Mild cognitive impairment (MCI), considered to be the earliest detectable form of Alzheimer's disease (AD), converts to AD at the annual rate of 10–15% [1]. Post-translational modification of brain proteins, specifically nitration, is reported in both MCI and late-stage AD [2–4]. Protein nitration is an important oxidative modification in neurodegenerative disease [2, 3, 5–7]. Nitric oxide, produced by the conversion of L-arginine to L-citrulline via nitric oxide synthase, can react with superoxide anion to form the powerful oxidant, peroxynitrite (ONOO⁻). Peroxynitrite can then react with carbon dioxide (CO₂) to form the intermediate nitrosoperoxycarbonate, which is spontaneously rearranged and homolytically cleaved to form carbonate (CO₃²⁻) and nitrite radical (NO₂⁻). NO₂⁻ can react with a tyrosine residue to form 3-nitrotyrosine (3-NT). Upon nitration, protein inactivation occurs in several key proteins including glyceraldehyde 3-phosphate dehydrogenase, tyrosine hydroxylase, heme oxygenase, manganese superoxide dismutase and glutamine synthetase. Proteins are significantly nitrated in MCI and late-stage AD, making this study the first to our knowledge to link nitrated proteins in all stages of AD. These results are discussed in terms of potential involvement in the progression of this dementing disorder.

**Keywords:** protein nitration • early Alzheimer's disease • oxidative stress • proteomics

**Abstract**

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive decline in multiple cognitive domains. Its pathological hallmarks include senile plaques and neurofibrillary tangles. Mild cognitive impairment (MCI) is the earliest detectable stage of AD with limited symptomology and no dementia. The yearly conversion rate of patients from MCI to AD is 10–15%, although conversion back to normal is possible in a small percentage. Early diagnosis of AD is important in an attempt to intervene or slow the advancement of the disease. Early AD (EAD) is a stage following MCI and characterized by full-blown dementia; however, information involving EAD is limited. Oxidative stress is well-established in MCI and AD, including protein oxidation. Protein nitration also is an important oxidative modification observed in MCI and AD, and proteomic analysis from our laboratory identified nitrated proteins in both MCI and AD. Therefore, in the current study, a proteomics approach was used to identify nitrated brain proteins in the inferior parietal lobule from four subjects with EAD. Eight proteins were found to be significantly nitrated in EAD: peroxiredoxin 2, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, neuropolypeptide h3, phosphoglycerate mutase1, H⁺-transporting ATPase, α-enolase and fructose-1,6-bisphosphate aldolase. Many of these proteins are also nitrated in MCI and late-stage AD, making this study the first to our knowledge to link nitrated proteins in all stages of AD. These results are discussed in terms of potential involvement in the progression of this dementing disorder.

**Introduction**

Mild cognitive impairment (MCI), considered to be the earliest detectable form of Alzheimer’s disease (AD), converts to AD at the annual rate of 10–15% [1]. Post-translational modification of brain proteins, specifically nitration, is reported in both MCI and late-stage AD [2–4]. Protein nitration is an important oxidative modification in neurodegenerative disease [2, 3, 5–7]. Nitric oxide, produced by the conversion of L-arginine to L-citrulline via nitric oxide synthase, can react with superoxide anion to form the powerful oxidant, peroxynitrite (ONOO⁻). Peroxynitrite can then react with carbon dioxide (CO₂) to form the intermediate nitrosoperoxycarbonate, which is spontaneously rearranged and homolytically cleaved to form carbonate (CO₃²⁻) and nitrite radical (NO₂⁻). NO₂⁻ can react with a tyrosine residue to form 3-nitrotyrosine (3-NT). Upon nitration, protein inactivation occurs in several key proteins including glyceraldehyde 3-phosphate dehydrogenase, tyrosine hydroxylase, heme oxygenase, manganese superoxide dismutase and glutamine synthetase. Proteins are significantly nitrated in MCI and AD [2, 3, 5–7] as well as other neurodegenerative diseases including Parkinson’s disease (PD) [5] and amyotrophic lateral sclerosis [10].

Early AD (EAD) is the next step beyond MCI and thus intermediate between MCI and more advanced AD. However, information involving this disease stage is limited. Nitrosative stress is well-documented in MCI and late AD as indexed by an increase in 3-NT [19, 20]. Increased protein nitration also can result in detrimental cellular effects [2, 3, 21]. In the current study, a proteomics
approach was used to identify nitrated proteins in EAD to determine if any proteins overlap with proteins already known to be nitrated in this early stage of the disease following MCI.

