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Neurobiology of Aging 27 (2006) 1564–1576

NEUROBIOLOGY OF AGING

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Redox proteomics identification of oxidized proteins in Alzheimer's disease hippocampus and cerebellum: An approach to understand pathological and biochemical alterations in AD

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Received 25 March 2005; received in revised form 19 September 2005; accepted 20 September 2005
Available online 4 November 2005

Abstract

Alzheimer's disease (AD) is characterized by the presence of neurofibrillary tangles, senile plaques and loss of synapses. There is accumulating evidence that oxidative stress plays an important role in AD pathophysiology. Previous redox proteomics studies from our laboratory on AD inferior parietal lobule led to the identification of oxidatively modified proteins that were consistent with biochemical or pathological alterations in AD. The present study was focused on the identification of specific targets of protein oxidation in AD and control hippocampus and cerebellum using a redox proteomics approach. In AD hippocampus, peptidyl prolyl *cis–trans* isomerase, phosphoglycerate mutase 1, ubiquitin carboxyl terminal hydrolase 1, dihydropyrimidinase related protein-2 (DRP-2), carbonic anhydrase II, triose phosphate isomerase, α-enolase, and γ-SNAP were identified as significantly oxidized protein with reduced enzyme activities relative to control hippocampus. In addition, no significant excessively oxidized protein spots were identified in cerebellum compared to control, consistent with the lack of pathology in this brain region in AD. The identification of oxidatively modified proteins in AD hippocampus was verified by immunochemical means. The identification of common oxidized proteins in different brain regions of AD brain suggests a potential role for these oxidized proteins and thereby oxidative stress in the pathogenesis of Alzheimer's disease.

Keywords: Alzheimer's disease; Redox proteomics; Protein oxidation; Hippocampus; Cerebellum; Enolase; Triose phosphate isomerase; Peptidyl prolyl *cis–trans* isomerase; Phosphoglycerate mutase 1; Ubiquitin c-terminal hydroxylase 1; γ-SNAP; Carbonic anhydrase II; Dihydropyrimidinase related protein-2

1. Introduction

Alzheimer's disease (AD) is characterized clinically as a progressive dementia and pathologically by the presence of neurofibrillary tangles (NFT), senile plaques (SP), and loss of synapses [30]. SP consist of a core of amyloid beta-peptide (Aβ), surrounded by dystrophic neurites [36]. Hippocampal pathology plays a major role in memory and cognitive dysfunction early in AD [3]. The neurobiological mechanisms influencing the progressive impairments in memory and intellectual performance that are the hallmarks of AD are not well understood. There is accumulating evidence that oxidative stress plays an important role in this disease pathophysiology, manifested by protein oxidation, lipid peroxidation, DNA oxidation, advanced glycation end products, and ROS formation [1,12,13,33,42,48–50,77,78]. ROS can bring about different kinds of protein oxidation [79].

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doi:10.1016/j.neurobiolaging.2005.09.021

Several sources of free radical are important in AD brain, including Aβ, redox metal ions, inflammation, microglia activation, etc. [10,26,62,71,72,90]. In AD brain protein oxidation occurs in Aβ-rich regions, such as inferior parietal lobule, cortex, and hippocampus, but not in cerebellum where Aβ levels are negligible [33]. The most widely used marker for oxidative damage to proteins is the presence of protein carbonyl groups, which can be introduced into proteins by direct oxidation of certain amino acid side chains, peptide backbone scission, or by Michael addition reactions with products of lipid peroxidation or glyco-oxidation [12,77]. Elevation in the total levels of protein carbonyls has been reported in AD [13,18,33,77,78].

Previous studies from our laboratory and others identified oxidized proteins using a redox proteomics approach in this disorder [9,10,14,15,17,19], consistent with biochemical and pathological alterations in AD. In essentially all cases examined thus far, oxidative modification of brain proteins is associated with loss of function [1,11,16,33,39], suggesting a possible link between oxidative stress of key proteins and mechanisms for neurodegeneration in AD brain. Identification of modified proteins is crucial for establishing a relationship between oxidative modification and neuronal death in AD brain, and proteomics aids in identifying potential new therapeutic targets for in this dementing disease.

In the present study, specific targets of protein oxidation and expression were studied in control and AD hippocampus and cerebellum, using a redox proteomics approach. The identified oxidized proteins play key roles in ATP synthesis, protein degradation, axonal growth, pH regulation, and vesicular transport. In contrast, no excessively oxidized proteins were revealed in the cerebellum compared to basal oxidation in control cerebellum. Our data support the notion that oxidative stress plays an important role in protein oxidation in AD brain as evinced by increased protein oxidation in hippocampus compared to cerebellum, and given the regional distribution of protein oxidation and Aβ levels [33], the results suggest an important role of Aβ in oxidative stress and pathology in AD.

2. Materials and methods

2.1. Control and AD brains

Frozen hippocampal samples were obtained from six AD patients and six age matched controls for the present study. The Rapid Autopsy Program of the University of Kentucky Alzheimer's Disease Research Center (UK ADRC) provided autopsy samples with average postmortem intervals (PMIs) of 2.1 h for AD patients and 2.9 h for control subjects (Table 1). All AD patients displayed progressive intellectual decline and met NINCDS-ADRDA Workgroup criteria for the clinical diagnosis of probable AD [54]. Hematoxylin–eosin and modified-Bielschowsky staining and 10-D-5, and α-synuclein immunohistochemistry were used on multiple neocortical, hippocampal, entorhinal, amygdala, brainstem, and cerebellum sections for diagnosis. Some patients were also diagnosed with AD plus dementia with Lewy bodies. Control subjects underwent annual mental status testing and semi-annual physical and neurological exams, as a part of the UK ADRC normal volunteer longitudinal aging study and did not have a history of dementia or other neurological disorders. All control subjects had test scores in the normal range (Table 1). Neuropathologic evaluation of control brains revealed only age-associated gross and histopathologic alterations. Other characteristics of AD and control patients that were available from medical records are provided in Table 1.

