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Research Report

Proteomic identification of HNE-bound proteins in early Alzheimer disease: Insights into the role of lipid peroxidation in the progression of AD

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ABSTRACT

Early Alzheimer's disease (EAD) is the intermediary stage between mild cognitive impairment (MCI) and late-stage Alzheimer's disease (AD). The symptoms of EAD mirror the disease advancement between the two phases. Dementia, memory deficits, and cognitive decline are more pronounced as the disease progresses. Oxidative stress in brain is reported in MCI and AD, including lipid peroxidation indexed by protein-bound 4-hydroxy-2-nonenal (HNE). There are limited data regarding the proteomics analysis of brain from subjects with EAD and even less concerning the possible relationship of EAD HNE-modified brain proteins with HNE-modified proteins in MCI and AD. Proteomics was utilized to investigate excessively HNE-bound brain proteins in EAD compared to those in control. These new results provide potentially valuable insight into connecting HNE-bound brain proteins in EAD to those previously identified in MCI and AD, since EAD is a transitional stage between MCI and late-stage AD. In total, six proteins were found to be excessively covalently bound by HNE in EAD inferior parietal lobule (IPL) compared to age-related control brain. These proteins play roles in antioxidant defense (manganese superoxide dismutase), neuronal communication and neurite outgrowth (dihydropyriminidase-related protein 2), and energy metabolism (α-enolase, malate dehydrogenase, triosephosphate isomerase, and F1 ATPase, alpha subunit). This study shows that there is an overlap of brain proteins in EAD with previously identified oxidatively modified proteins in MCI and late-stage AD. The results are consistent with the hypothesis that oxidative stress, in particular lipid peroxidation, is an early event in the progression of AD, and is the first to identify in EAD identical brain proteins previously identified as HNE-modified in MCI and late-state AD.

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1. Introduction

Early Alzheimer's disease (EAD) is the intermediate state between mild cognitive impairment (MCI) and Alzheimer's disease (AD) (Markesbery et al., 2006). There is a progressive deterioration of the brain as demonstrated by increased frontal lobe atrophy and ventricular widening, which contribute to representative memory deficits (Markesbery et al., 2006). The order of cognitive decline from minor to major agrees with the three-stage development of neurofibrillary tangles described by Braak and Braak (1991). There is a significant increase in the number of neurofibrillary tangles in EAD subjects compared to MCI subjects in the frontal and temporal lobes (Markesbery et al., 2006). Other impairments include: verbal abilities, visuospatial functions, attention, and executive functions. As expected, brains from subjects with EAD also demonstrate synapse loss, which correlates to AD pathology (Scheff et al., 2006). One synaptic protein in particular, synaptophysin, is greatly reduced in AD hippocampus (Sze et al., 1997). Synaptophysin is a synaptic vesicle protein involved in synapse formation and exocytosis, and its dysfunction can lead to poor neurotransmission and altered learning and memory.

Oxidative stress in brain, characterized by protein oxidation (indexed by protein carbonyls; 3-nitrotyrosine), lipid peroxidation (indexed by free or protein-bound 4-hydroxy-2-nonenal (HNE)), and DNA and RNA oxidation (indexed by 8-hydroxy-2-deoxyguanosine and 8-hydroxyguanosine, respectively) have been reported from subjects with AD and MCI (Butterfield et al., 2006b,c, 2007; Hensley et al., 1995; Lauderback et al., 2001). The lipid peroxidation product, HNE, covalently binds to proteins via Michael addition to Cys, His, and Lys residues (Butterfield and Stadtman, 1997).

Normal aging, MCI, and AD can be distinguished by pathological differences in nicotinic acetylcholine receptor binding (Sabbagh et al., 2006). Nicotinic acetylcholine receptors, located in the postsynaptic membrane, are bound not only by acetylcholine but also by amyloid β-peptide (Aβ) as well. Acetylcholine and Aβ participate in well publicized hypotheses of the etiology of AD (Coyle et al., 1983; Glenner et al., 1984; Nordberg, 1992). There is a growing hypothesis of neurotrophic factors involved in AD pathogenesis (Arancio and Chao, 2007). One such neurotrophin of interest is brain-derived neurotrophic factor (BDNF). BDNF increases synaptic plasticity and prevents neuronal cell death in certain neurodegenerative diseases such as Huntington's disease (HD) (Ciammola et al., 2007), Parkinson's disease (PD) (Garcia de Yebenes et al., 2000) and AD (Siegel and Chauhan, 2000). Hippocampal neurons treated with BDNF significantly increased synapse stability (Bamji et al., 2006). The serum concentration of brain-derived neurotrophic factor is decreased in EAD; however, these levels were higher than those found in late-stage AD patients (Laske et al., 2006), which correlates with previous results that showed post mortem BDNF mRNA levels are decreased in hippocampus and cortex in AD (Durany et al., 2000).

Finding the specific points of conversion from MCI to EAD is difficult. By using a proteomics approach to identify HNE-bound proteins that overlap these two disease stages, the protein profiles produced can potentially provide insight into the progression of the disease and lead to improved therapeutics to slow the progression of AD.