## Materials and methods

Inferior parietal lobule (IPL) samples from four EAD patients and four age-matched controls were provided by the Rapid Autopsy Program of the University of Kentucky Alzheimer’s Disease Center (UK ADC). The normal control subjects in this study were two women and two men, whose average age at death was 79 ± 2.4 years (Table 1). The EAD patients were three women and one man, whose average age at death was 86 ± 4.0 years (Table 1). All subjects underwent neuropsychological testing and neurological and physical examinations annually. All of the samples came from the UK ADC’s normal control group. Normal control subjects had no cognitive complaints, normal cognitive test scores and normal neurological examinations. EAD patients met the following criteria: progressive memory loss as reported by an informant, deficits in two or more cognitive domains, altered activities of daily living, clinical dementia rating score of 0.5 to 1.0 (mild dementia), a nonfocal neurological evaluation and no other cause for dementia [22, 23]. The post-mortem interval (PMI) at autopsy was 2.9 hrs for control and 2.4 hrs for EAD patients (Table 1). EAD patients met the National Institute on Aging/Regan Institute [24] intermediate or high likelihood criteria for the neuropathological diagnosis of AD. Controls met the low likelihood criteria for the neuropathological diagnosis of AD and had no other significant neuropathological alterations. Braak staging (score) characterizes severity of disease based on distribution pattern of neurofibrillary tangles and neurofibril threads [25]. There are six levels of Braak staging, whereby the higher the stage, the more severe the stage of AD. Although, ideally a greater number of subjects would be desirable, it is rare to obtain brain from EAD subjects because most patients live long enough to convert to late-stage AD. Therefore, only four independent EAD samples were available through the UK ADC Brain Bank.

### Table 1 Profile of EAD subjects

| Age (year) | Gender | Brain weight (g) | PMI (hrs) | 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|                 | 1217.5 ± 41 | 2.4 ± 0.3   | 5.0         |

### Sample preparation

Brain samples were minced and suspended in Media I buffer (10 mM HEPES buffer (pH 7.4), 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH₂PO₄, 0.1 mM EDTA, 0.6 mM MgSO₄, 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)). To remove debris, brain homogenates were centrifuged at 14,000 × g for 10 min. Protein concentration of the supernatant was determined by the BCA protein assay. 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 kept and washed with 1 ml of 1:1 (v/v) ethyl acetate/ethanol three times. The resultant 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 15 sec. intervals.

### Measurement of 3-nitrotyrosine

Levels of total 3-NT were determined immunochemically [3]. Samples (5 μl) were incubated with 5 μl Laemmli buffer without reducing agents (0.125 M Tris base pH 6.8, 4% (v/v) sodium dodecyl sulfate (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 for 2 hrs and incubated with a 1:2000 dilution of anti-3-NT polyclonal antibody (Sigma Aldrich, St. Louis, MO, USA) in wash blot for 2 hrs. Following completion of the primary antibody incubation, the membranes were washed three times in wash blot for 5 min. each. An anti-rabbit IgG alkaline phosphatase secondary antibody (Sigma Aldrich) was diluted 1:3000 in wash blot and added to the membrane for 1 hr. The membrane was washed in wash blot three times for 5 min. and developed using Sigmafast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets (Sigma). Blots were dried, scanned into TIFF files with Adobe Photoshop (San Jose, CA, USA), and
Two-dimensional gel electrophoresis

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 hrs linear gradient, 1200 V for 4 hrs slow gradient, 8000 V for 8 hrs linear gradient and 8000 V for 10 hrs rapid gradient. Gel strips were equilibrated for 10 min. prior to second-dimension separation in solution B (0.375M Tris-HCl, pH 8.8 containing 6M urea (Bio-Rad, Hercules, CA, USA), 2% (w/v) SDS, 20% (v/v) glycerol and 0.5% DTT (Bio-Rad), followed by re-equilibration for 10 min. in solution B (0.375M Tris-HCl pH 8.8 containing 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, and 4.5% iodoacetamide (IA) (Bio-Rad)). 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. 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). Gels were then destained with 50 ml deionized water overnight.

Immunological detection

For immunoblotting analysis, electrophoresis was performed as stated previously, and gels were transferred to a nitrocellulose membrane. The membranes were blocked with 3% BSA in phosphate-buffered saline containing 0.01% (v/v) sodium azide and 0.2% (v/v) Tween 20 (PBST) overnight at 4°C. The membranes were incubated with anti-nitrotyrosine polyclonal antibody (3-NT) (Sigma-Aldrich), diluted 1:100 in wash blot for 2 hrs at room temperature with rocking. Following completion of the primary antibody incubation, the membranes were washed three times in wash blot for 5 min. each. An anti-rabbit IgG alkaline phosphatase secondary antibody (Sigma) was diluted 1:3000 in wash blot and incubated with the membranes for 1 hr at room temperature. The membranes were washed in wash blot three times for 5 min. and developed using Sigmafast Tablets (BCIP/NBT substrate) (Sigma) to yield 2D Western blots.

Image analysis

Analysis of gel maps and membranes comparing protein levels and tyrosine nitration content between control and EAD IPL samples was performed with PDQuest image analysis software (Bio-Rad). The immunoreactivity of the Western blot was normalized to the actual protein content as measured by the intensity of a SYPRO Ruby (Bio-Rad) protein stain. Images from SYPRO Ruby-stained gels, used to measure protein content, were obtained performed with a UV transilluminator (λex = 470 nm, λem = 618 nm, Molecular Dynamics, Sunnyvale, CA, USA). Nitrocellulose membranes were scanned with Adobe Photoshop on a Microtek Scanmaker 4900.

