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Identification of nitrated proteins in Alzheimer's disease brain using a redox proteomics approach

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Nitric oxide (NO) has been implicated in the pathophysiology of a number of neurodegenerative diseases including Alzheimer's disease (AD). In the present study, using a proteomics approach, we identified enolase, glyceraldehyde-3-phosphate dehydrogenase, ATP synthase alpha chain, carbonic anhydrase-II, and voltage-dependent anion channel—protein as the targets of nitration in AD hippocampus, a region that shows a extensive deposition of amyloid β-peptide, compared with the age-matched control brains. Immunoprecipitation and Western blotting techniques were used to validate the correct identification of these proteins. Our results are discussed in context of the role of oxidative stress as one of the important mechanisms of neurodegeneration in AD.

Keywords: Alzheimer's disease; Hippocampus; ATP synthase alpha chain; Voltage-dependent anion-selective channel protein 1; Carbonic anhydrase II; Alpha enolase; Glyceraldehyde-3-phosphate dehydrogenase; Redox proteomics

Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder associated with progressive impairment of memory and cognition. AD is characterized by three pathological lesions, senile plaques, neurofibrillary tangles, and loss of synapses. Plaques are extracellular and consist of deposits of a fibrillous protein referred to as amyloid beta peptide surrounded by dying neurites. Tangles are intracellular and are abnormally altered deposits of tau protein, whose normal function involves intracellular axonal transport.

Several mechanisms have been proposed to underlie AD pathogenesis; however, there is accumulating evidence that oxidative stress plays an important role in this disease pathophysiology. Either the oxidants or the products of oxidative stress could modify the proteins or activate other pathways that may lead to additional impairment of cellular functions and neuronal loss (Butterfield et al., 2001, 2002a; Keil et al., 2004; Koppal et al., 1999; Lovell et al., 2001; Mark et al., 1997; Markesbery, 1997; Smith et al., 1994). Oxidative stress is manifested by protein oxidation, lipid peroxidation, DNA oxidation, advanced glycation end products, ROS (reactive oxygen species), and RNS (reactive nitrogen species) formation. Protein nitration has been reported in AD (Smith et al., 1997, Tohgi et al., 1999; Castegna et al., 2003; Hensley et al., 1998), Parkinson's disease (PD) (Good et al., 1998), amyotrophic lateral sclerosis (ALS) (Cookson and Shaw, 1999), and ischemia–reperfusion (Hall et al., 1995a,b, 2004; Walker et al., 2001; Zou and Bachschmid, 1999).

Oxidative stress could also stimulate the additional damage via the overexpression of inducible and neuronal specific nitric oxide synthase (NOS: iNOS and nNOS, respectively). Previous studies suggested that an increase production of peroxynitrite, a product of reaction of nitric oxide (NO) with superoxide, could cause nitration of proteins that may lead to irreversible damage to the proteins (Koppal et al., 1999; Yamakura et al., 1998). Peroxynitrite is an extremely strong oxidant with a half-life of <1 s, and the homolytic cleavage of peroxynitrite results in the production of hydroxyl radicals which have much more deleterious effect than peroxynitrate itself (Beckman, 1996; Pryor and Squadrito, 1995). Peroxynitrite can nitrate tyrosine (Halliwell, 1997) at the ortho position that, by steric effects, could prevent the phosphorylation of tyrosine

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residues, thereby rendering that protein dysfunctional and could lead to cell death (Lafon-Cazal et al., 1993; Yamakura et al., 1998). Peroxynitrite can oxidatively modify both membrane and cytosolic proteins, affecting both their physical and chemical nature (Koppal et al., 1999).

Peroxynitrite can also avidly react with thiols to form nitrosothiols, affecting the function of proteins (Halliwell, 1997; Ottesen et al., 2001). In addition, peroxynitrite can affect the energy status of a cell by inactivating key mitochondrial enzymes (Radi et al., 1994) and triggering calcium release from the mitochondria. However, the RNS generated within a physiological-relevant concentration by Ca^{+2}-activated constitutive NOS are not toxic; rather, RNS so generated are relatively specific in their cellular targets (Lafon-Cazal et al., 1993). In addition, NOS activities are modulated by phosphorylation and protein–protein interactions (Dreyer et al., 2004; Osuka et al., 2002).

Recently, several studies suggested that protein nitration could be a cellular signaling mechanism, as is often a reversible and selective process, similar to protein phosphorylation (Aulak et al., 2004; Koeck et al., 2004). In addition, proteins that are nitrated are more prone to proteosomal degradation than their counterparts (Gow et al., 1996). Ubiquitin carboxyl-terminal hydrolase L-1 (UCH L-1), one of the components of the proteosomal pathway, was found to be oxidized in the IPL and hippocampus of AD, a finding that could be one of the reasons for the observed increase in nitrated proteins in this disorder (Castegna et al., 2002a; Sultana et al., in press-b).

Tyrosine residues in a protein play an important role in regulating the function of the protein. Tyr is a site of phosphorylation, a prominent regulation function. Addition of nitrite to the protein at the tyrosine residue prevents the phosphorylation of tyrosine and also may change the structure of protein, thereby rendering a protein inactive. In the present study, we focused on identification of specific targets of protein nitration in AD and age-matched control hippocampus using a proteomics approach, similar to our prior investigation of nitrated proteins in AD inferior parietal lobule (IPL) (Castegna et al., 2003). We report specific nitration of alpha enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATP synthase alpha chain, voltage-dependent anion channel protein 1, and carbonic anhydrase II in AD hippocampus. Our data support the notion that perturbation in energy metabolism, pH regulation, and mitochondrial functions by specific protein nitration could be one of the mechanisms for the onset and progression of AD.