2.2. Sample preparation

Brain samples were 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, 0.1 mM EDTA, and 0.6 mM $MgSO_4$ as well as proteinase inhibitors: leupeptin (0.5 mg/mL), pepstatin (0.7 μg/mL), type II S soybean trypsin inhibitor (0.5 μg/mL), and PMSF (40 μg/mL). Homogenates were centrifuged at 14,000 × *g* for 10 min to remove debris. Protein concentration in the supernatant was determined by the BCA method (Pierce, Rockford, IL, USA).

Table 1
Characteristics of AD and control subjects (means ± S.D.)

Parameters	Groups	
	Normal	AD
Demographic variables		
Number of subjects	6	6
Gender (male/female)	4/2	4/2
Age at death (years)	85.8 ± 4.1	84.5 ± 5.2
Postmortem interval (h)	2.9 ± 0.23	2.1 ± 0.47
MMSE; number of months prior to death test taken	28 ± 0.8; 6.6 ± 1.4	15.7 ± 2.6; 19.7 ± 1.0
APOE genotype if known (*N*)	3/3 (3) 3/4 (2)	ND
Cause of death	Complications of surgery, cardiac failure; COPD	Complications of AD
Location at death if known	Home (3); hospital (2)	Home (1); hospital (2)

Abbreviations: AD, Alzheimer's disease; MMSE, Mini-Mental State Examination; APOE, apolipoprotein E; ND, not determined; *N*, number of individuals; S.D., standard deviation; COPD, chronic obstructive pulmonary disease.

2.3. Two-dimensional electrophoresis

Samples (150 μg) were incubated at room temperature for 30 min in four volumes of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in either 2 M HCl for protein carbonyl derivatization/oxyblots or 2 M HCl for gel maps and mass spectrometry analysis, according to the method of Levine et al. [41]. This was followed by precipitation of proteins by addition of ice-cold 100% trichloroacetic acid (TCA) to a final concentration of 15% and samples were placed on ice for 10 min. Precipitates were centrifuged for 2 min at 14000 × *g* at 4 °C. The pellet was washed with 500 μL of 1:1 (v/v) ethyl acetate/ethanol three times. The final pellet was dissolved in rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 0.2% (v/v) biolytes, 50 mM dithiothreitol (DTT), and bromophenol blue). Samples were sonicated in rehydration buffer on ice three times for 20 s intervals and were applied to a Ready Strip IPG (pH 3–10) (Bio-Rad, Hercules, CA, USA). The strip was then actively rehydrated at 50 V for 16 h in a protean IEF cell (Bio-Rad). Isoelectric focusing was performed at 20 °C as follows: 800 V for 2 h linear gradient, 1200 V for 4 h slow gradient, 8000 V for 8 h linear gradient, and 8000 V for 10 h rapid gradient. The strips were stored at −80 °C until second dimension electrophoresis was performed. Gel strips were equilibrated for 10 min prior to second dimension separation in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate, 30% (v/v) glycerol, and 0.5% dithiothreitol, and followed by re-equilibration for 10 min in the same buffer containing 4.5% iodoacetamide in place of dithiothreitol. Linear gradient precast criterion Tris–HCl gels (8–16%; Bio-Rad) were used to perform second dimension electrophoresis. Precision protein standards (Bio-Rad) were run along with the sample at 200 V for 65 min.

2.4. SYPRO ruby staining

The gels from control and AD hippocampus and cerebellum were fixed in a solution containing 10% (v/v) methanol, 7% (v/v) acetic acid for 20 min, and stained overnight at room temperature with agitation in 50 mL of SYPRO Ruby gel stain (Bio-Rad). The gels were placed in deionized water overnight and scanned.

2.5. Immunoprecipitation

To confirm the correct identification of the proteins identified by mass spectrometry control or AD samples (250 μg) were first precleared by incubation with protein A-agarose (Pharmacia) for 1 h at 4 °C. Samples were then incubated overnight with the relevant antibody followed by 1 h of incubation with protein A-agarose, then washed three times with buffer B (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 1% NP40). Proteins were resolved by SDS–PAGE followed by immunoblotting on a nitrocellulose membrane (Bio-Rad). Proteins were detected by the alkaline phosphate (Sigma) [83].

2.6. Post-derivatization of proteins

Samples were post-derivatized with DNPH on membrane and probed with anti-DNPH antibody to identify the oxidized proteins. The nitrocellulose membranes were equilibrated in solution A (20% (v/v) methanol:80% (v/v) wash blot buffer) for 5 min, followed by incubation of membranes in 2N HCl for 5 min. The proteins on blots were then derivatized in solution B (0.5 mM DNPH in 2 N HCl) for exactly 5 min as described by Conrad et al. [22]. The membranes were washed three times in 2N HCl for 5 min each and then five times with 50% methanol and two times with wash blot each for 5 min. We also treated a set of control and AD hippocampal samples with $NaBH_4$, a reducing agent that coverts carbonyls to alcohols, followed by DNPH and antibody treatment, to check the specificity of protein-DNP hydrazone antibody [1].

2.7. Western blotting (oxyblot)

Protein oxidation was indexed by elevated protein carbonyls [4,8,10]. For immunoblotting analysis, 2,4-dinitrophenyl hydrazine derivatized or non-derivatized samples were separated by electrophoresis as described in sample preparation followed by transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) using the Transblot-Blot SD semi-dry transfer cell at 45 mA per gel for 2 h. The membranes were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween-20 (PBST) at 4 °C for 1 h. The membranes were incubated with anti-2,4-dinitrophenylhydrazone (DNP) polyclonal antibody (1:100) or anti-Pin 1 (1:1000) (Stressgen Biotech, USA) or anti-UCH-L1 antibody (1:1000) (Stressgen Biotech, USA) in PBST for 2 h at room temperature with gentle rocking. After washing the blots three times in PBST for 5 min each, the anti-rabbit or anti-goat IgG alkaline phosphatase secondary antibody (1:3000) in PBST was incubated 1 h at room temperature. The membranes were washed in PBST three times for 5 min and developed using Sigma-Fast 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) tablets.

