samples (Fig. 2).

Increased protein carbonyl immunoreactivity in AD was observed in neurons without visual pathomorphological changes and degenerating neurons in HPG and SMT. Prominent protein carbonyl immunoreactivity in neurons in AD HPG and SMT sections was located in a perikaryon. In neurons with neurofibrillary changes, neurofibrillary tangles (NFT) surrounding the nucleus exhibited intense anti-DNP immunoreactivity (Fig. 3, panel I–II). Anti-DNP staining of a few senile plaques was observed in SMT of several, but not all AD patients (33% of total AD group used in the study, Fig. 3, panel III).

Robust staining was present in the walls of brain blood vessels in HPG, SMT, and cerebellar sections from AD and control subjects (Fig. 3, panel IV). Some positive anti-DNP staining, which could be attributed to reactive carbonyls in brain matrix proteins, was observed in the neuropil.

The specificity of anti-DNP immunostaining was confirmed in a set of control experiments. Results presented in Fig. 4 demonstrate that the reduction of reactive carbonyl groups with sodium borohydride, as well as the omission of primary antibody or DNPH-treatment, abolished carbonyl immunostaining in brain slides. Staining of brain slides with polyclonal or monoclonal anti-DNP antibodies produced the same results (not shown).

Because protein carbonyl immunohistochemistry detected prominent protein carbonyl formation in the cytoplasm of neurons in brain regions severely affected in AD, we investigated the spectrum of anti-DNP positive soluble proteins extracted from SMT samples of AD and control subjects by 2D variant of Oxyblot analysis. A representative result is shown in Fig. 5. Many, but not all, proteins resolved by 2D PAGE of AD and control extracts were recognized by anti-DNP antibodies. Major anti-DNP-positive protein spots were located between 111 kDa and 34 kDa. The intensity of anti-DNP staining of individual proteins did not always reflect their abundance. Some major protein spots in Coomassie Blue-stained 2D gels were faintly stained or completely undetectable on AD and control 2D blots stained for protein carbonyl immunoreactivity.

To determine which individual proteins may contain carbonyl groups in AD and control brain extracts, we identified locations of several brain proteins on 2D protein maps using specific antibodies and matched them with 2D patterns of anti-DNP positive proteins. Positions of GFAP, tau, β-tubulin, β-actin and CKBB-immunoreactive spots are shown in Fig. 6. Monoclonal anti-β-tubulin and anti-β-actin antibodies recognized single polypeptide spots on 2D blots. Molecular weight of the β-tubulin-positive spot was 52 kDa and the p*I* was