Materials and methods

Frozen hippocampal samples were obtained from 6 AD patients and 6 age-matched controls for the present study. Three of the six AD samples that were used in the current study were from the subjects whose IPL region was previously studied for nitrated proteins in our laboratory (Castegna et al., 2003). 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). This short PMI offers a distinct advantage for proteomics analysis since postmortem changes in brain, a common problem in many studies in AD, are minimal. All AD patients displayed progressive intellectual decline and met NINCDS-ADRDA Workgroup criteria for the clinical diagnosis of probable AD (McKhann et al., 1984). 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, but the results of this study showed no difference between AD patients with or without the presence of Lewy bodies. Control subjects underwent annual mental status testing and semi-annual physical and neurological examinations 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. Neuropathologic evaluation of control brains revealed only age-associated gross and histopathologic alterations.

Table 1
Demographic characteristics of AD and control subjects

Sample $n = 6$	Age (years)	Gender (M/F)	Postmortem interval (h)
Control	85.8 ± 4.1	4/2	2.9 ± 0.23
AD	84.5 ± 5.2	4/2	2.1 ± 0.47

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, 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).

Two-dimensional electrophoresis

Proteins from samples (150 μg) were precipitated 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 14,000×*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 strips were 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.

SYPRO Ruby staining

The gels were fixed in a solution containing 10% (v/v) methanol and 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 then were placed in deionized water overnight and scanned.

Immunoprecipitation

Control or AD samples (250 μg) were first precleared by incubation with protein A–agarose (Pharmacia, USA) 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 or IEF followed by immunoblotting on a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Proteins were detected by the alkaline-phosphatase-linked secondary antibody (Sigma, St. Louis, MO, USA) as previously described (Sultana and Butterfield, 2004).

Western blotting

For immunoblotting analysis, samples were separated by electrophoresis as described in the sample preparation section above 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-VDAC-1 polyclonal antibody (1:100) (Stressgen Biotech, USA), anti-actin polyclonal antibody (Stressgen Biotech, USA) or anti-nitrotyrosine polyclonal (Chemicon, Temecula, CA, USA) (1:1000) or anti-GAPDH (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 blots were incubated with the anti-rabbit or anti-goat IgG alkaline phosphatase secondary antibody (1:3000) (Sigma, St. Louis, MO, USA) in PBST for 1 h at room temperature. The membranes then 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. Blots were dried and scanned with Adobe Photoshop.

Image analysis

The gels and nitrocellulose membranes were scanned and saved in TIFF format using a Scanjet 3300C (Hewlett Packard, Palo Alto, CA, USA). PD Quest software (Bio-Rad) was used to compare protein expression and protein nitration between control and AD samples. Protein expression was measured using SYPRO Ruby stained gels that were scanned using a UV transilluminator (λ_{ex} = 470 nm, λ_{em} = 618 nm, Molecular Dynamics, Sunnyvale, CA, USA). Blots, used to measure nitrated proteins immunoreactivity, were scanned with a Microtek Scanmaker 4900. The average mode of background subtraction was used to normalize intensity values, which represent the amount of protein (total protein on gel and nitrated protein on blot) per spot. After completion of spot matching, the normalized intensity of each protein spot from individual gels (or blots) was compared between groups using statistical analysis.

Trypsin digestion

Samples were prepared according to the method described by Thongboonkerd et al. (2002). Based on the data obtained from image analysis, only the protein spots that showed a significant increase nitration in AD compared to control hippocampal 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 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.

Mass spectrometry

Mass spectra of the sample were determined by a TofSpec 2E (Micromass, UK) MALDI-TOF mass spectrometer in reflectron mode. The tryptic digest (1 μL) was mixed with 1 μL α-cyano-4-hydroxy-*trans*-cinnamic acid (10 mg/mL in 0.1% TFA (trifluoroacetic acid):ACN (acetonitrile), 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). The spectra reported in this study are a summation of 100 laser shots. External calibration of the mass axis, used for acquisition and internal calibration, employed either trypsin autolysis ions or matrix clusters and 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). Peptide mass fingerprinting used the assumption that peptides are monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Up to 1 missed trypsin cleavage was allowed. Mass tolerance of 100 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 $-10 * \mathrm{Log}_{10}(P)$, 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 p*I* range based on positions in the gel.