2.8. Image analysis

The gels and nitrocellulose membranes were scanned and saved in TIFF format using a Scan jet 3300C scanner (Hewlett Packard, Palo Alto, CA, USA). PD Quest software (Bio-Rad) was used to compare protein expression and protein oxidation between control and AD samples. Protein expression was measured using SYPRO ruby-stained gels that were scanned using a UV transilluminator ($\lambda_{ex} = 470$ nm, $\lambda_{em} = 618$ nm, Molecular Dynamics, Sunnyvale, CA, USA). Oxyblots, used to measure carbonyl immunoreactivity, were scanned with a Microtek Scanmaker 4900. Average mode of background subtraction was used to normalize intensity value, which represents the amount of protein (total protein on gel and

oxidized protein on oxyblot) per spot. After completion of spot matching, the normalized intensity of each protein spot from individual gels (or oxyblots) was compared between groups using statistical analysis.

2.9. Trypsin digestion

Samples were prepared according to the method described by Thongboonkerd et al. [85]. Based on the data obtained from image analysis, the protein spots that showed a significant increase in oxidation in AD compared to control brain samples were excised from the gel with a clean razor blade and transferred to clean 1.5 mL microcentrifuge tubes. The gel pieces were washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) for 15 min at room temperature under a flow hood, followed by addition of acetonitrile and incubation at room temperature for 15 min. The solvents were removed and the gel pieces were allowed to dry. The gel pieces were incubated with 20 μL of 20 mM DTT in 0.1 M NH_4HCO_3 and incubated for 45 min at 56 °C. The DTT solution was removed and 20 μL of 55 mM iodoacetamide (IA) in 0.1 M NH_4HCO_3 was added and incubated for 30 min in the dark at room temperature. The liquid was drawn off and the gel pieces were incubated with 200 μL of 50 mM NH_4HCO_3 at room temperature for 15 min. Acetonitrile was added to the gel pieces for 15 min at room temperature. The solvents were removed and the gel pieces were allowed to dry for 30 min. The gel pieces were rehydrated with 20 ng/μL modified trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3. The gel pieces were chopped into small pieces and placed in shaking incubator overnight (∼18 h) at 37 °C.

2.10. Mass spectrometry

Mass spectra of the sample were determined by a Tof-Spec 2E (Micromass, UK) MALDI-TOF mass spectrometer in reflectron mode. Tryptic digest (1 μL) was mixed with 1 μL α-cyano-4-hydroxy-*trans*-cinnamic acid (10 mg/mL in 0.1% TFA:ACN, 1:1, v/v) directly on the target and dried at room temperature. The sample spot was then washed with 1 μL of 1% TFA solution for approximately 60 s. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) using 1 μL of a solution of ethanol:acetone:0.1% TFA (6:3:1 ratio). Reported spectra are a summation of 100 laser shots. External calibration of the mass axis, used for acquisition and internal calibration using either trypsin autolysis ions or matrix clusters, was applied post-acquisition for accurate mass determination.

The MALDI spectra used for protein identification from tryptic fragments were searched against the NCBI protein databases using the MASCOT search engine (http://www.matrixscience.com). Data base searches were based on the assumption that peptides are monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed. Mass tolerance of 150 ppm was the window of error allowed for matching the peptide mass values. Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as $-10\log_{10}(p)$, where p is the probability that the identification of the protein is not correct. MOWSE scores greater than 59 were considered to be significant ($p < 0.05$). Protein identification was consistent with the expected size and pI range based on position in the gel.

2.11. Assay for enzymes

Brain homogenates (10%) from control ($n = 6$) and AD ($n = 6$) hippocampus and cerebellum were prepared in media-I, and used freshly for all the enzyme assays. The enzyme activity for enolase was determined by slight modification of the method described by Wager et al. [86]. Briefly, brain homogenate was added to 100 μL of assay mixture (20 mM Na_2HPO_4, pH 7.4, 400 mM KCl, 0.01 mM EDTA, 2 mM 2-phospho-D-glycerate) in a UV-transparent microtiter plate (Corning, MA, USA) and the change of absorption at A_{240} was monitored in powerwave X plate reader (Bio-Tek Instrument Inc., winooshi, Vermont) for 5 min. Carbonic anhydrase activity was measured as described in [2] with modification. For assay of carbonic anhydrase activity, a decrease in absorbance at 560 nm was recorded after the addition of 5 μL of the samples to CO_2 saturated Tris buffer (pH 8.3, 0.2 M Tris–HCl, phenol red). UCHL assay was performed according to Dang et al. [24] with slight modifications. The assay was carried out in 96-well black assay plate at room temperature. Briefly, samples were incubated in assay buffer (20 mM HEPES, 0.5 mM EDTA, pH 7.8, containing 0.1 mg/ml ovalbumin, and 5 mM dithiothreitol) for 2 h followed by the addition of the fluorogenic substrate ubiquitin-7-amino-4-methylcoumarin (Ub-AMC) (Boston Biochem, Cambridge, MA, USA). The AMC fluorophore was excited at 380 nm and the rates of release of free AMC were measured at 25 °C by determining the increase in fluorescence emission at 460 nm using a fluorescence plate reader.

2.12. Statistics

The data of protein level and protein specific carbonyl level were analyzed by Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

3. Results

The demographic data (Table 1) showed that some patients had Lewy bodies and the results of this study showed no difference between AD patients with or without the presence of Lewy bodies. Oxidized proteins in the AD and control hippocampus and cerebellum were identified immunochemically using 2D-oxyblot.