2. Results

2.1. *Increase in total level of protein-bound HNE*

Fig. 1 shows that EAD had significantly elevated levels of HNE-modified proteins in the inferior parietal lobule (IPL) compared to age-matched controls. A 20% increase in HNE-bound proteins was observed in brain of subjects with EAD vs. control. This increased lipid peroxidation, indexed by protein-bound HNE, is consistent with the finding of elevated free HNE in EAD, particularly in the hippocampus/parahippocampal gyrus (HPG), superior and middle temporal gyrus (SMTG) (Williams et al., 2006).

2.2. *Identification of HNE-bound proteins in EAD-IPL*

Six proteins were found to be excessively bound to HNE in EAD-IPL, compared to control, by 2D gel electrophoresis and Western blot analysis. These identified proteins include manganese superoxide dismutase (MnSOD), alpha enolase (α-enolase), dihydropyriminidase-related protein 2 (DRP-2), malate dehydrogenase, triosephosphate isomerase, and F1 ATPase, alpha subunit (Figs. 2a and b). Coincidentally, many of these proteins also are reported to be oxidatively modified in brain of subjects with MCI and AD. Table 1 gives a summary of the HNE-bound proteins in EAD inferior parietal lobule. These gels and blots were analyzed and compared using PDQuest software to locate the specific protein spots that were found to have significantly elevated HNE bound in EAD compared to aged-matched control based on HNE immunoreactivity (Figs. 3a and b).

2.3. *Reduction of alpha enolase activity*

As a confirmation of the proteomics-identified brain proteins in EAD, enzyme activities of selected proteins were determined. Fig. 4 shows there is a significant decrease (~22%) in enolase activity in brain of EAD subjects compared to age-matched control. This finding is consistent with previous data that demonstrated loss of enzymatic activity in oxidatively modified proteins (Butterfield et al., 2006b; Newman et al., 2007) and decreased energy metabolism observed in AD (Parihar and Brewer, 2007).

2.4. *Reduction of superoxide dismutase activity*

Superoxide dismutase activity is significantly reduced in EAD patients compared to age-matched control (Fig. 5). These results extend data of others (Casado et al., 2008; Marcus et al., 1998), who demonstrated SOD activity is diminished in AD patients. Manganese superoxide dismutase also has been found to be less expressed in hippocampal neurons from AD subjects compared to non-AD subjects, conceivably contributing to neuronal loss observed in late-stage AD (Marcus et al., 2006).

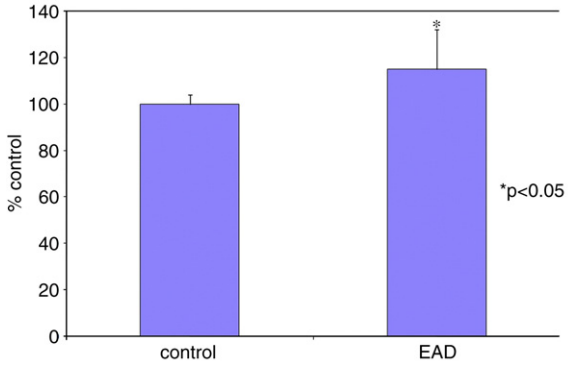

Fig. 1 – Increased protein-bound HNE in brain of subjects with EAD versus aged-matched control. The slot-blot results show a significant increase in the level of protein-bound HNE in EAD samples compared to age-matched controls ($p<0.05$). See text for experimental details.

2.5. *Increase in malate dehydrogenase activity*

The enzymatic activity of malate dehydrogenase was markedly increased in EAD (Fig. 6), which bolsters the previous research of others (Bubber et al., 2005; Op den Velde and Stam, 1976), who reported increased MDH activity in late-stage AD brain.

3. Discussion

Early AD links MCI to AD. Proteins become oxidatively modified during the aging process and in various neurodegenerative diseases. The imbalance of prooxidants and antioxidants leads to oxidative stress. Oxidative stress has been thoroughly established in MCI (Butterfield et al., 2006a,b,c, 2007; Pratico et al., 2002) and AD (Butterfield et al., 2001; Butterfield and Lauderback, 2002; Lauderback et al., 2001; Lovell and Markesbery, 2007; Montine et al., 2002; Pratico and Sung, 2004). Covalent modification of proteins by HNE is one such oxidative modification. Through Michael addition and Schiff base formation, HNE binds to proteins, and the current finding, the first to report elevated protein-bound HNE in EAD, is consistent with increased lipid peroxidation in brain of subjects with EAD. Therefore, lipid peroxidation has now been demonstrated in three stages of Alzheimer's disease: MCI (Butterfield et al., 2006c; Lovell and Markesbery, 2007; Williams et al., 2006; Keller et al., 2005), EAD (Lovell and Markesbery, 2007; Williams et al., 2006; this work), and AD (Butterfield and Lauderback, 2002; Lauderback et al., 2001; Montine et al., 2002; Pratico and Sung, 2004). Covalent modification of proteins by HNE leads to changes in protein conformation (Subramaniam et al., 1997) and activity of enzymes (Eliuk et al., 2007).