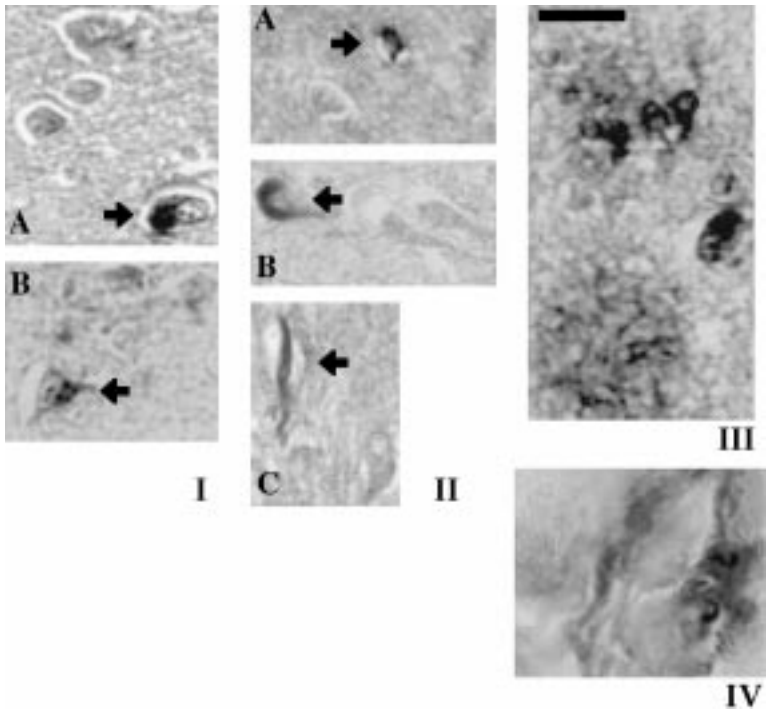

Fig. 3. Fragments of microscopic images of AD brain sections from severely affected regions showing localization of protein carbonyl immunoreactivity at the cytological level. (I) Arrows point at the intense protein carbonyl immunoreactivity in the cell body of neurons without visual pathomorphological changes in sections from the AD SMT (A) and HPG (B). (II) Arrows point at intensively stained NFT in tangle-bearing neurons in the AD SMT (A) and HPG (B, C). (III) Anti-DNP-positive senile plaques observed in the SMT of two AD subjects (33% of all AD cases used in the study). (IV) Intense protein carbonyl immunoreactivity localized in the wall of a brain blood vessel (fragment). In contrast to the other images shown in this figure, this localization of the intense protein carbonyl immunoreactivity was not specific for the AD pathology. All images were taken at the same magnification. Scale bar = 50 μm.

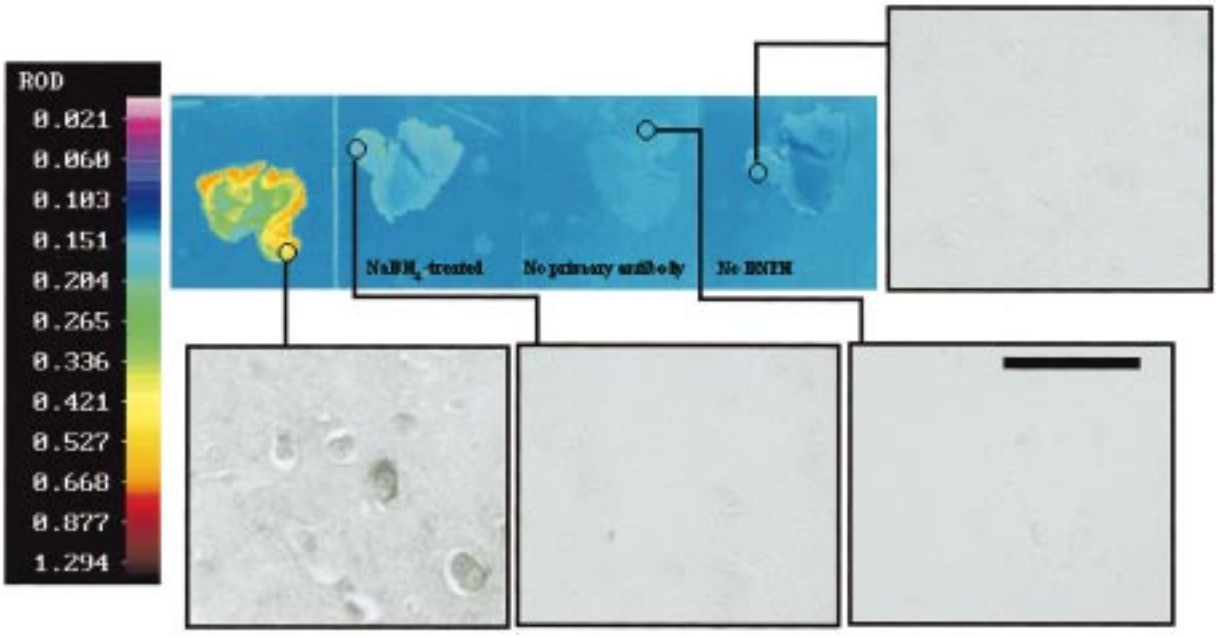

Fig. 4. Images of four adjacent sections of the AD SMT, which illustrate the specificity of anti-DNP immunostaining. Images in boxes are representative microscopic images from encircled regions of the AD section immunostained for protein carbonyls and the adjacent sections, which served as specificity controls (sodium borohydride-treated, no primary antibody and no DNPH, respectively). All images were captured at the same lighting conditions and magnification. Scale bar = 100 μm. ROD, relative optical density.