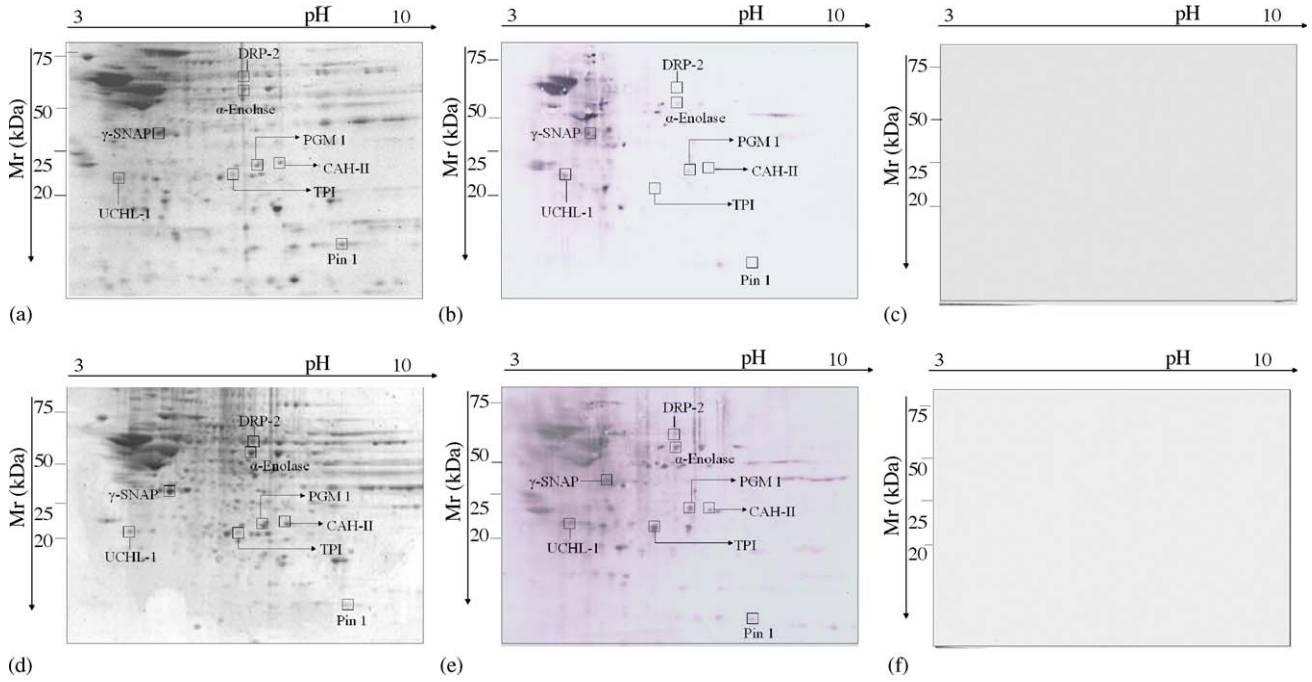

Fig. 1. SYPRO ruby-stained gels from control (a) and AD hippocampus (d). (b and e) Western blots for detection of the level of protein carbonyls from control and AD hippocampus. (c and f) Control and AD hippocampus blots treated with $NaHB_4$. In hippocampus, total protein oxidation was significantly increased in AD brain compared to that of control. One hundred micrograms of protein were loaded per gel for detection of protein expression and oxidation.

To identify oxidized proteins, images of the blots and gels of the samples were compared by the PD Quest software, and individual protein spots were normalized to the protein content in the 2D-PAGE (Fig. 1). Using this approach, we confirmed that not all of the protein spots with increased immunoreactivity are excessively modified proteins in AD brain [14–16,64]. The oxyblot of AD hippocampus revealed a number of oxidized protein spots compared to that of age-matched control hippocampus (Fig. 1d). However, we identified seven significantly excessively oxidized proteins in AD hippocampus (Fig. 1e). In contrast, AD cerebellum did not reveal any increase in protein oxidation over basal level in controls, and no protein spots were found to be significantly oxidized compared to control cerebellum (Fig. 2d). Further, the hippocampal samples from control and AD that were treated with $NaBH_4$ did not show any positive immunoreactivity on the blot confirming the specificity of the antibody for protein–DNP adducts (Fig. 1c and f). The identified oxidized proteins spots were subjected to mass analysis using MALDI mass spectrometry for protein identification after in-gel trypsin digestion. Table 2 shows the proteins that were successfully identified by mass spectrometry along with the peptides matched, percentage coverage, and pI and Mr values. Peptidyl prolyl *cis–trans* isomerase (Pin 1), dihydropyrimidinase-related protein-2 (DRP2), carbonic anhydrase II (CA II), phosphoglycerate mutase 1 (PGM 1), α-enolase, triose phosphate isomerase (TPI), gamma soluble NSF attachment protein (γ-SNAP), and ubiquitin carboxy terminal hydrolase L-1 (UCHL-1) were identified by quantitative redox proteomics to be oxidatively modified proteins in AD hippocampus compared to control brain. The increase in protein carbonylation and protein expression compared to control for the identified protein spots are shown in Table 3. Further, validation of the correct identification of these proteins was performed by immunoprecipitation of two of the oxidized proteins, i.e., Pin 1 and UCHL-1. The position of these protein spots on blots probed with anti-Pin 1 and anti-UCHL-1 antibodies were found to be same as observed on derivatized blots (Fig. 3). In addition, Pin 1 and UCHL-1 proteins were immunoprecipitated from control and AD brain samples and the oxidation status of these proteins were determined by using post-DNPH derivatization of proteins. As reported in Fig. 4, Pin 1 protein showed a significant increase ($p < 0.05$) in protein oxidation, and significant ($p < 0.05$) decrease in protein expression. UCHL-1 protein showed a significant increase ($p < 0.05$) in protein oxidation as well as in protein expression, confirming the redox proteomics results.

The measurement of enzymatic activity of CA II, UCHL-1, and enolase from AD hippocampus revealed decreased activity compared to control (Fig. 5), while no difference was observed in the activities of these enzymes in cerebellum (data not shown).

A comparison between the previously reported oxidized proteins in AD inferior parietal lobule [14,15] and the currently identified oxidized proteins in AD hippocampus revealed α-enolase, UCHL-1, TPI and DRP-2 as the common targets of oxidation in both regions of the brain in this disorder (Fig. 6).