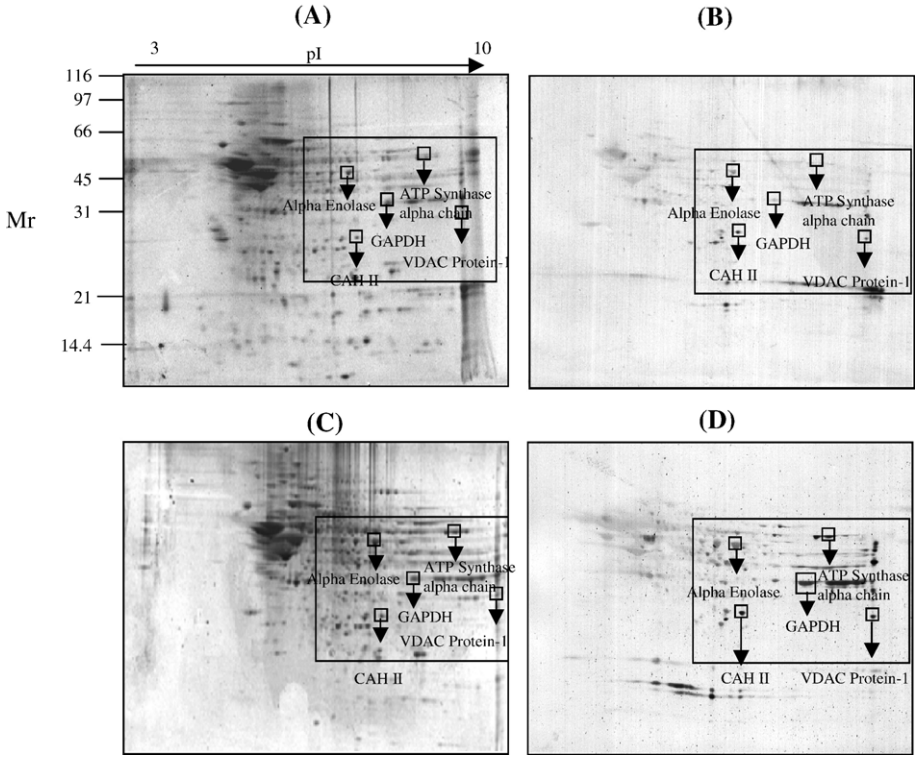

Fig. 1. SYPRO Ruby 2-DE images of the hippocampus from control (A) or AD (C) brain. Panels (B) and (D) represent Western blots probed with anti-3-NT antibody in control and AD brains, respectively. Protein (150 μg) was separated on immobilized pH 3–10 IPG strips followed by separation on an 8–16% gradient SDS-PAGE gels. Protein nitration was significantly increased in AD brain compared to age-matched control. See text. Box area is the enlarged area shown in Fig. 2.

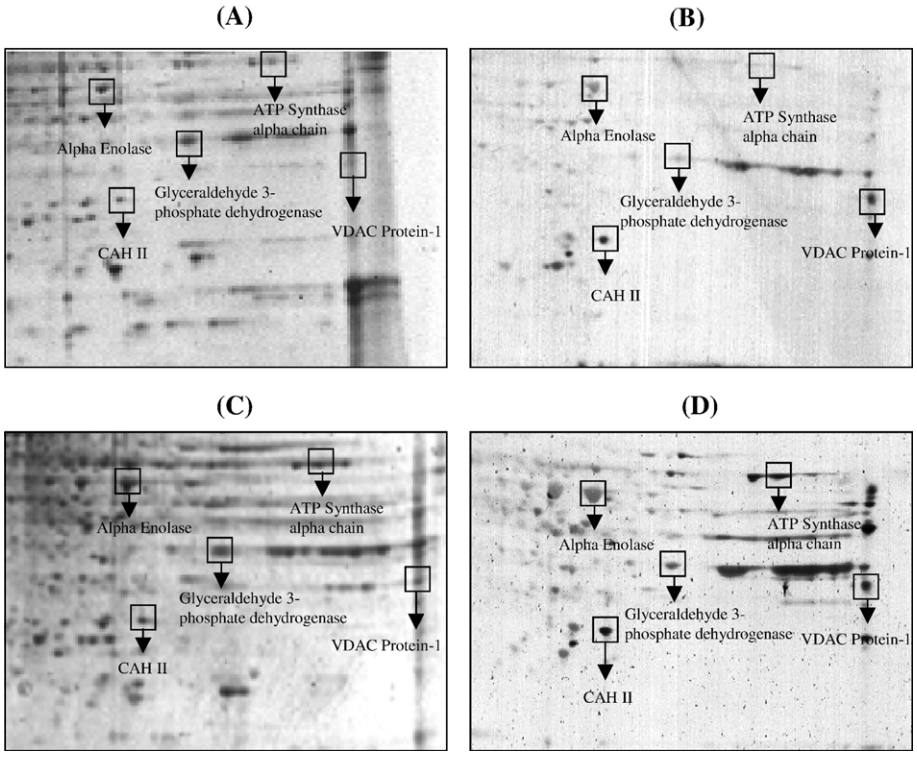

Fig. 2. Enlargement of boxed area from Fig. 1.

Protein–protein interactions

Interaction of the proteins that were nitrated with other proteins was searched using the Stratagene database (http://www.stratagene.com).

Statistics

The data of protein levels and protein-specific nitration levels were analyzed by two-tailed Student's t test. A value of $P < 0.05$ was considered statistically significant. A similar statistical analysis is usually used for proteomics data analysis (Castegna et al., 2002a; Korolainen et al., 2002; Maurer et al., 2005).

Results

Specific nitrated proteins in the AD and age-matched control hippocampus were identified immunochemically using a redox proteomics approach. 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 gels (Fig. 1). The numbers of the protein spots that are nitrated in AD are more when compared to the age-matched control blots (Fig. 1D). Using this approach of normalization, we confirmed that all the immunopositive spots for 3-NT are not excessively modified proteins (Castegna et al., 2002a,b, 2003; Poon et al., 2004). However, we identified five significantly excessively nitrated proteins in AD hippocampus (Fig. 2D). The identified nitrated proteins spots were in-gel trypsin-digested and subjected to mass analysis using MALDI mass spectrometry for protein identification. Table 2 shows the proteins that were successfully identified by mass spectrometry along with the peptides matched, percentage coverage, and pI and Mr values.

The proteins that were identified to be excessively nitrated proteins in AD hippocampus compared to age-matched control brain by MS analysis include: alpha enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), carbonic anhydrase II (CAH II), ATP synthase alpha chain, and voltage-dependent anion channel protein 1 (VDAC-1). The increase in 3-NT levels in AD compared to age-matched control was significant for ATP synthase alpha chain (326 ± 170% of control, $P < 0.04$), CAH II (253 ± 72% of control, $P < 0.009$), GAPDH (218 ± 64% of control, $P < 0.04$), alpha enolase (347 ± 90% of control, $P < 0.006$), VDAC protein-1 (511 ± 120% of control, $P < 0.04$). Protein expression was found to be significantly increased for enolase (210 ± 28% of control, $P < 0.05$), GAPDH (157 ± 31% of control, $P < 0.03$), ATP synthase alpha chain (187 ± 43% of control, $P < 0.02$), and CAH II (227 ± 50% of control, $P < 0.04$). No significant change in expression in AD hippocampus was observed for VDAC-1 protein.