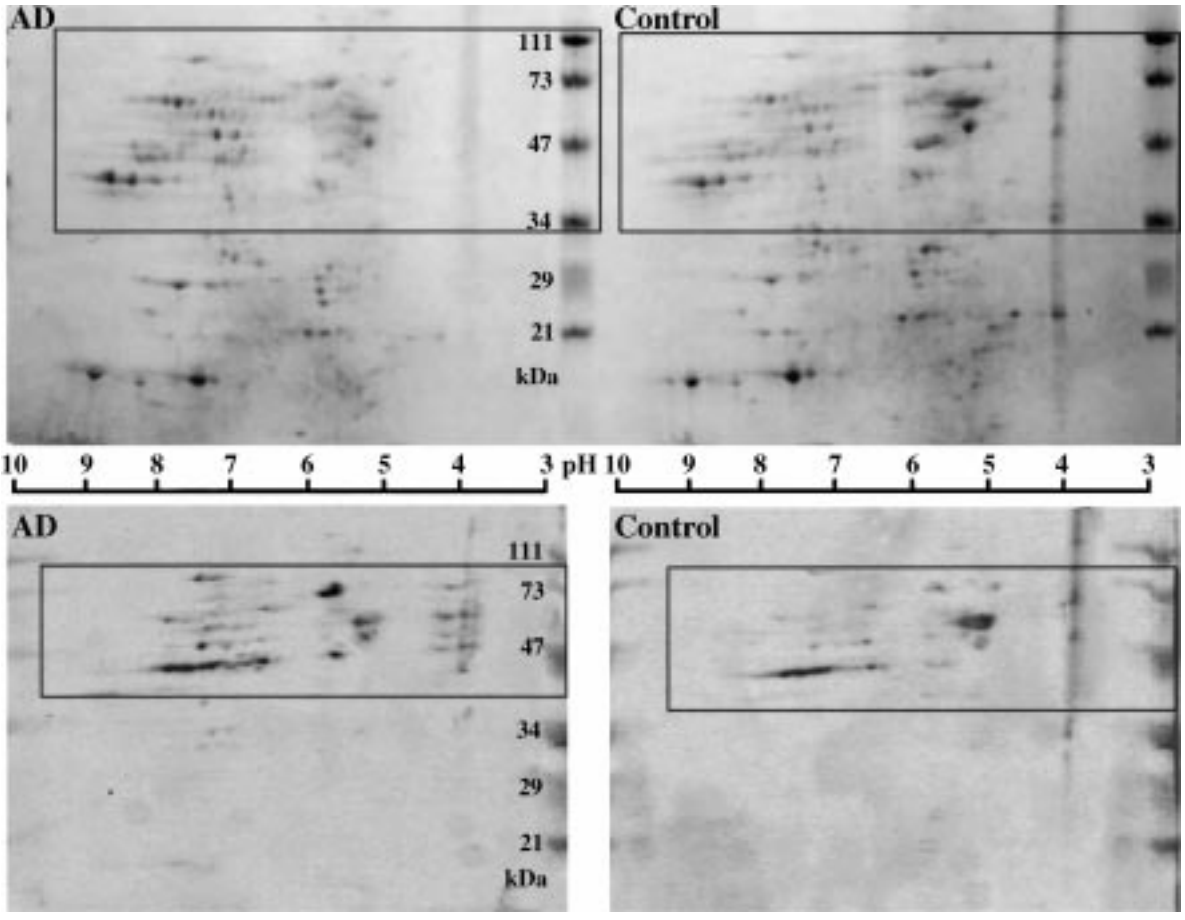

Fig. 5. Coomassie Blue-stained 2D gel (top panel) and 2D Oxyblots (bottom panel) from AD and control brain extracts. Boxes mark image areas that are shown enlarged in Fig. 6.