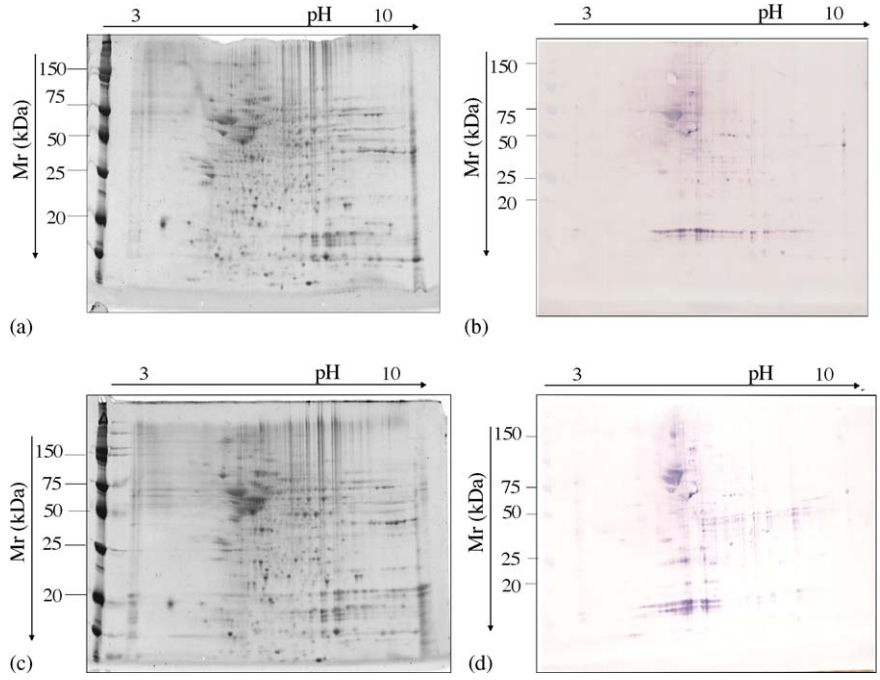

Fig. 2. SYPRO ruby-stained gels from control (a) and AD cerebellum (c). (b and d) Western blots for detection of the level of protein carbonyls from control and AD cerebellum. In cerebellum, total protein oxidation was not significantly increased in AD brain compared to that of control.

Table 2
Summary of the identified oxidatively modified proteins in AD hippocampus

gI Accession number; identity of oxidatively modified proteins in AD hippocampus	# Peptides matched of the identified protein	Percent coverage of the matched peptides	pI, Mr (kDa)	Mowse score
Q13526; Pin 1	5/22	32	7.82, 18	60
Q16555; DRP2	11/32	42	6.12, 62	75
P00918-00-01-00; CA II	9/19	44	6.89, 29	75
P18669; PGM1	8/29	39	6.75, 28	81
P06733; alpha-enolase	18/36	47	6.99, 47	194
P60174-00-00-00; TPI	10/33	28	6.5, 26	65
P09936-00-01-00; UCHL-1	14/44	72	5.33, 25	165
Q99747; gamma-SNAP	9/28	32	5.33, 35	85

Abbreviations: Pin 1, peptidyl prolyl *cis–trans* isomerase 1; DRP2, dihydropyrimidinase-like protein 2; CA II, carbonic anhydrase II; PGM1, phosphoglycerate mutase 1; TPI, triose phosphate isomerase; UCHL-1, ubiquitin carboxyl terminal hydrolase L-1; Gamma-SNAP, gamma synaptosomal protein like soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment proteins; pI, isoelectric point; Mr, relative mobility; kDa, kilo dalton.

Table 3
Oxidization and expression of identified proteins in the AD hippocampus

Protein	Protein oxidation (percent control ± S.E.M.)	*p*-value	Protein expression (percent control ± S.E.M)	*p*-value
Pin 1	136 ± 55	<0.05	40.2 ± 8.2[a]	<0.03
DRP-2	126 ± 45	<0.01	26 ± 4.8[a]	<0.02
PGM1	21230 ± 2668	<0.05	30 ± 5.3[a]	<0.01
CA II	327 ± 85	=0.05	124 ± 5.7[b]	=0.05
ENO1	255 ± 62	<0.05	135 ± 5.4[b]	<0.05
TPI	644 ± 228	<0.05	138 ± 10[b]	<0.05
γ-SNAP	315 ± 132	<0.007	255 ± 62[c]	NS
UCHL-1	210 ± 45	<0.05	131 ± 3.8[b]	<0.02

NS, non-significant.
[a] Decreased protein expression.
[b] Increased protein expression.
[c] No change in protein expression.

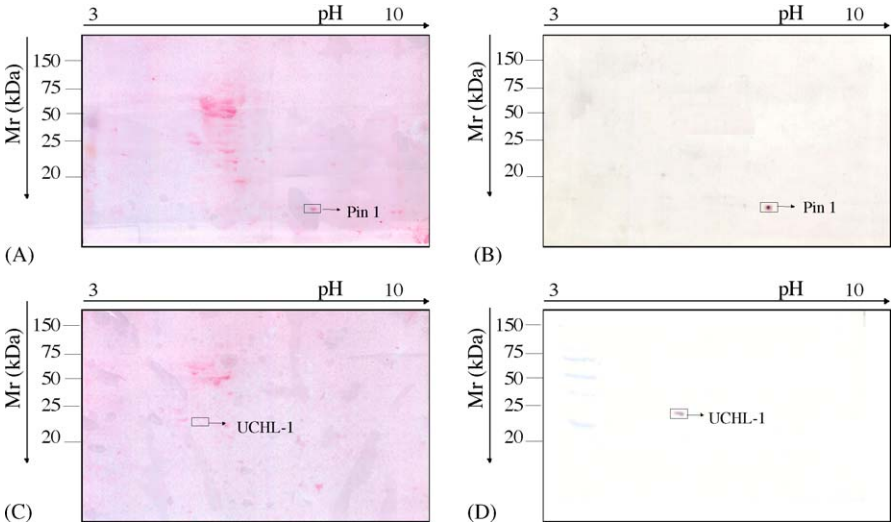

Fig. 3. Confirmation of correct identification of Pin 1 and UCHL-1 proteins in hippocampus by Western blot analysis: (A and C) blots stained with ponceau S stain. (B and D) Blots probed with anti-Pin 1 and anti-UCHL-1 antibodies, respectively. A box is drawn around the protein spots of interest.