Furthermore, to ensure correct identification of these proteins, immunochemical selection of two proteins, VDAC-1 protein and GAPDH, was undertaken. The blot probed with anti-GAPDH antibody (Fig. 3) showed four spots that are likely the isoforms of this protein (Fig. 3C). The position of the nitrated GAPDH protein spot on the blot probed with anti-GAPDH antibody was the same as that observed on the nitrated protein blot (Fig. 3C), further validating the identification of this protein. In addition, VDAC-1 protein was immunoprecipitated from age-matched control and AD brain samples (Fig. 4). The immunoprecipitated VDAC-1 protein further confirmed the protein expression and nitration status of this protein as suggested by proteomics results. Fig. 4A shows a significant increase ($P < 0.03$) in protein nitration of VDAC-1 protein in AD, and no significant increase was observed in protein expression (Fig. 4B), a result reported in Table 2. Consistent with these results, we immunoprecipitated the protein actin. The results did not show any change in the nitration and expression (Figs. 5E, F) between the control and AD hippocampus, thereby confirming the lack of significant difference in the nitration of this protein.

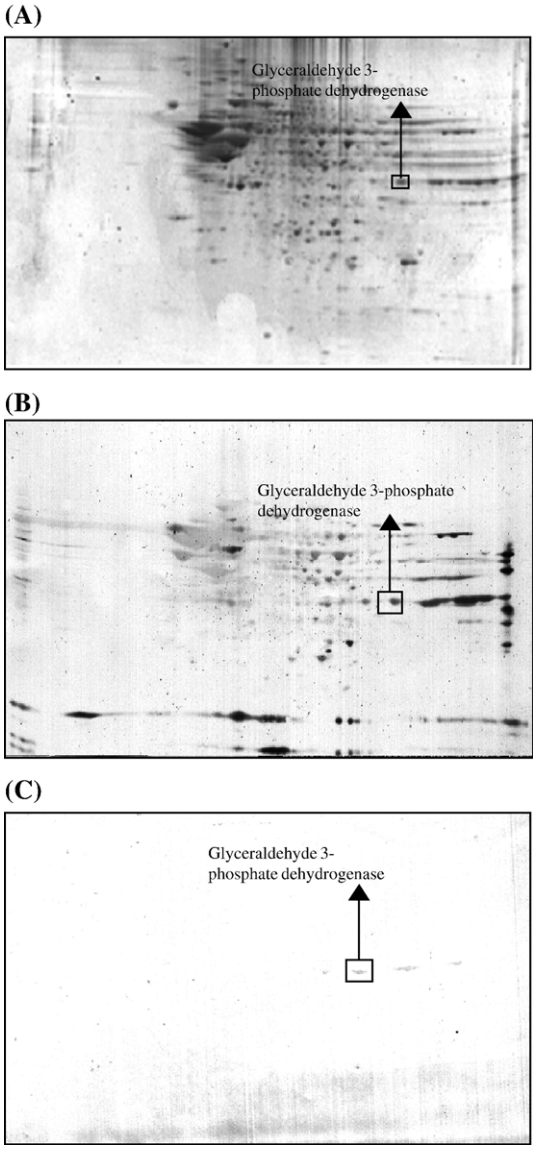

Fig. 3. Validation of protein identified by MS using Western blot analysis: A—gel, B—blot probed with 3-NT antibody, C—blots probed with anti-GAPDH respectively. A box is drawn around the protein spot of interest. $n = 3$.

Nitration of a protein affects not just the function of the modified protein, but nitration may also affect the interaction of the nitrated protein with other proteins or the pathways. Utilization of the Stratagene database for protein–protein interactions suggests that the nitration of identified proteins could affect various cellular processes, such as proliferation, secretion, motility, energy metabolism, as well as apoptosis induction and pH buffering alterations (Fig. 5).