5.0–5.1. Anti-β-actin antibodies recognized the spot of 45 kDa and p*I* = 5.0. Creatine kinase BB immunoreactivity matched with one to three closely located spots of 43 kDa and p*I* from 5.5 to 6.0. GFAP was usually represented by many spots on 2D western blots and in 2D Coomassie Blue-stained gels, which were located at p*I* = 5.1 and migrating at 50 kDa and 45–44 kDa. Occasionally, additional minor GFAP-positive spots of 40–38 kDa were observed in brain extracts of AD and control subjects. Anti-tau antibodies used in this study recognized neutral (55 kDa, pI = 7.0) and basic (p*I* = 9.0) tau isoforms (50–55 kDa) on 2D blots from control samples. Additional abnormal acidic (p*I* = 4.9–5.1; 65–70 kDa) tau-positive spots were observed in AD samples, but not in controls. Basic tau isoforms were undetectable in Coomassie Blue-stained 2D gels of AD and control samples, probably because of their low abundance in brain extracts.

The comparison of 2D protein maps with 2D Oxyblots from AD and control brain extracts demonstrated that three major anti-DNP-positive signals in the range of p*I* from 4.5 to 6.0 and molecular weight from 60 to 40 kDa belong to β-tubulin, β-actin and CKBB. Protein spots recognized by anti-tau and GFAP-specific antibodies on AD and control 2D western blots did not match anti-DNP-positive spots on 2D Oxyblots (Fig. 6 A, B). To determine whether the identified anti-DNP-positive proteins were more oxidized in AD than in control, the intensities of anti-DNP staining were normalized to the specific protein content. Significant increases in specific protein carbonyl levels were observed in β-actin (442 ± 23% of control, $P < 0.01$) and in CKBB (336 ± 24% of control, $P < 0.01$). The specific protein carbonyl level in β-tubulin in AD was not significantly different from control (115 ± 28% of control, $P > 0.05$) (Table 1).

DISCUSSION

This study used immunohistochemical and 2D immunoblotting analyses together for the first time to study protein carbonyl formation in the brain of AD patients. We observed that in the severely affected regions of the AD brain, increased protein carbonyl immunoreactivity occurred in the cell bodies of neurons without visual pathomorphological changes and neurons with neurofibrillary abnormalities. Our results of the immunohistochemical analysis of protein carbonyls in brain regions severely affected by AD pathology are generally consistent with the report of Smith *et al.*,[43] who studied protein carbonyl immunoreactivity in the AD hippocampus. We demonstrated in this study that the localization of protein