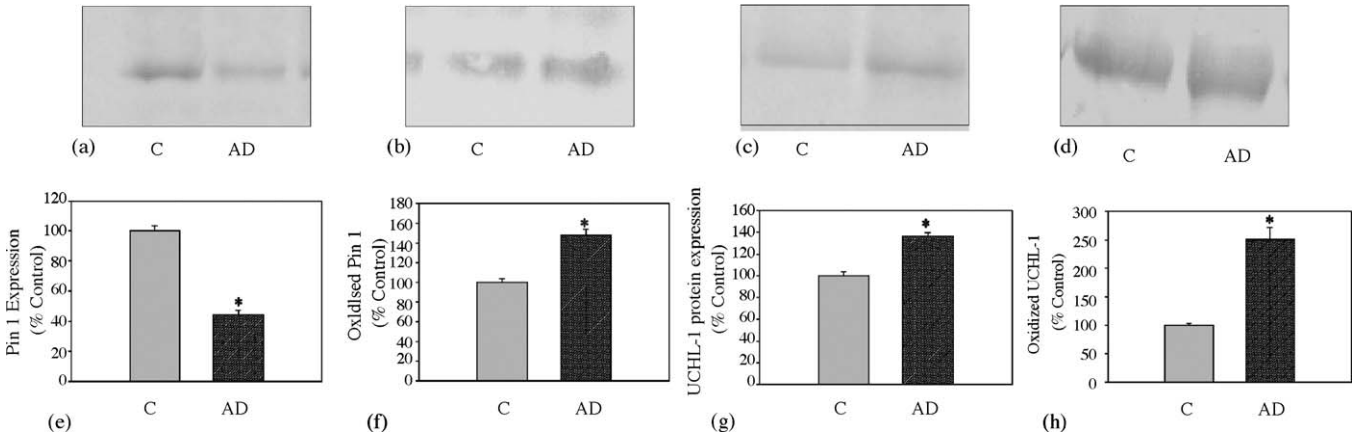

Fig. 4. Immunoprecipitation followed by Western blot analysis was performed to confirm the carbonylation of Pin 1 and UCHL-1 proteins in hippocampus. Pin 1 or UCHL-1 proteins were immunoprecipitated using anti-Pin 1 and anti-UCHL-1 antibodies and probed for protein carbonyl levels. (a) and (c) represent gels showing the immunoprecipitated Pin 1 and UCHL-1 proteins, respectively, whereas (b) or (d) represent blots probed with anti-Pin 1 or anti-UCHL-1 antibody. Histograms for individual blots or gels are shown below them. (e) Pin 1 protein expression, (f) Pin 1 protein oxidation, (g) UCHL-1 protein expression, and (h) UCHL-1 protein oxidation. $^{*}p < 0.05$; $N = 6$ for both control and AD brain.

4. Discussion

In Alzheimer disease, neuronal and synaptic loss occur in a region-specific manner. An understanding of why some regions are more sensitive in AD and the identification of common targets of oxidative damage would enhance our understanding of disease pathogenesis and thereby enable clinicians to develop more specific therapeutic strategies. In the present study, we analyzed the AD hippocampus and cerebellum to identify the specific targets of oxidation. AD cerebellum did not show any significantly oxidized protein spots compared to the basal level of protein oxidation in normal cerebellum. However, TPI, α-enolase, PGM1, γ-SNAP, DRP-2, CA II, Pin 1, and UCHL-1 were found as the specific targets of protein oxidation in AD hippocampus. UCHL-1, α-enolase, TPI are the common proteins of oxidation in both AD hippocampus and inferior parietal regions of the brain, the latter a brain region that was previously studied by our group (Fig. 6). Oxidative modification of proteins impairs protein function, as observed in the present study and reported previously, thereby affecting neuronal functions and survival [33,39]. Such functional decline conceivably may be critically involved in the etiology of AD [9,14,15,17].

4.1. Pin 1, UCHL-1

Peptidyl prolyl *cis–trans* isomerases (PPIases) are highly conserved proteins from yeast to human [28,43,71,72]. Pin 1 plays an important role as a chaperone protein and also in cell cycle regulation [71]. Pin 1 also catalyzes the isomerization

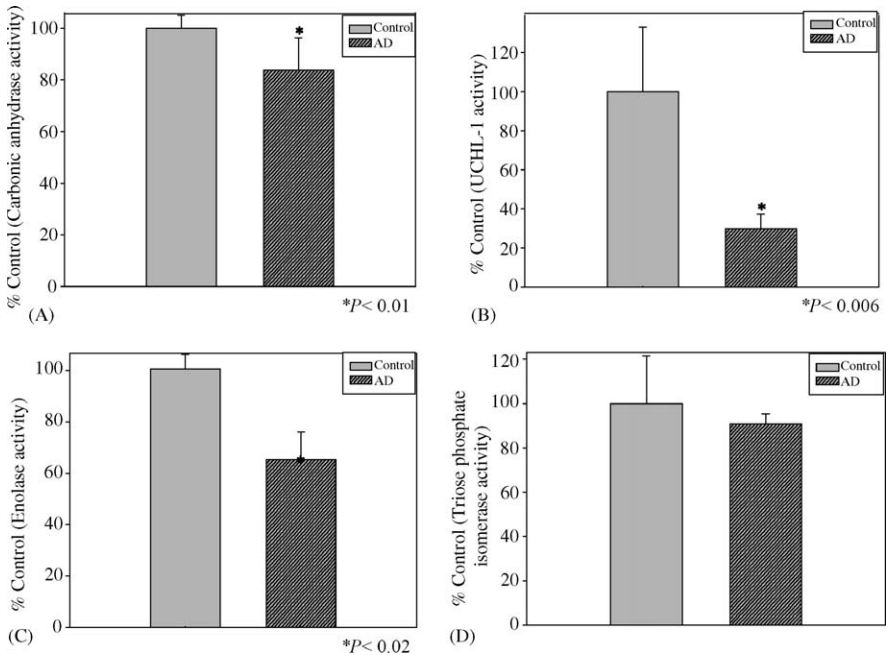

Fig. 5. Enzyme activities were measured in the hippocampus of control and AD samples using the protocols as described in methodology. (A) Carbonic anhydrase, (B) UCHL, (C) enolase, and (D) triose phosphate isomerase. Enzyme activities are represented as percent of control. $N = 6$ for both control and AD brain.