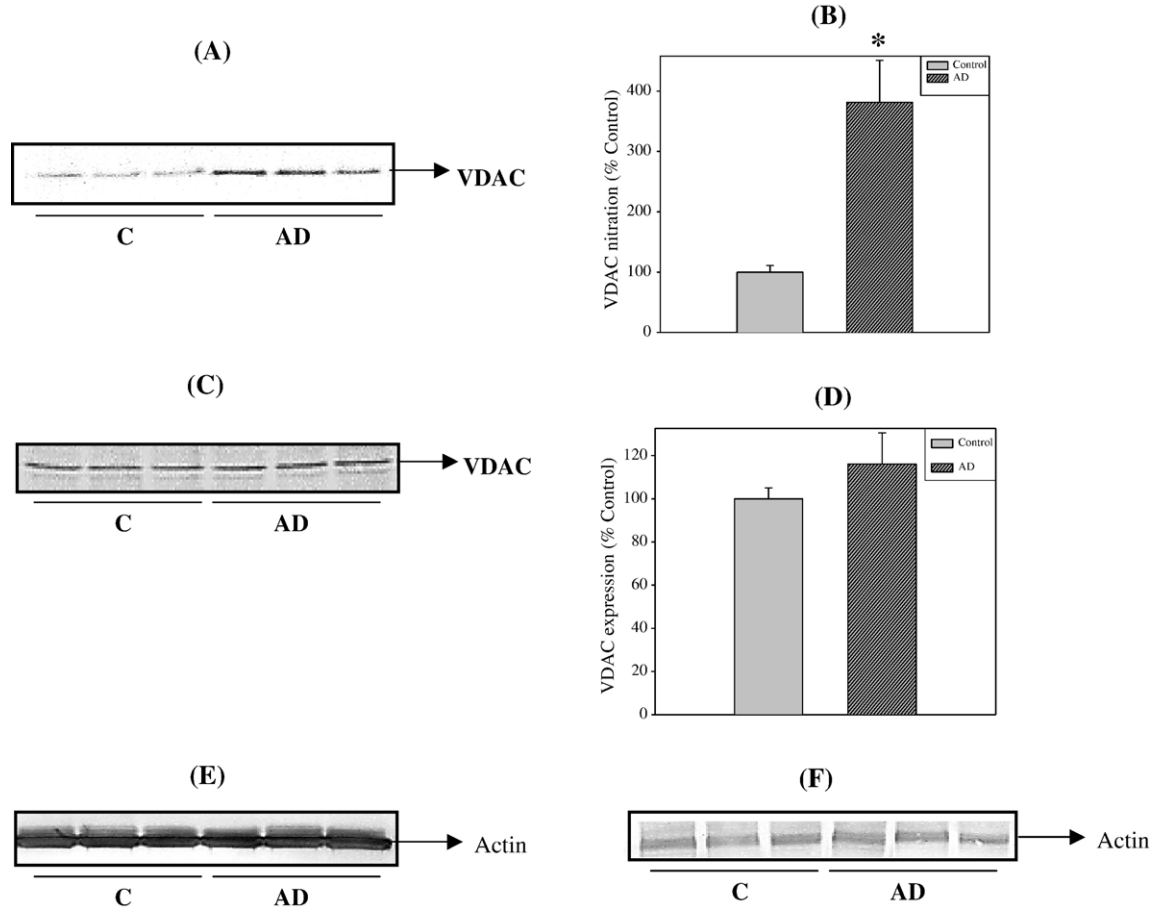

Fig. 4. Western blot analysis. VDAC and actin proteins were immunoprecipitated using anti-VDAC and anti-actin antibodies respectively from control and AD hippocampus followed by determination of nitration and protein expression. Panels (A) and (F) represent blots probed with anti-3-NT, and panels (C) and (E) represent blots probed with anti-VDAC and anti-actin antibody respectively, whereas panels (B) or (D) represent histograms for panels (A) and (C) blots. *$P <$ 0.03. $n = 3$.

As shown in Fig. 6, a comparison was made between the previously reported oxidized proteins in inferior parietal lobule (Castegna et al., 2003) and the identified nitrated proteins in AD hippocampus of the current study. Alpha enolase is identified as a common target of nitration in both the regions of brain (Fig. 6A). Carbonic anhydrase II and alpha enolase were identified as the common targets of both carbonylation and nitration in hippocampus (Fig. 6B), whereas alpha enolase, triose phosphate isomerase, and UCH L-1 were identified as the common targets of protein oxidation (carbonylation and nitration) in IPL and hippocampus.

Discussion

In AD brain, the hippocampus is one of the first brain regions to be affected due to functional isolation from the entorhinal cortex and subiculum, regions that convey information into and out of hippocampus, leading to loss of memory (Ball, 1977; Jones, 1993). However, until now, it is not clear what causes the loss of synaptic connections in the brain. Several lines of evidence point towards oxidative stress as an underlying mechanism that could trigger all these downstream events, as indicated by decreased antioxidant systems and increased oxidative stress products including protein

Table 2
Nitrated proteins in AD hippocampus[a]

Identity of nitrated proteins in AD hippocampus	# Peptide matched of the identified protein	Percent coverage of the matched peptides	p*I*, Mr (kDa)	Mowse score	% Oxidation	*P* values
pH regulation						
1. Carbonic anhydrase II	9/33	40%	6.86, 29	96	253 ± 72	0.009
Energy metabolism						
1. Enolase	18/42	49%	6.0, 47	163	347 ± 90	0.006
2. Glyceraldehyde-3-phosphate dehydrogenase	15/50	51%	8.5, 36	150	218 ± 64	0.04
3. ATP synthase alpha chain	24/40	52%	9.16, 59.8	263	326 ± 170	0.04
4. Voltage-dependent anion-channel protein-1	12/39	51%	8.6, 30.7	130	511 ± 120	0.04

[a] $n = 6$ each for control and AD hippocampus.

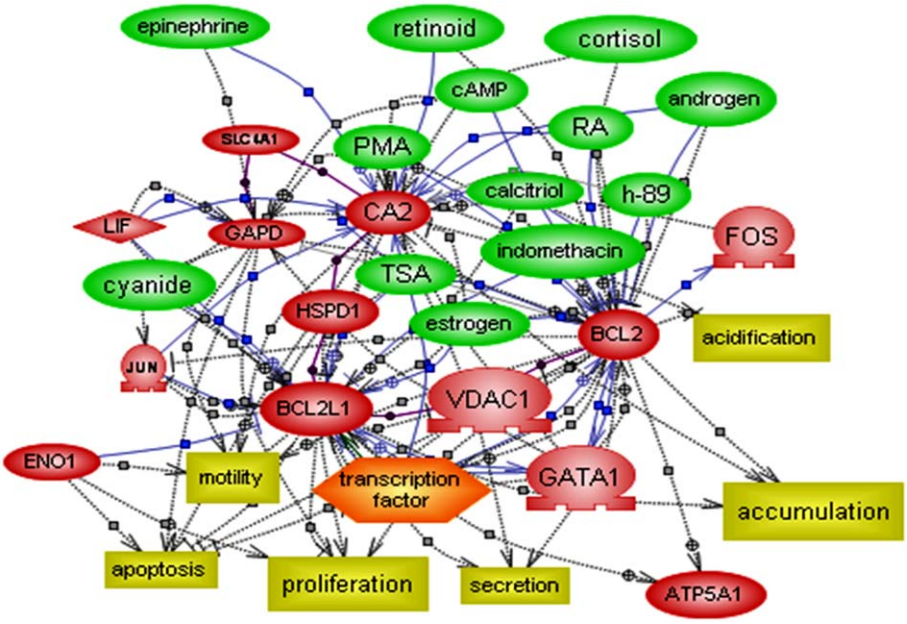

Fig. 5. Diagrammatic representation of protein–protein interactions of the nitrated proteins in AD hippocampus. Also note the functions regulated by these proteins.