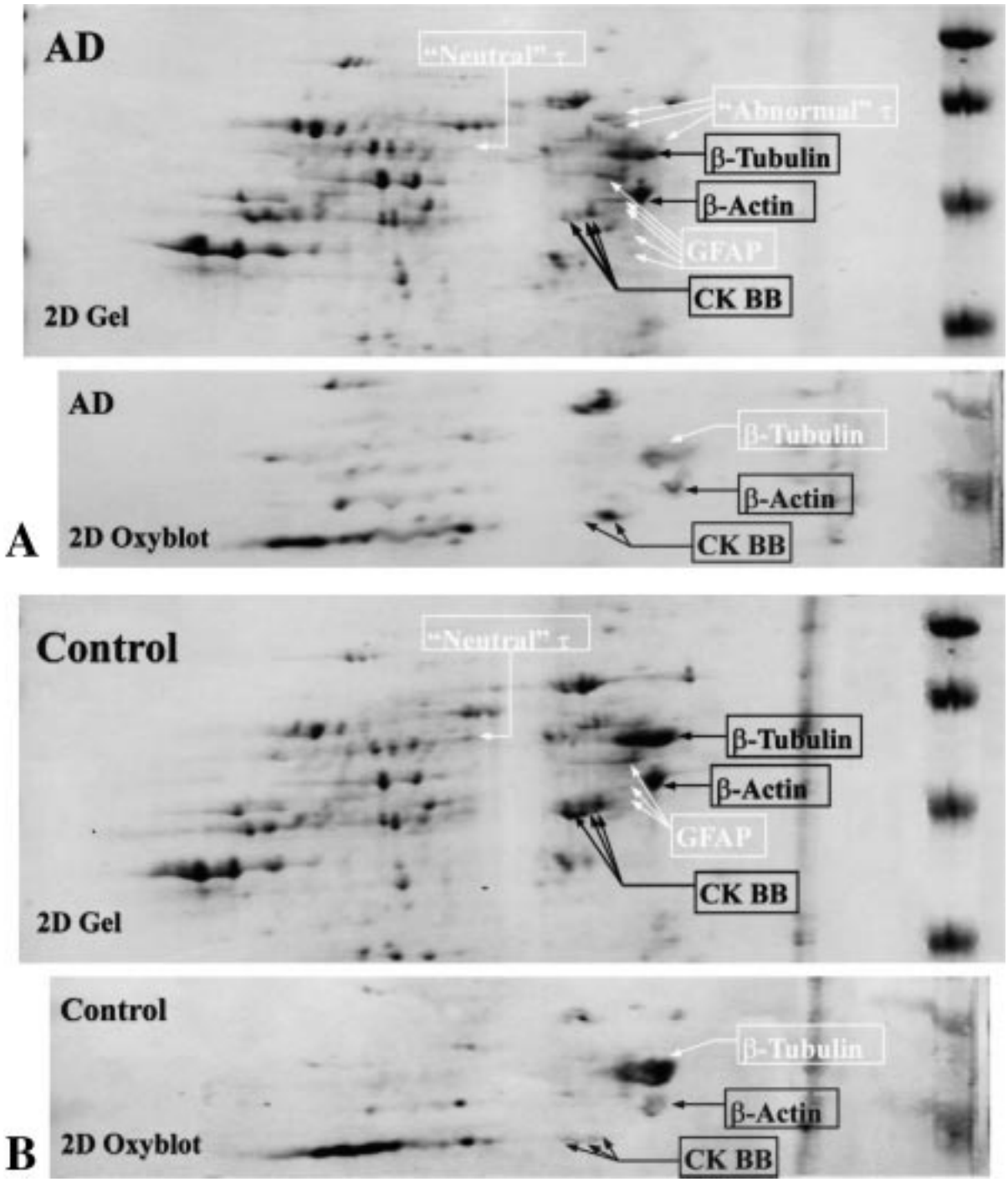

Fig. 6. Partial images of 2D protein maps and 2D Oxyblots of AD (A) and control (B) brain extracts. Partial 2D gel images (boxed areas in Fig. 5, top panel) show the position of protein spots recognized by specific antibodies against β-tubulin, β-actin, tau, GFAP and CKBB. Identified proteins that were not detected on 2D blots immunostained for protein carbonyls are marked in white. Partial 2D Oxyblot images (boxed areas in Fig. 5, bottom panel) show protein carbonyl immunoreactivity in β-tubulin, β-actin and CKBB. To determine specific levels of carbonyl immunoreactivity in individual proteins, the intensities of anti-DNP immunostaining had to be normalized to the content of the subsequent proteins. Relative changes in specific protein carbonyl immunoreactivity in β-tubulin, β-actin and CKBB are given in Table 1. Proteins, which exhibited statistically significant increase in protein carbonyl content (β-actin and CKBB), are marked in black. β-Tubulin, which did not demonstrate significant increase in protein carbonyls, is marked in white.

carbonyl immunoreactivity in neural cells in affected (SMT and HPG) and unaffected (cerebellum) regions of the AD brain was qualitatively different, which confirms that protein oxidation is a part of the neurodegenerative process in AD.