of tau, a neuronal cytoskeleton protein, which is hyperphosphorylated in AD brain [38]. Recently, it has been reported that Pin 1 restores the function of tau protein, and Pin 1 also shows an inverse relationship to expression of tau protein in AD. Pin 1 is co-localized with phosphorylated tau [34,44,68]. Taken together, the result reported in the present and previous studies [33,39] suggest that the oxidation of Pin 1 might lead to the decreased Pin 1 activity. Therefore, the oxidation of Pin 1 could be one of the initial events that trigger tangle formation. Redox proteomics analysis of Pin 1 and reduction in Pin 1 activity in AD hippocampus are thoroughly discussed elsewhere [82]. UCHL-1 plays a crucial role for proteolytic degradation of misfolded or damaged proteins by the proteasome. UCHL-1 was observed to be oxidatively modified in the present study and previously in AD inferior parietal lobule [14]. Oxidative modification of UCHL-1 in AD, which was confirmed by others [19], may lead to dysfunction of the ubiquitination/de-ubiquitination machinery, causing accumulation of damaged proteins and formation of protein aggregates that could lead to synaptic deterioration and degeneration in AD hippocampus. Consistent with this idea, examination of Fig. 5 shows that the activity of UCHL-1 is markedly depressed in AD hippocampus. Similarly, the activities of the 26S proteasome, ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme are reversibly depressed under conditions of oxidative stress [37,73]. Taken together, these different lines of evidence support a role for dysfunction of the ubiquitin-proteasome pathway in the pathogenesis of AD. Consistent with this notion, a recent in vitro study showed that the hydrolase activity of recombinant UCHL-1 was decreased by treatment with 4-hydroxynonenal, a lipid peroxidation product that

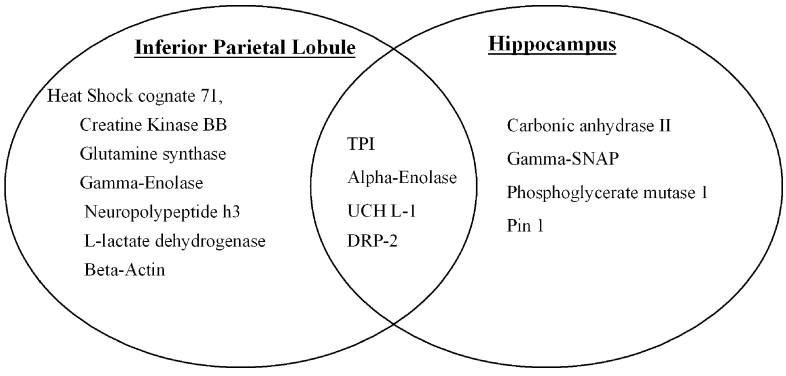

Fig. 6. Schematic representation of the oxidized proteins in AD hippocampus and inferior parietal lobule.

is elevated in AD brain [39,50] and formed by Aβ-induced lipid peroxidation [39,47]. Further, dysfunctional UCHL-1 contributes to the oxidative environment in brain [16].

NFTs are filamentous deposits consisting of ubiquitinated and hyper-phosphorylated tau protein [81]. Recently, it was reported that CHIP Hsc70 complex ubiquitinates phosphorylated tau and promotes the aggregation of tau protein [29]. The association of UCHL-1 with NFT and the inverse correlation between UCHL-1 level and number of NFT reported recently [17], suggest a possible role of UCHL-1 in preventing NFT formation in control brain by de-ubiquitination of phosphorylated tau. Both Pin 1, which normally catalyzes dephosphorylation of tau protein, and UCHL-1, which conceivably could de-ubiquitinate phosphorylated tau, are oxidized and dysfunctional in AD hippocampus [82]. Such oxidation-induced enzymatic dysfunction in Pin 1 and UCHL-1 is consistent with the observed formation and accumulation of tangles in the AD brain.

4.2. TPI, PGM1, and enolase

Glucose metabolism is the basis of cerebral energy under normal conditions. Hence, the necessity for glucose in brain function had been considered solely due to ATP production. These lines of evidence suggest that glycolysis plays an important role in maintaining normal synaptic function. In the present study, we found TPI, PGM1, and enolase, each of which participates in the glycolytic pathway, to be significantly oxidized in AD hippocampus.

Phosphoglycerate mutase (D-phosphoglycerate 2,3-phosphomutase; EC 5.4.2.1; PGM1) is a glycolytic enzyme that catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate. In the present study, we observed a significant increase in oxidation of PGM1 and a decrease in protein expression that is consistent with the reported decreased expression and activity of PGM1 in AD brain compared to the age-matched controls [35,55].

Enolases have been characterized as highly conserved cytoplasmic glycolytic enzymes that catalyze the formation of phosphoenolpyruvate from 2-phosphoglycerate, the second of the two high-energy intermediates that generate ATP in glycolysis [31]. Three isoforms of enolase have been identified and named as α-, β-, and γ-enolase that exist as homodimer or heterodimers. In the current study, α-enolase is identified as an oxidatively modified protein with reduced activity in AD hippocampus with no change in cerebellum (Fig. 5). Meier-Ruge et al., reported a similar significant decrease in enolase activity in AD brain compared to age-matched control [55]. A proteomics method applied to AD brain showed that the protein level of the α-subunit is increased compared to control brain [70] and is specifically oxidized protein in inferior parietal lobule of AD brain [15,17]. In addition, we found TPI, another glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate in glycolysis, was also oxidatively modified [17]. However, no change is TPI activity was observed in the present or a previous study [35]. A likely explanation for this observation could be the addition of carbonyl groups localized away from the catalytic site of this enzyme. Further studies are required to clarify this point.

The present finding that α-enolase, TPI, and PGM1 are significantly more oxidized in AD hippocampus compared with control hippocampus suggests a possible relationship between glycolytic enzymatic impairment and reduced glucose metabolism in AD [56,59]. Because glucose is the main source for ATP production in brain, the alteration in these key glycolytic enzymes may lead to cellular dysfunction such as impaired ion-motive ATPase to maintain potential gradients, operate pumps, and maintain membrane lipid asymmetry, etc. Such changes could lead to exposure of phosphatidylserine to the outer membrane leaflet, a signal for apoptosis [57]. Recently, we showed that Aβ [57] and HNE, which is produced by Aβ-mediated lipid peroxidation [39,40,47], lead to loss of synaptosomal membrane bilayer asymmetry. Alterations in glucose metabolism also can induce loss of membrane potential leading to the opening of voltage-gated Ca^{2+} channels, and metabolic reduction can also induce hypothermia leading to abnormal tau hyper-phosphorylation through differential inhibition of kinase and phosphatase activities [63]. Previous studies from our laboratory showed the oxidization of glycolytic enzymes and creatine kinase BB in inferior parietal of AD subjects [9,11,14,15,17].