One of the oxidatively modified brain proteins found in EAD by proteomics in this study, superoxide dismutase (SOD) catalyzes the conversion of two superoxide anions to hydrogen peroxide and oxygen. Maintenance of this enzyme is critical to achieving oxidative balance; otherwise the cell would be in a continual state of oxidative stress. There are several distinct forms of SOD including Cu/ZnSOD (SOD1), MnSOD (SOD2), NiSOD, and FeSOD. Mutations in SOD1 can cause familial ALS (Potter and Valentine, 2003) and overexpression of SOD1 is related to Down's syndrome (Lee et al., 2001). These data are relevant because the SOD1 gene resides on chromosome 21, the same chromosome for APP (amyloid precursor protein), the source of the toxic peptide, Aβ(1–42). This is equally important because SOD1 knockout mice do not develop motor neuron disease and have a normal lifespan (Reaume et al., 1996), but SOD2 knockout mice die shortly after birth (Leibovitz and Siegel, 1980) due to increased oxidative stress. This observation demonstrates how essential SOD2 is. Located in the mitochondria, impairment of MnSOD can greatly affect the proteasome causing an oxidized protein "overload" with the inability to correctly ubiquitinate and degrade oxidized proteins. This notion is further supported by research demonstrating that oxidative modification of manganese superoxide dismutase inactivates the enzyme (MacMillan-Crow and Thompson, 1999; Tangpong et al., 2007). The current study demonstrates that the activity of MnSOD is significantly reduced in EAD compared to aged-matched control, which is consistent with this hypothesis. MnSOD was also found to be HNE-modified in brain in late-stage AD (Perluigi et al., in press), consonant with the results presented here in EAD. In addition, MnSOD is modified by HNE in PC6 neural cells subjected to oxidative insults (Keller et al., 1998).

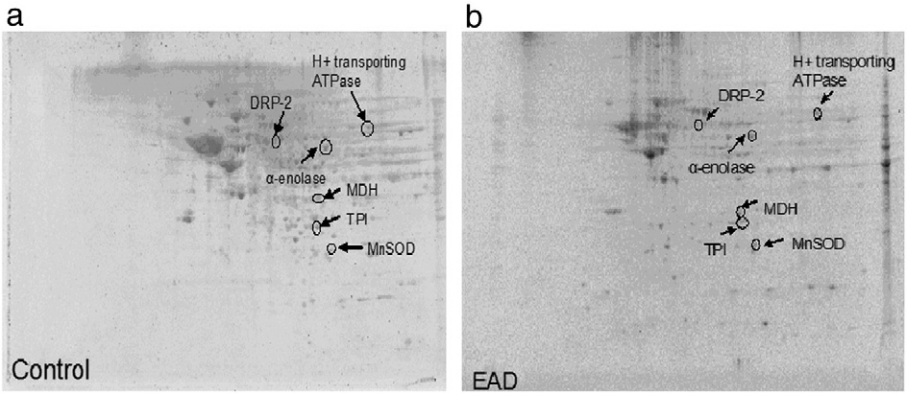

Fig. 2 – Representative 2D gels of HNE-bound proteins in age-matched control (a) and EAD-IPL (b). 2D gels of brain proteins from EAD and control subjects were obtained as described in Experimental procedures. Six proteins from IPL in EAD were found to have protein levels significantly different than those compared to aged-matched control.

Table 1 – Protein summary of excessively HNE-bound proteins identified in EAD-IPL.

Protein	Mowse score	pI	Apparent molecular wt. (M_r)	HNE adduction (% control ± SEM)	Peptide coverage (%)	Probability
Manganese superoxide dismutase (MnSOD)	81	7.96	19945	120 ± 17.0	33	0.04
Alpha enolase	143	6.99	47350	123 ± 16.0	29	0.03
F1 ATPase, alpha subunit	225	9.16	59828	118 ± 27.3	34	0.03
Dihydropyrimindase-related protein 2(DRP-2)	177	5.95	62711	319 ± 7.00	39	0.05
Triose phosphate isomerase (TPI)	88	6.51	26807	1380 ± 23.8	30	0.04
Malate dehydrogenase (MDH)	80	6.92	36620	520 ± 9.08	26	0.04

Superoxide dismutase acts as an antioxidant; therefore HNE adduction impairs this protein and reduces its antioxidant ability.

Altered expression of mitochondrial proteins and lowered activity in different complexes of the electron transport chain (ETC) are observed in AD (Chen et al., 2006a). F1 ATPase, alpha subunit (ATP synthase) is necessary in proton transport for phosphorylation of ADP to produce ATP. ATP synthase is commonly known as complex V in the ETC. This protein has been shown to be modified by HNE (Perluigi et al., in press) and of lower levels (Sergeant et al., 2003) in brain of subjects with late-stage AD (Perluigi et al., in press) and ATP synthase is oxidized in synaptosomes treated with the peptide Aβ (1–42) (Boyd-Kimball et al., 2005). Covalent modification of HNE bound to ATP synthase, alpha subunit can disrupt complex V. A decrease in the activity of the entire electron transport chain and impaired ATP production could be a result of this oxidative modification. This event, coupled with the changes in complex I, III, and IV, may cause electron leakage from the mitochondria to produce ROS. This action can also affect the mitochondrial proton gradient and its pH. ROS generation by reason of mitochondrial dysfunction suggests a different hypothesis (Mancuso et al., 2006) for the widely accepted existence of oxidative stress in AD (Butterfield et al., 2001, 2006a; Butterfield and Lauderback, 2002; Lauderback et al., 2001; Lovell and Markesbery, 2007; Markesbery, 1997; Montine et al., 2002; Pratico and Sung, 2004; Sultana et al., 2006a; Zhu et al., 2005).