Protein carbonyl immunoreactivity associated with the extracellular matrix and blood vessel walls was present in all brain regions studied in AD and age-matched control subjects and could reflect age-related

Table 1. Relative changes in specific carbonyl levels in β-tubulin, β-actin and creatine kinase BB in Alzheimer's disease and control brain samples

Identified protein	Specific oxidation*
β-Tubulin	115 ± 28
β-Actin	442 ± 23**
CK BB	336 ± 24**

*For each identified anti-DNP positive protein, individual anti-DNP immunostain/ protein stain ratios (calculated for each of six AD and six age-matched control subjects) were averaged and results expressed as percentage of control ± S.E.M.
**Differences that were significant at $P < 0.05$.

accumulation of oxidative modifications in long-lived matrix proteins.[43]

We observed intense protein carbonyl immunoreactivity in the perikaryal space associated with NFT. Fiber-like structures in neurites and neuropil did not exhibit intense staining. We suggest that the presence of protein carbonyl immunoreactivity in NFT may be related to the stage of their development. Carbonyl groups introduced into NFT-associated proteins at the initial stages of tangle formation may participate in cross-linking reactions or may be transformed into non-carbonyl derivatives. Consistent with this explanation are the results of Horie *et al.*,[16] who reported that the advanced glycation end-product (AGE) pentosidine, but not the carbonyl-containing AGE product *N*-ε-(carboxymethyl)lysine, was present in extraneuronal tangles and in cores of classic senile plaques in the AD brain.

Accumulating evidence suggests that amyloid β-peptides (Aβ) play a role in neuronal oxidative damage in AD.[7,9,15,26] However, studies on the association of oxidative markers with amyloid deposits produce controversial results. In transgenic mice that overexpress amyloid precursor protein, oxidative markers, such as pentosidine and 4-hydroxynonenal (HNE) pyrrole adduct were detected in periplaque regions and in neuronal cell bodies distant from β-amyloid deposits.[44] In the AD brain, positive HNE immunoreactivity was found in perivascular areas where amyloid deposition was confirmed by Congo Red staining and in some, but not all, senile plaques.[6] Other investigators did not detect lipid peroxide adducts,[42] AGEs[46] or protein carbonyls[43] in amyloid deposits in the AD brain. Our study did not demonstrate that increased protein carbonyl formation in the AD brain is consistently associated with senile plaques. A few protein carbonyl-positive plaques were found in SMT sections only in some AD cases. Absence of increased carbonyl formation around mature senile plaques indirectly supports the view that a soluble pool of beta-amyloid rather than insoluble Aβ determines the severity of neuronal damage.[3,30,35,51,52] A recent study in transgenic mouse models of familial AD revealed that the decrease in synaptic density and prominent deficits in synaptic transmission, which were associated with increased Aβ production, preceded amyloid deposition.[17] It is tempting to speculate that diffusible, small-sized Aβ aggregates may induce plaque-independent oxidative damage of neurons in the AD brain. Neurotoxic small Aβ oligomers (Aβ-derived diffusive ligands; protofibrils) were reported to induce oxidative stress and kill mature neurons *in vitro*.[23,35,51] However, evidence for the presence of toxic amyloid protofibrils *in vivo* has yet to be obtained.

Increased protein carbonyl immunoreactivity in some morphologically unchanged neurons in brain regions affected by AD pathology indicates that oxidative modification of brain proteins may precede degenerative changes in neurons. Therefore, protein oxidation is not a simple consequence of neuronal degeneration. Supportive of this are the results of La Fontaine *et al.*,[22] who observed that protein oxidation induced by the injection of 3-nitropropionic acid, a specific inhibitor of succinate dehydrogenase, into the rat striatum precedes lesion formation. Demonstration of protein carbonyl localization in the neuronal cytoplasm suggests that intracellular proteins may be affected by oxidative modifications in AD. However, the immunohistochemical staining for protein carbonyls cannot determine which individual proteins are oxidized. This study shows that 2D fingerprinting of proteins with carbonyls may be a useful tool for detailed studies of protein damage induced by oxidative stress in the AD brain. Our results demonstrate that many proteins in brain extracts from AD and age-matched control subjects have detectable amounts of carbonyl groups. Protein carbonyl immunoreactivity in control subjects may reflect accumulation of oxidized proteins associated with normal brain aging.[40] Thus, it is necessary to compare specific carbonyl levels in individual proteins in AD and control brains to detect which proteins are specifically susceptible to oxidative modifications in AD.