carbonyl, lipid peroxidation, RNA and DNA oxidation, ROS and RNS (Butterfield et al., 2001, 2002a,b; Keil et al., 2004; Koppal et al., 1999; Lovell et al., 2001; Mark et al., 1997; Markesbery, 1997; Markesbery and Carney, 1999; Smith et al., 1994; Stadtman and Berlett, 1997; Varadarajan et al., 2000). In AD brain and CSF, increased levels of nitrated proteins have been found (Castegna et al., 2003, Smith et al., 1997; Tohgi et al., 1999), implying a role for RNS in AD pathology.

Increased levels of 3-nitrotyrosine (NT) immunoreactivity in neurons from AD brain when compared to aged-matched controls were observed (Smith et al., 1997), and dityrosine and 3-nitrotyrosine levels were reported to be elevated consistently in the hippocampus, IPL, and neocortical regions of the AD brain and in ventricular cerebrospinal fluid (VF) (Su et al., 1997; Hensley et al., 1998).

In the present study, we identified the specific nitrated proteins in AD hippocampus in order to gain insight into mechanisms of disease progression and also to gain insight into potential pharmacological strategies to combat this disease. Previous studies from our laboratory and others found alpha enolase, gamma enolase, beta actin, lactate dehydrogenase (LDH), triose phosphate isomerase (TPI), carbonic anhydrase II, gamma-SNAP, ubiquitin carboxyl terminal hydrolase L-1 (UCH L-1), neuropolypeptide h3, phosphoglycerate mutase 1 (PGM1), glutamine synthase (GS), dihydropyrimidinase related protein-2 (DRP-2), glutamate transporter-1 (GLUT-1), heat shock cognate 71, peptidyl prolyl *cis–trans* isoemerase (Pin 1), glutathione-*S*-transferase, and creatine kinase BB as oxidized and functionally impaired proteins, further supporting the hypothesis of oxidative stress as a mediator of synaptic loss and a presumed factor for the formation of tangles and plaques (Aksenov et al., 1999; Castegna et al., 2002a,b, 2003, Choi et al., 2004, Lauderback et al., 2001; Sultana et al., in press-a-b); Sultana and Butterfield, 2004).

In the present study, we identified alpha enolase, glyceraldehyde-3-phosphate dehydrogenase, ATP synthase alpha chain, voltage-dependent anion channel protein 1, and carbonic anhydrase II as specifically nitrated hippocampal proteins in AD brain. These proteins are grouped in Table 2 based on the functional pathways in which they are involved.

Since glucose is the main source for the production of ATP in the normal brain (Vannucci and Vannucci, 2000), impaired glucose uptake or utilization not only decreases the ATP levels, but also has other deleterious effects on the cell. For example, disturbances in cholesterol homeostasis, cholinergic defects, ion homeostasis, altered protein synthesis, sorting, transport and degradation of proteins, and maintenance of synaptic transmission—all of which are physiological hallmarks of AD (Castegna et al., 2002a,b, 2003; Hoyer, 2004; Sultana et al., in press-b). Hypometabolism of glucose can also lead to altered ion homeostasis, impaired folding of proteins, loss of cell potentials, etc. (Erecinska and Silver, 1989). Previous studies from our laboratory and others have shown altered function of the enzymes involved in glucose metabolism (Castegna et al., 2002b; Iwangoff et al., 1980, Meier-Ruge et al., 1984; Sultana et al., in press-b). And, the identification of alpha enolase and glyceraldehyde-3-phosphate dehydrogenase each of which participates in the glycolytic pathway to be significantly nitrated in the present study correlated with the altered energy metabolism in AD brain (Geddes et al., 1996; Messier and Gagnon, 1996; Vanhanen and Soininen, 1998). PET studies also show a pattern consistent with the reduced cerebral glucose utilization in AD brain (Erecinska and Silver, 1989; Hoyer, 2004; Rapoport, 1999).

Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, the second of the two-energy intermediates that generates ATP in glycolysis. Several pathologies are linked to enolase-dependent pathways, especially autoimmune and neurodegenerative disorders (Pancholi, 2001, Parnetti et al., 1995). In AD brain, identification of alpha enolase as a nitrated protein reflects an impaired glucose metabolism (Castegna et al., 2002b; Parnetti et al., 1995; Sultana et al., in press-b; Verbeek et al., 2003). Oxidation of enolase decreases the activity of this enzyme (Meier-Ruge et al., 1984; Sultana et al., in press-b) in AD brain compared to the age-matched controls. A proteomics method