Dihydropyriminidase-related protein 2 (DRP-2) is important for maintaining neuronal communication and forming neuronal connections. DRP-2 guides proteins for axonal growth by interacting with and modulating the protein, collapsin, to elongate and guide dendrites. Dendritic length is shortened in AD (Coleman and Flood, 1987), and this could be attributed to the fact that DRP-2 is oxidatively modified in late-stage AD (Sultana et al., 2006b; Perluigi et al., in press), ALS (Perluigi et al., 2005a), and Aβ(1–42)-treated synaptosomes (Boyd-Kimball et al., 2005). DRP-2 has decreased expression in AD brain (Lubec et al., 1999; Sultana et al., 2007a). Shortened dendritic length would lead to less neuronal communication with adjacent neurons that could contribute to memory loss and cognitive decline associated with AD. DRP-2 is closely associated with neurofibrillary tangles, one of the hallmarks of AD (Yoshida et al., 1998). Soluble DRP-2 becomes depleted due to phosphorylation and leads to abnormal neuritic and axonal outgrowth thereby accelerating neurodegeneration in AD (Gu et al., 2000).

Several energy-related proteins are covalently modified by HNE in EAD. α-Enolase is a glycolytic enzyme responsible for the hydrolysis of 2-phosphoglycerate to phosphoenolpyruvate in the penultimate step of glycolysis, thus affecting ATP production. Glucose is the primary source of energy for the brain even though the brain encompasses a diminutive amount of the body mass but accounts for 20% of glucose metabolism. Glucose metabolism is required for proper brain function. Nominal interruption of glucose metabolism causes brain dysfunction and memory loss (Hoyer, 1996; Meier-Ruge et al., 1994). Energy metabolism is altered and this can contribute to neurodegeneration. Alpha enolase in brain is modified by HNE in late-stage AD (Perluigi et al., in press) and is

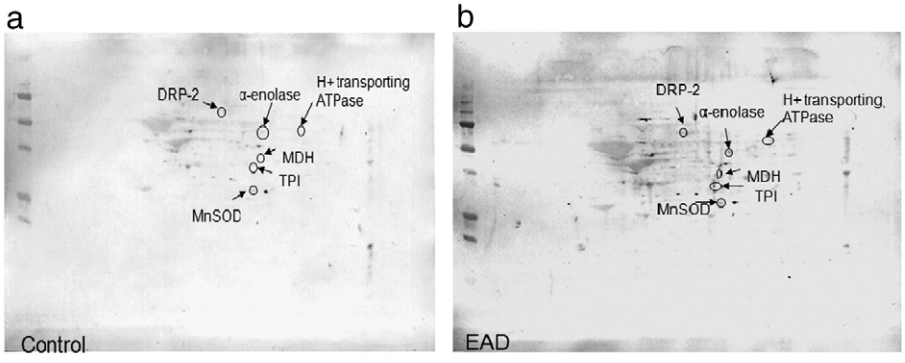

Fig. 3 – HNE immunoreactivity in representative 2D immunoblots for age-matched control (a) and EAD-IPL (b). 2D Western blots of brain proteins from EAD and control subjects that were oxidatively modified by HNE were obtained as described in Experimental procedures. Six proteins from IPL in EAD were found to have an increase in the level of protein-bound HNE compared to aged-matched controls.

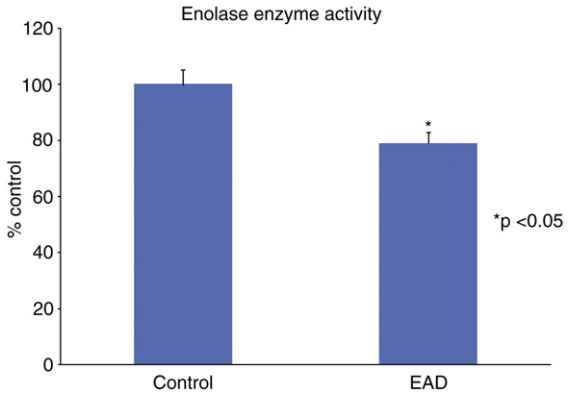

Fig. 4 – Enolase activity in EAD-IPL versus aged-matched control. The enolase activity assay was performed as described in Experimental procedures. Enzymatic activity is significantly reduced in EAD-IPL compared to age-matched controls ($p<0.05$). Bars represent mean ± S.E.M; $n=4$ for each group.

a common target for HNE adduction, as this protein is oxidatively modified in other neurodegenerative diseases such as PD (Poon et al., 2005) and HD (Perluigi et al., 2005b). α-enolase is one of only a small number of oxidatively modified proteins found to overlap all three stages of AD (Butterfield et al., 2006b; Castegna et al., 2002, 2003). This result supports the notion that energy metabolism is a critical component in the progression of AD pathogenesis from MCI to AD.

Triosephosphate isomerase (TPI) catalyzes the isomerization of dihydroxyacetonephosphate (DHAP) to glyceraldehyde-3-phosphate (G3P) in glycolysis. Transformation of DHAP to G3P is imperative to continue glycolysis and generate ATP. ATP is essential in maintaining ATPases, ion motive pumps and potential gradients. In AD brain, TPI is oxidatively modified as shown by Castegna et al. (2003) and Sultana et al. (2006c). In the senescence accelerated, SAMP8 mouse model of Abeta deposition, TPI undergoes excessive carbonylation (Poon et al., 2004). In triosephosphate isomerase deficient persons, low TPI activity causes increased glycolytic kinase activity, therefore increasing another post-translational modification, phosphorylation (Olah et al., 2005). Although this enzyme is oxidatively modified its activity is not diminished in AD patients (Meier-Ruge et al., 1984). Oxidized TPI levels are increased in neurodegenerative models (Chen et al., 2006b; Poon et al., 2004).