In this study, increased protein carbonyl levels were detected in β-actin in the AD SMT. Specific protein carbonyl level in β-actin was higher than in cytosolic CKBB, which was previously identified as a target of protein oxidation in AD.[5] In the CNS, actin is distributed widely in neurons, astrocytes and blood vessels.[12] Some investigators report that actin immunoreactivity in neuronal cell bodies is primarily concentrated along the periphery of the neuronal perikaryon, including the perikaryal projections.[36] Thus, at the cytological level, localization of actin overlaps with protein carbonyl immunoreactivity in AD brain sections. Oxidative stress-associated carbonylation of muscle actin was reported recently.[13] Oxidative injury on actin during prolonged oxidative stress may affect actin filament architecture and lead to severe disarrangement of the cytoskeleton.[31]

Our results demonstrate that not all proteins which are recognized by anti-DNP antibodies in brain extracts exhibit increased protein carbonyl content in AD. We did not detect a statistically significant increase in the specific protein carbonyl level in β-tubulin, which was identified as one of the major anti-DNP-positive spots on AD and control 2D Oxyblots. β-Tubulin is a lysine-rich protein and is known to react with aldehydes.[49] Therefore, it was not unexpected that carbonyls may be present in this brain protein in AD and aged controls. Absence of the increase of protein carbonyl content in β-tubulin

suggests that potentially sensitive proteins can be selectively affected by oxidation in AD. It is possible that excessively oxidized tubulin can be removed by specific proteinases, while poor handling of some other oxidized proteins in the cell may lead to their accumulation in AD.

Positive staining of interneuronal NFT suggests that some protein components of fibrils within the neuronal cytoplasm may contain increased amounts of carbonyl groups. Neurofibrillary tangles in AD and normal aged brains are composed predominantly of paired helical filaments (PHF), which contain aggregated isoforms of microtubule-associated protein tau. In this study, protein carbonyl immunoreactivity was not detected in protein spots matching with tau isoforms on 2D protein maps of AD and control brain extracts. This indicates that soluble tau may not have reactive carbonyl groups. Our results do not rule out the possibility that oxidatively modified tau may present in PHF. If oxidation of tau occurs in AD, it may result in cross-linking of oxidized tau proteins and formation of insoluble aggregates, which could be detected as anti-DNP positive in brain sections, but lost during the preparation of brain extracts. In an *in vitro* study, Troncoso *et al*.[48] demonstrated that oxidation of tau induces its dimerization and polymerization into insoluble filaments. It was shown recently that antibody against *N*-ε-(carboxymethyl)lysine labels predominantly tau proteins in PHF aggregates, which suggested that tau becomes oxidatively modified in PHF and oxidative glycation of tau may stabilize PHF aggregation and lead to NFT formation in AD.[19] Ledesma *et al*.[24] demonstrated the presence of AGEs in tau in a fraction of PHF in AD, whereas soluble tau from either AD or non-demented control brains was not glycated. Oxidative modifications of tau may be an intermediate step in the progress of oxidative stress-induced neuronal degeneration. Alternatively, oxidative modification of other cytosolic proteins, which may associate with filaments in cell bodies of neurons with neurofibrillary changes, may be the source of protein carbonyl immunoreactivity of intraneuronal tangles in AD.

CONCLUSIONS

Immunohistochemical detection of oxidative damage markers demonstrates their preferential localization in neurons versus glia[39,43] in AD. In this study, we did not identify glial-specific GFAP as carbonyl-containing protein in AD or control brain extracts, which is consistent with the suggestion that glia are less sensitive to oxidative stress than neurons. Nevertheless, our results cannot imply that glial cells in the AD brain completely escape protein oxidation. Individual brain proteins, which were identified as targets of protein carbonyl formation in AD, were not neuronal specific. Our study could not demonstrate that excessively carbonylated CKBB or β-actin was present only in oxidatively stressed neurons. We suggest that oxidative stress-induced injury may involve selective modification of different intracellular proteins, including key enzymes and structural proteins, which precedes and may lead to the neurofibrillary degeneration of neurons in the AD brain.

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