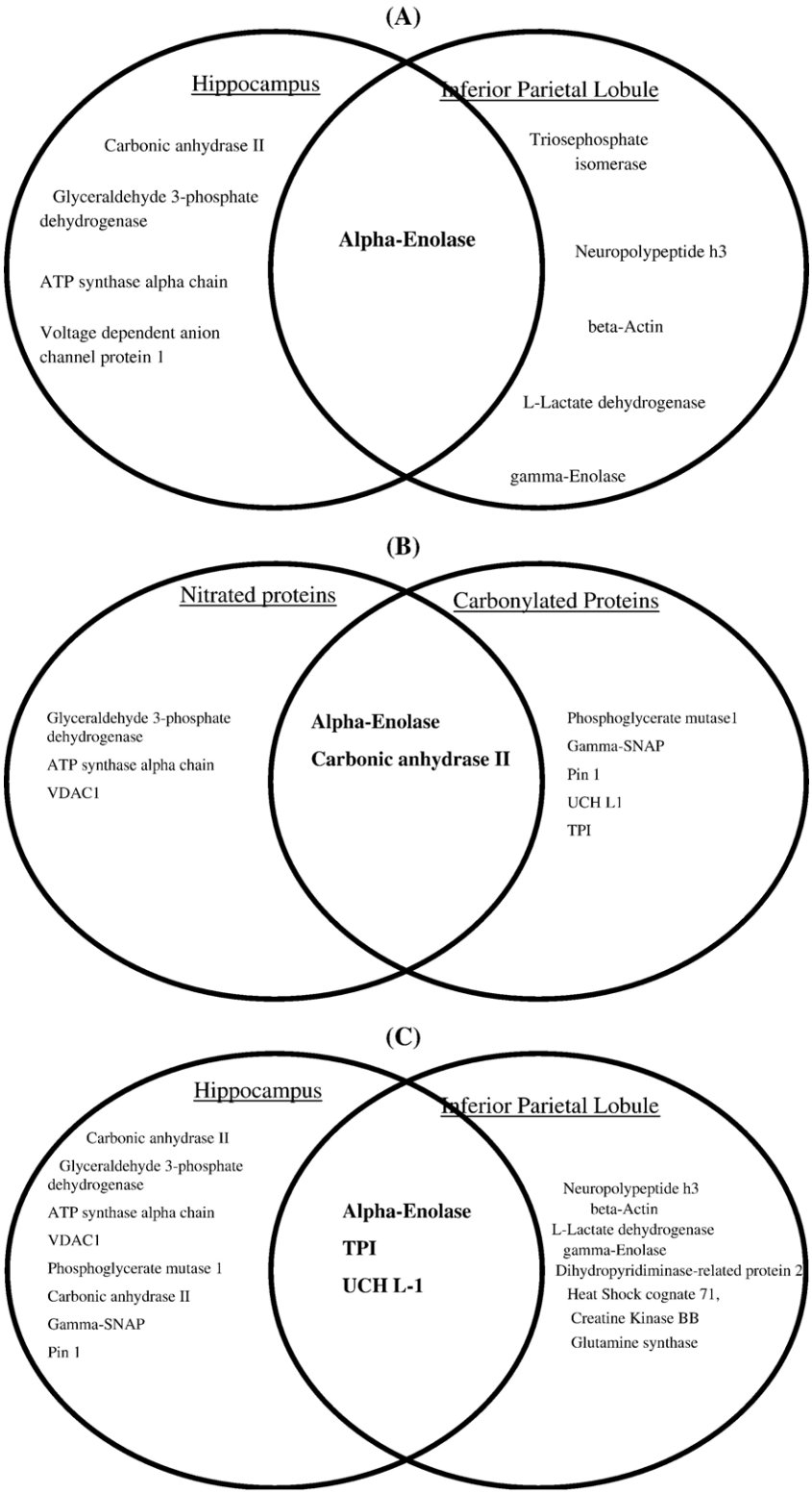

Fig. 6. Comparison of the commonly oxidized protein using Venn diagram. A—comparison of common nitrated proteins in AD hippocampus and IPL. B—common targets of nitration and carbonylation in AD hippocampus. C—common targets of oxidation (carbonylation and nitration) in AD IPL and hippocampus.

applied to AD brain showed that the protein level of the α-subunit is increased compared to control brain (Castegna et al., 2002b; Sultana et al., in press-b). In addition, we also found glyceraldehyde-3-phosphate dehydrogenase, another glycolytic enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-phosphoglycerate, to be an excessively nitrated protein in AD

hippocampus. Previous studies reported an accumulation of this enzyme along with α-enolase and γ-enolase and also a decrease in enzyme activity in AD brain (Mazzola and Sirover, 2001). GAPDH was found to be oxidized in rat brain following intracerebral injection of beta-amyloid peptide (1–42) (Boyd-Kimball et al., 2005). Oxidation and subsequent loss of function of glyceraldehyde-3-phosphate dehydrogenase and enolase could result in decreased ATP production, a finding consistent with the altered glucose tolerance and metabolism of AD patients (Messier and Gagnon, 1996; Rapoport et al., 1991; Vanhanen and Soininen, 1998).

Carbonic anhydrase II is another enzyme found to be nitrated in AD hippocampus. Since this enzyme catalyzes the reversible hydration of CO_2, a reaction fundamental to many cellular and systemic processes including glycolysis and acid and fluid secretion, CAH II is a fundamentally important enzyme for brain function. The catalytically active CAH II is confined to oligodendrocytes and subtypes of protoplasmic astrocytes in the CNS. The physiological functions of CAH II are involved in cellular pH regulation, CO_2 and HCO_3^- transport, and maintaining H_2O and electrolyte balance (Sly and Hu, 1995). Production of CSF and the synthesis of glucose and lipids (Maren, 1988) also involve CAH II. Deficiency of CAH II results in osteoporosis, renal tubular acidosis, and cerebral calcification. Patients with CAH II deficiency also demonstrate cognitive defects varying from disabilities to severe mental retardation (Sly et al., 1983, 1985). Consistent with previous studies of other enzymes and transporters (Aksenova et al., 1999; Lauderback et al., 2001; Sultana and Butterfield, 2004), oxidative modification of CAH II likely explains its diminished activity that has been reported in AD brain compared to age-matched control brain (Meier-Ruge et al., 1984; Sultana et al., in press-b). Consequently, oxidized CAH 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 CAH II may be involved in the progression of AD.