Malate dehydrogenase (MDH) catalyzes the reversible oxidation of malate to oxaloacetate by NAD^+ in the TCA cycle. MDH links glycolysis to the ETC by transferring NADH to complex I through the malate–aspartate shuttle thus stimulating ATP production. This enzyme has increased nitration in the hippocampus of MCI subjects (Sultana et al., 2007b), but reduced nitration in caloric-restricted rat hippocampus from aged rodents (Poon et al., 2006). Activity of MDH increases during aging (Bubber et al., 2005; Op den Velde and Stam, 1976), which can further bolster the hypothesis of mitochondrial dysfunction in AD. Our finding of elevated MDH activity in brain of EAD subjects is consistent with elevated MDH activity in late-stage AD (Korolainen et al., 2006). We speculate that HNE modification of brain MDH in aging and EAD leads to conformational changes of the MDH active site, since elevated activity occurs.

Several key proteins found to be excessively bound by 4-hydroxynonenal are modified in MCI and AD. In brain of subjects with EAD, six proteins were identified as being HNE-modified. Their functions include antioxidant defense, neuronal communication, and energy metabolism. SOD2 is a normal antioxidant that reduces toxic reactive oxygen species to hydrogen peroxide and water. Loss of SOD2 function causes oxidative balance to favor prooxidants and increase the level of oxidative stress. Impairment of DRP-2 can trigger shortened dendritic length, leading to decreased neuronal communication. Maintaining neuronal communication is vital in preserving cognitive function as memory loss is a criterion for amnestic MCI and AD. In EAD, glycolytic enzymes showed reduced activity, which supports previous research involving enzyme activity in MCI and AD (Meier-Ruge et al., 1984). Lowered activity reflects protein dysfunction. Loss of protein functionality in these oxidatively modified proteins results in ATP depletion which is detrimental to the cell. ATP is necessary for operating pumps, pH regulation, and maintaining cell potential gradients, and lipid asymmetry. Altered energy metabolism appears to be a recurrent theme in neurodegenerative disease as it is present in AD. The results of this study have identified potential therapeutic targets that conceivably could be important in slowing progression of AD.

4. Experimental procedures

Having brain from EAD subjects is a rare opportunity, since typically AD patients live about 8 years following diagnosis. Accordingly, we have only 4 different specimens due to sample availability. The normal control subjects in this study were two females and two males, whose average age at death was 79 ± 2.4 years. The EAD patients were three females and one male, whose average age at death was 86 ± 4.0 years (Table 2). All subjects came from the UK Alzheimer's Disease Clinical

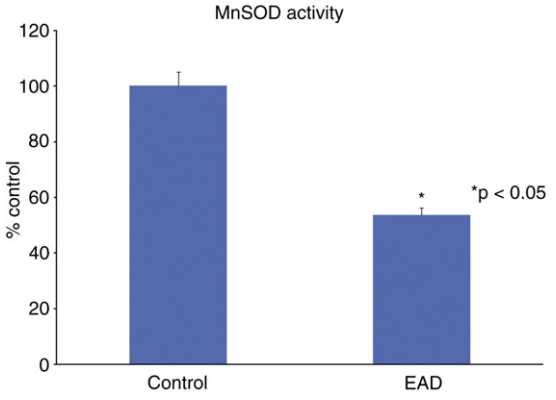

Fig. 5 – Activity of MnSOD in EAD-IPL versus aged-matched control. The SOD activity assay was performed as described in Experimental procedures. Superoxide dismutase activity is notably decreased in EAD samples compared to age-matched controls ($p<0.05$). Bars represent mean ± S.E.M; $n=4$ for each group.

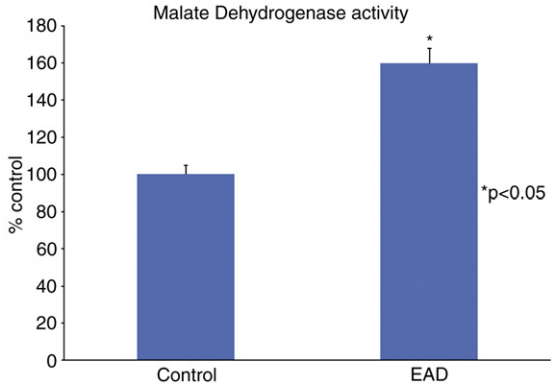

Fig. 6 – Malate dehydrogenase activity in EAD-IPL compared to age-matched control. The MDH activity assay was performed as described in Experimental procedures. Enzymatic activity is considerably reduced in EAD samples compared to age-matched controls ($p<0.05$). Bars represent mean ± S.E.M; $n=4$ for each group.