4.3. Dihydropyrimidinase-related protein-2

Dihydropyrimidinase-related protein-2 (DRP2) is a member of the dihydropyrimidinase-related protein family. These proteins are involved in axonal outgrowth and path-finding through the transmission and modulation of extracellular signals. These proteins are found abundantly in the nervous system, especially during development, and have also been found in adult brain, suggesting their role in repair and regeneration of adult neurons. Previously it has been shown that mutation in the *unc*-33 gene of *Caenorhabditis elegans* (*C. elegans*) leads to severely uncoordinated movements, with swelling and premature termination of axonal endings [27,32]. In addition, DRP-2 is oxidatively modified in AD and has been shown to have decreased expression in AD and fetal Down's syndrome (DS) brain, and Aβ(1–42) treated culture [5,15,45]. Such changes may interfere with synaptogenesis and neuronal differentiation and migration. It is thus conceivable that oxidation of DRP-2 could be related to the neuronal inability to regenerate the neurons that were damaged and could also interfere with synaptic connections leading to loss of synaptic plasticity as observed in AD brain. Consistent with this notion, dendritic length is shortened in AD brain compared to control [21]. In AD brain, DRP-2 is associated with neurofibrillary tangles. Taken together with the current study, the cytosolic DRP-2 findings are consistent with the shortened neuritic and axonal outgrowth of tangle-bearing neurons in AD.

4.4. Carbonic anhydrase II

Carbonic anhydrase II is one of the most widespread of the CA isozymes, which catalyze the reversible hydration of CO_2, a reaction fundamental to many cellular and systemic processes including glycolysis and acid and fluid secretion. The physiological functions of CA II are involved in cellular pH regulation, CO_2 and HCO_3^- transport, and maintaining H_2O and electrolyte balance [75]. Production of CSF and the synthesis of glucose and lipids [29,46] also involve CA II. CA II deficiency results in osteoporosis, renal tubular acidosis, and cerebral calcification. Patients with CA II deficiency also demonstrate cognitive defects varying from disabilities to severe mental retardation [74,76]. Consistent with previous studies of other enzymes and transporters [1,39,83], oxidative modification of CA II likely explains its diminished activity that has been reported in AD brain compared to age-matched control brain [55] and confirmed in the present study (Fig. 5). Consequently, oxidized CA II may not be able to balance both the extracellular and intracellular pH and may lead to pH imbalance in the cell. Because pH plays such a crucial role for enzymes and mitochondria to function, oxidative modification of CA II may be involved in the progression of AD. Moreover, altered pH would promote a tendency of proteins to aggregate, a phenomenon found readily in AD brain.

4.5. γ-SNAP

Studies have shown that synaptic pathology is central to the pathogenesis of AD [69], and relationships among synaptic alterations, amyloid deposits, cytoskeletal abnormalities, and cognitive deficits in individuals with AD reportedly exist [51]. Synaptic loss in the hippocampus occurs early in the development of AD [53] and Aβ oligomers causes synaptic dysfunction [87]. In the present study, one of the oxidized proteins in hippocampus is γ-SNAP. This protein is a member of synaptosomal protein like soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment proteins (SNAPs). These proteins are highly conserved and play an important role in vesicular transport in the constitutive secretory pathway as well as in neurotransmitter release and hormone secretion [4,80]. In the mammalian system, there are three individual isoforms of SNAPs: α-, β-, and γ-SNAP [88]. Gamma-SNAP was shown to play a role in vesicular transport and in control of mitochondrial organization [20]. Gamma-SNAP can activate the ATPase activity of NSF when it is initially bound to a hydrophobic surface [58,89]. The oxidation of this protein may lead to loss of synaptic integrity in AD [25,52,84]. Based on these results, we propose that oxidation of γ-SNAPs may be involved in the known altered neurotransmitter systems in AD brain and may be related to the observed synaptic pathology in this disorder. Consistent with previous reports, we did not observe any change in the expression of this protein in hippocampus [83].

In the current study using redox proteomics, we demonstrated markedly elevated levels of protein carbonyls of specific proteins in AD hippocampus with no change in the AD cerebellum compared to age-matched controls. Redox proteomics has numerous advantages, the chief one being the ease of detecting post-translationally modified proteins [6–8,23]. Indeed, this technique has been used successfully in our laboratory to identify oxidatively modified proteins in models of AD [5–8], Parkinson's disease [66], amyotrophic lateral sclerosis [60,67], Huntington's disease [61], and accelerated aging [64,65]. However, there are limitations to this method as well, including the inability to detect low-abundance proteins, the difficulty of detecting membrane-bound proteins, and the unlikelihood of detecting proteins with high isoelectric points [9–11].

These current findings are consistent with our previous reports on oxidative stress in AD hippocampus and the lack of oxidative stress in AD cerebellum [33], which correlated with amyloid β-peptide levels, NFT and reduced glucose metabolism in AD brain [33,56]. In addition, we reported oxidation of UCHL-1, α-enolase, and TPI in inferior parietal lobule (IPL) [14,15,17], and the current study demonstrates these proteins to be oxidized in AD hippocampus as well. The appearance of oxidation of common proteins in two different brain regions (Fig. 6), suggests a potentially important link between oxidative stress-related protein modification, amyloid β-peptide, NFT, and neurodegeneration in AD brain. As sequelae of these results, hippocampus-specific oxidized proteins may be related to memory deficits in AD. Thus, the presence of oxidatively modified proteins follows the brain regional distribution of Aβ in those areas thus far examined.

The present study implies that oxidation of key proteins in AD brain may account, in part, for AD pathology and may be a potential mechanism of neurodegeneration in AD. Studies are in progress using animal models of AD to delineate further potential mechanisms of neurodegeneration relevant to this devastating dementing disorder.

Acknowledgements

The authors thank the University of Kentucky ADRC Clinical and Neuropathology Cores for providing the brain specimens used for this study. This work was supported in part by grants from NIH to D.A.B. [AG-05119; AG-10836], to W.R.M. [5P01-AG0-5119], and to J.B.K. [HL-66358].

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