Increasing evidence suggests an important role of mitochondrial dysfunction in the pathogenesis of AD brain. The activity of many of the different mitochondrial enzymes appears to be reduced in AD brain (Bosetti et al., 2002, Hirai et al., 2001).Several other studies indicate that Aβ decreases the activity of mitochondrial respiratory chain complexes (Hirai et al., 2001, Lovell et al., 2005, Molina et al., 1997). The mitochondrial respiratory chain is sensitive to both NO- and peroxynitrate-mediated damage. In the present study, we found ATP synthase alpha chain and voltage-dependent anion channel proteins that belong to the mitochondrial membrane as nitrated proteins that could play an important role in mitochondrial dysfunction and cell death in AD.

ATP synthase α-chain is localized in the inner membrane of mitochondria and is a part of the complex V of oxidative phosphorylation that plays a key role in energy production. ATPase, by complex rotational movements of its subunits, couples the proton gradient generated by the respiratory chain which promotes ATP synthesis and release (Junge et al., 1997). The cytosolic accumulation of ATP synthase α-chain with neurofibrillary tangles in AD has been reported previously (Sergeant et al., 2003). On isolated mitochondria, a lower protein content of the complex V has been described in AD (Schagger and Ohm, 1995). It was recently suggested that intact complex V is required for apoptosis (Matsuyama et al., 1998). Moreover, the identification of ATP synthase alpha as a nitrated protein suggests impaired function and also interactions between the subunits leading to reduced activity of F_1F_0-ATPase (ATP synthase, complex V) that could compromise brain ATP synthesis and induce damaging ROS production and, if severe, could lead to neuronal death. Moreover, the dysfunction of mitochondria has been recently described to alter APP metabolism, enhancing the intraneuronal accumulation of amyloid β-peptides and enhancing the neuronal vulnerability (Busciglio et al., 2002). Our data, in addition to previous studies, suggest that the function of ATP synthase α-chain is altered in AD degenerating neurons that could participate in the neurodegenerative process of AD (Sergeant et al., 2003).

The voltage-dependent anion channel (VDAC) is the outer pore component of the mitochondrial permeability transition pore (MPTP), a structure that plays an essential role in movement of metabolites like ATP in and out of mitochondria by passive diffusion, synaptic communication, and in the early stages of apoptosis. ATP production and mitochondrial calcium buffering are essential for normal synaptic transmission (Csordas and Hajnoczky, 2003; Jonas et al., 2003; Mattson and Liu, 2002). Furthermore, VDAC1-deficient mice were reported to show deficits in learning behavior and synaptic plasticity (Weeber et al., 2002). In addition, VDAC also plays an important role in apoptotic process involving release of several apoptogenic factors such as cytochrome C (Liu et al., 1996), apoptosis inducing factor (Lorenzo et al., 1999), smac (Du et al., 2000), and caspases (Susin et al., 1999) from mitochondria. Caspase-3 and caspase-8 were found to be involved in vivo in the proteolytic cleavage of APP in hippocampal neurons following toxic or ischemic brain injury (Gervais et al., 1999) and in apoptosis of neuronal cells induced by beta-amyloid, respectively (Ivins et al., 1999). Identification of VDAC1 protein as a nitrated protein in AD hippocampus suggests that the nitration of this protein could alter the function of the MPTP leading to mitochondrial depolarization and altered signal transduction pathways, which could be crucial in synaptic transmission and plasticity. In addition, this alteration may also induce apoptotic events leading to cell death. Yoo et al. (2001) showed a decrease expression of VDAC1 protein (pI 10.0) in the temporal, frontal, and occipital cortex of AD patient, whereas we did not observe any significant change in the expression of VDAC protein in AD hippocampus (Fig. 2). Furthermore, nitration of this protein could prevent the interaction of BCL-xL with VDAC, leading to increase BAX and BAK levels that are associated with VDAC (Shimizu et al., 1999) and facilitating release of cytochrome C through VDAC (Fig. 5). As can be seen in Fig. 5, the proteins we identified as oxidized interact with other proteins and the oxidation of these proteins could also influence the protein–protein interactions leading to cellular alterations (Poon et al., 2004; Sultana et al., in press-b). The protein–protein interaction network suggests that modified proteins regulate glycolysis, proliferation, secretion, accumulation, acidification, motility, and apoptosis, and all these functions are vital to neuronal survivability.

Comparative analysis of nitrated proteins between the previously studied AD IPL region (Castegna et al., 2003) and the results of the present study in AD hippocampus showed enolase as the common target of nitration (Fig. 5A), suggesting that alterations in cellular bioenergetics could be involved in the progression of AD. CAH II and enolase are the common targets for carbonylation and nitration in AD hippocampus (Fig. 6B), which implicates changes in cellular pH and bioenergetics that could impair enzyme activities leading to cognitive impairment and neurodegeneration. Furthermore, the identification of alpha enolase, TPI, and UCH L-1 as the

target of oxidation in hippocampus and IPL implicates a common mechanism operating in the two different regions of brain (Fig. 6C). However, further studies are required to confirm this hypothesis.

Taken together, the nitration of proteins in AD hippocampus suggests impaired energy metabolism, synaptic loss, and mitochondrial function, consistent with the observed pathology of AD brain. From this study, we conclude that nitration of these proteins may be involved in the complex mechanisms of AD brain pathology. Additional studies are underway using animal models of AD to understand further the mechanisms of neurodegeneration in this dementing disorder.

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