Center's longitudinally followed normal control group. Each subject had neuropsychological testing and neurological and physical examinations every two years. Control subjects had no cognitive complaints, normal cognitive test scores, normal objective memory test scores, and normal neurological examinations. EAD patients met the criteria set by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association. These criteria include: progressive memory loss, two or more cognitive deficits, altered activities of daily living, onset of disease between age 40 and 90, Clinical Dementia Rating score of 0.5 to 1.0 (mild dementia), and a clinical evaluation. As also indicated in Table 2, the postmortem interval (PMI) prior to the acquisition of brain samples was approximately 2.9 h for control and 2.4 h for EAD patients. This short PMI is an added advantage in studies of human brain. Four EAD samples and their controls were provided by the Rapid Autopsy Program of the University of Kentucky Alzheimer's Disease Clinical Center (UK ADC). Demographic data for all subjects are shown in Table 2.

4.1. *Chemicals*

All chemicals were of the highest purity and most were obtained from Sigma-Aldrich (St. Louis, MO, USA). The rabbit anti-HNE antibody was purchased from Alpha Diagnostics (San Antonio, TX, USA).

4.2. *Sample preparation*

Brain samples were minced and suspended in 10 mM HEPES buffer (pH 7.4) containing 137 mM NaC1, 4.6 mM KC1, 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 protein assay (Pierce, Rockford, IL, USA).

Protein samples (200 μg) were precipitated by addition of ice-cold 100% trichloroacetic acid (TCA) to a final concentration of 15% for 10 min on ice. Precipitates were centrifuged for 2 min at 14,000 ×*g* at 4 °C. The pellet was retained and washed three times with 1 m1 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 three times for 15s intervals.

4.3. *Measurement of protein-bound 4-hydroxynoneal (HNE)*

Levels of protein-bound HNE were determined immunochemically (Sultana et al., 2006c). Samples (5μl) were incubated with an equal volume of modified Laemmli buffer containing 0.125 M Tris base pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol. The resulting sample (250 ng) was loaded per well in the slot blot apparatus. Samples were loaded onto a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in wash blot buffer, consisting of 8.8 M NaCl, 3 mM NaH_2PO_4, 17 mM $NaHPO_4$, and 0.04% Tween-20 (v/v), for 2 h and incubated with a 1:5000 dilution of anti-HNE polyclonal antibody (Alpha Diagnostics, San Antonio, TX, USA) in wash blot buffer for 2 h. Following completion of the primary antibody incubation, the membranes were washed three times in wash blot buffer for 5 min each. An anti-rabbit IgG alkaline phosphatase secondary antibody (Sigma, St. Louis, MO, USA) was diluted (1:3000) in wash blot buffer and added to the membrane for 1 h. The membrane was washed in wash blot buffer three times for 5 min and developed using Sigmafast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets (Sigma, St. Louis, MO, USA). Blots were dried, scanned with Adobe Photoshop (San Jose, CA, USA), and quantitated with Scion Image.

4.4. *Two-dimensional gel electrophoresis and Western blotting*

Two-dimensional polyacrylamide gel electrophoresis was performed with a Bio-Rad IEF Cell system using 110-mm pH 3–10 immobilized pH gradients (IPG) strips and Criterion 8–16% resolving gels. IPG strips were actively rehydrated at 50 V 20 °C followed by isoelectric focusing: 300 V for 2 h linear gradient,

Table 2 – Profile of EAD subjects.

	Age (y)	Sex	Brain weight (g)	PMI (h)	Braak score
Control 1	79	Male	1300	2.25	II
Control 2	75	Female	1080	3.50	I
Control 3	86	Female	1310	2.25	II
Control 4	77	Male	1310	3.50	I
Average	79±2.4		1250±57	2.9±0.4	1.5
EAD 1	83	Female	1160	2.25	V
EAD 2	96	Female	1180	1.60	V
EAD 3	88	Male	1340	2.75	V
EAD 4	77	Female	1190	3.00	V
Average	86±4.0		1220±41	2.4±0.3	5.0

1200 V for 4 h slow gradient, 8000 V for 8 h linear gradient, and 8000 for 10 h rapid gradient. Gel strips were equilibrated for 10 min prior to second-dimension separation in solution A [0.375 M Tris–HCl (pH 8.8), 6 M urea (Bio-Rad, Hercules, CA), 2% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, and 0.5% dithiothreitol (Bio-Rad, Hercules, CA)], and then re-equilibrated for 10 min in solution A containing 4.5% iodoacetamide (IA) instead of dithiothreitol. Control and EAD strips were placed on the 8–16% Criterion gels, unstained molecular standards were applied, and electrophoresis was performed at 200 V for 65 min.

4.5. *SYPRO Ruby staining*

Gels 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, Hercules, CA, USA). Gels were then destained with 50 ml deionized water overnight.

4.6. *HNE immunochemical detection*

Immunoblotting with HNE antibody allows specific detection of HNE adducts in the IPL region of EAD subjects compared with age-matched controls. We measured the specific HNE-bound levels by dividing the HNE level of a protein spot on the membrane by the protein level of its corresponding protein spot on the gel. Western blot and subsequent immunochemical detection of HNE allowed identification of HNE-modified proteins in the IPL of EAD subjects compared with age-matched controls. The same amount of protein sample (200 μg) was used for 2D-immunoblotting analysis, and the electrophoresis was carried out as described above. The proteins from the 2D electrophoresis gels were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) using a Transblot-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA) at 15 V for 2 h. HNE-protein adducts were detected on the nitrocellulose membrane using a primary anti-HNE rabbit antibody (Alpha Diagnostics, San Antonio, TX, USA) specific for HNE-bound protein (1:5000) for 2 h at room temperature while rocking, followed by a secondary goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) antibody (1:3000) diluted in wash blot buffer for 1 h at RT. The resultant membrane was developed using 5-bromo-4-chloro-3indolyl phosphate/nitro-blue tetrazolium (BCIP/NBT) solution (SigmaFast tablets; Sigma, St. Louis, MO, USA).

4.7. *In-gel digestion*

Samples were prepared according to the method described by Thongboonkerd et al. (2002). Briefly, the protein spots were cut and removed from the gel with a clean razor blade. The gel pieces were placed into individual, clean 1.5 ml microcentrifuge tubes and kept overnight at −20 °C. The gel pieces were thawed and washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) (Sigma, St. Louis, MO, USA) for 15 min at room temperature. Acetonitrile (Sigma, St. Louis, MO, USA) was added to the gel pieces and incubated for an additional 15 min. The liquid was removed and the gel pieces were allowed to dry. The gel pieces were rehydrated with 20 mM DTT (Bio-Rad, Hercules, CA, USA) in 0.1 M NH_4HCO_3 (Sigma, St. Louis, MO, USA) and incubated for 45 min at 56 °C. The DTT was removed and replaced with 55 mM IA (Bio-Rad, Hercules, CA, USA) in 0.1 M NH_4HCO_3 for 30 min in the dark at room temperature. The liquid was drawn off and the gel pieces were incubated with 50 mM NH_4HCO_3 at room temperature for 15 min. Acetonitrile was added to the gel pieces for 15 min at room temperature. All solvents were removed and the gel pieces were allowed to dry for 30 min. The gel pieces were rehydrated with addition of a minimal volume of 20 ng/μl modified trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3. The gel pieces were chopped and incubated with shaking overnight (~18 h) at 37 °C.

4.8. *Analysis of gel images*

The immunoreactivity of the oxyblot was normalized to the actual protein content as measured by the intensity of a SYPRO Ruby protein stain (Bio-Rad, Hercules, CA, USA). Images from SYPRO Ruby stained gels, used to measure protein content, were obtained using a UV transilluminator (excitation=470 nm, emission=618 nm, Molecular Dynamics, Sunnyvale, CA, USA). Western blots were scanned with Adobe Photoshop on a Microtek Scanmaker 4900. PDQuest 2-D Analysis Software (Bio-Rad, Hercules, CA, USA) was used to match and analyze visualized protein spots among differential gels and membranes in order to compare protein and HNE immunoreactivity content between EAD-IPL samples and controls. Powerful automatching algorithms quickly and accurately match gels or blots and sophisticated statistical analysis tools allow spots of interest to be identified. The principles of measuring intensity values by 2D analysis software were similar to those of densitometric measurement. The average mode of background subtraction was used to normalize intensity values, which represent the amount of protein (total protein on gel and HNE-bound protein on the membrane) per spot. After completion of spot matching, the normalized intensity of each protein spot from individual gels (or membranes) was compared between groups using statistical analysis.

4.9. *Mass spectrometry*

All mass spectra reported in this study were acquired at the Department of Pharmacology in the University of Louisville Mass Spectrometry Facility (ULMSF) and VAMC. A Bruker Autoflex MALDI-TOF (matrix assisted laser desorption ionization-time of flight) mass spectrometer (Bruker Daltonic, Billerica, MA, USA) operated in the reflectron mode was used to generate peptide mass fingerprints. Peptides resulting from in-gel digestion with trypsin were analyzed on a 384 position, 600 μm AnchorChip™ Target (Bruker Daltonics, Breman, Germany) and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2, Bruker Daltonics, Breman, Germany). One μl of digestate was mixed with 1 μl of alpha-cyano-4-hydroxycinnamic acid (0.3 mg/mL in ethanol: acetone, 2:1 ratio) directly on the target and allowed to dry at room temperature. The sample spot was washed with 1 μl of a 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 was used for acquisition and internal calibration using either trypsin autolysis ions or matrix clusters and was applied post acquisition for accurate mass determination.

4.10. *Analysis of protein sequences*

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 (Butterfield et al., 2003; Castegna et al., 2002). Database searches allow up to 1 missed trypsin cleavage. 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 protein identification is not correct. MOWSE scores greater than 68 were considered to be significant ($p<0.05$). All the protein identifications were in the expected size range based on their position in the gel. An example of a typical mass spectrum for DRP-2 is given in Fig. 7.

4.11. *Enzyme assays*

4.11.1. *α-enolase enzyme assay*

Enolase activity was measured by measuring the conversion of 2-phosphoglycerate to phosphoenolpyruvate spectrophotometrically at 340 nm. The reaction mixture (0.1 ml final volume) contained: 2 mM Na_2HPO_4, pH 7.4 (86.7 μl), 400 mM EDTA (5 μl), 400 mM KCl (5 μl), 2 mM 2-phosphoglycerate (3.3 μl) and 5 μl of protein sample. The assay was carried out at 25 °C in a microtiter plate reader (Bio-Tek Instrument Inc., Winooski, Vermont).

4.11.2. *MnSOD enzyme assay*

Superoxide dismutase activity was measured spectrophotometrically at 550 nm as the inhibition of cytochrome *c* by superoxide radical. Superoxide was produced by converting xanthine to uric acid via xanthine oxidase. A reaction "cocktail" consisting of 216 mM potassium phosphate buffer, pH 7.8 (48 μl), 10.7 mM EDTA (50 μl), 1.1 mM cytochrome *c* solution (2 μl), 0.108 mM xanthine solution (100μl) and 5 μl protein sample. To this cocktail, xanthine oxidase enzyme (0.05 U/ml) was added. The reaction (final volume of 0.2 mL) was monitored at 25 °C.

4.11.3. *Malate dehydrogenase enzyme assay*

Malate dehydrogenase activity was measured spectrophotometrically at 340 nm by following the rate of the oxidation of NADH in the presence of oxaloacetate. The reaction mixture

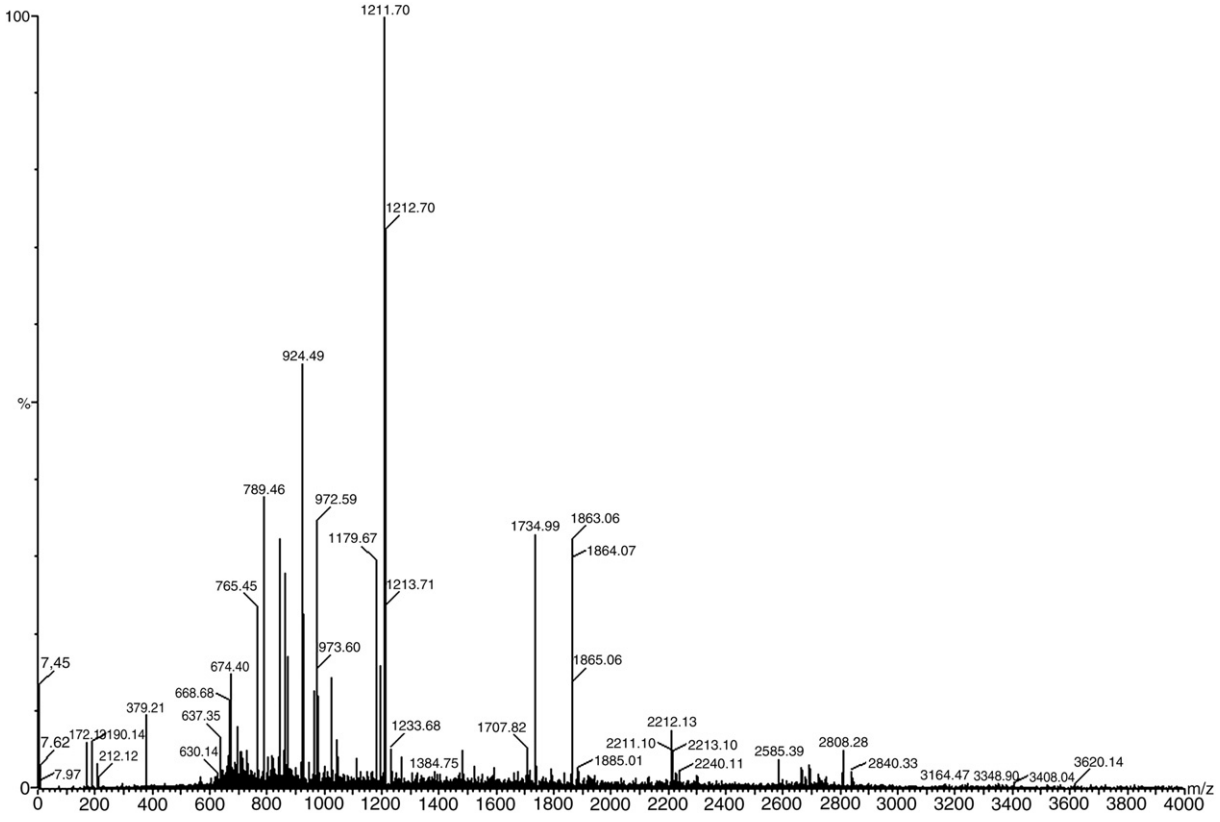

Fig. 7 – Typical MALDI mass spectrum for dihydropyrimidinase-related protein 2. MALDI-TOF mass spectrometry was performed as described in Experimental procedures.

(0.2 ml final volume) contained: 2 mM oxaloacetic acid in 100 mM potassium phosphate buffer, pH 7.5 (13.3 μl), 0.14 mM NADH (186.7 μl), and 5 μl of protein sample. The assay was carried out at 25 °C in a microtiter plate reader (Bio-Tek Instrument Inc., Winooski, Vermont).

4.12. *Statistical analysis*

The data of protein levels and protein specific HNE levels were analyzed by Student's *t*-test. Statistical comparison of HNE levels of proteins, matched with anti-HNE positive spots on Western blots from EAD subjects and their age-matched controls, was performed using Student's *t*-tests. A value of $p<0.05$ was considered statistically significant. For this analysis, the HNE level of each spot was normalized to the protein level of the corresponding spot on the gel. According to Maurer et al. (2005), generalized statistical tests are not applicable to proteomics data with small numbers of proteins identified, in contrast to microarray data with the large quantities of genes that are analyzed. Consequently, as recommended (Maurer et al., 2005), Student's *t*-test was used for analyses in the current study.

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