In-gel trypsin digestion

Samples were prepared according to the method described by Thongboonkerd et al. [27]. 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 (NH4HCO3) (Sigma) for 15 min. at room temperature. Acetonitrile (Sigma) 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) in 0.1 M NH4HCO3 and incubated for 45 min. at 56°C. The DTT was removed and replaced with 55 mM IA (Bio-Rad) in 0.1 M NH4HCO3 for 30 min. in the dark at room temperature. The liquid was drawn off and the gel pieces were incubated with 50 mM NH4HCO3 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 NH4HCO3. The gel pieces were chopped and incubated with shaking overnight (~18 hrs) at 37°C.

Mass spectrometry

All mass spectra were obtained at the University of Louisville Mass Spectrometry Facility on a Bruker Autoflex MALDI TOF (matrix assisted laser desorption-time of flight) mass spectrometer (Bruker Daltonic, Billerica, MA, USA) operated in the reflectron mode to generate peptide mass fingerprints. Peptides resulting from in-gel digestion were analysed on a 384 position, 600 μm Anchor-ChipTM Target (Bruker Daltonics, Bremen, Germany) and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2, Bruker Daltonics). Briefly, 1 μl of trypsic digest was mixed with 1 ml 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 1% TFA solution for approximately 60 sec. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) performed with 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 performed with either trypsin autolysis ions or matrix clusters and was applied after acquisition for accurate mass determination.

Protein identification

Peptide mass fingerprinting was used to identify proteins from trypsic peptide fragments by utilizing the MASCOT search engine (http://www.matrixscience.com) based on the entire NCBI protein database. Database searches were conducted allowing for up to one missed trypsic cleavage and using the assumption that the peptides were monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Mass tolerance of 100 ppm/g was the window of error allowed for matching the peptide mass values. Statistical comparisons of 3-NT protein levels on 2D gels and corresponding matched anti-3NT positive spots on 2D-Western blots from EAD IPL samples and age-matched control samples were performed using Student’s t-test.
Enzyme assays (ATP synthase and glutamate dehydrogenase)

Mitochondrial ATP synthase activity was measured spectrophotometrically at 340 nm by coupling the production of ADP to the oxidation of NADPH via the pyruvate kinase and lactate dehydrogenase reaction (coupled assay) as described [28]. The reaction mixture (0.2 ml final volume) contained: 100 mM Tris (pH 8.0), 4 mM Mg-ATP (substrate), 2 mM MgCl₂, 50 mM KCl, 0.2 mM EDTA, 0.23 mM NADH, 1 mM phosphoenolpyruvate, 1.4 unit pyruvate kinase, 1.4 unit lactate dehydrogenase and about 25–50 μg proteins (brain homogenates), and was assayed at 30°C. The assay was carried out in a microtitre plate reader (Bio-Tek Instrument Inc., Winooski, VT, USA).

Glutamate dehydrogenase catalyses the reversible conversion of L-ketoglutarate to L-glutamate, and its activity was determined by spectrophotometric methods to measure the oxidation of NADH to NAD⁺ at 340 nm in the presence of the ammonium ion. The reaction mixture consists of 90 mM triethanolamine HCl, 13 mM L-ketoglutarate, 53 mM ammonium acetate, 0.06 mM β-NADH (substrate), 0.25 mM EDTA and 5 μl sample. The reaction was assayed at 25°C.

Statistical analysis

The data were analysed by Student’s t-tests. According to Maurer et al. [29], generalized statistical tests applicable to proteomics data are unavailable, in contrast to microarray data. Because of the small number of proteins identified in this study, compared to the large quantities of genes analysed by microarrays, a value of p < 0.05 was considered significant. Similarly, after protein identification using the NCBI database, a MOWSE score of over 61 was considered significant. Probability-based MOWSE scores were estimated by comparing search results against estimated random match population and were reported as -10 * Log₁₀ (P), where P is the probability that the identification of the protein is not correct. All protein identifications were in the expected size and pI range based on position in the gel.

Results

The total level of nitrated proteins in the IPL of EAD patients yielded a significant 18% increase compared with age-matched controls (Fig. 1). Using proteomics analysis, 8 proteins were significantly nitrated (p < 0.05) in EAD IPL according to PDQuest software analysis of 2D gels (Fig. 2A and B) and 2D Western blots (Fig. 3A and B) probed appropriately with anti-3-NT antibody. These excessively nitrated brain proteins compared with control IPL include peroxiredoxin 2 (Prx2), triose phosphate isomerase (TPI), glutamate dehydrogenase (GDH), neuropolypeptide h3, phosphoglycerate mutase 1 (PGM1), H⁺ transporting ATPase, alpha enolase, and fructose 1,6-bisphosphate aldolase (ALDO1). Table 2 provides the proteomics results from which these proteins were identified.

Because our earlier research demonstrated that oxidatively modified proteins generally are dysfunctional [19, 30–35], the activities of a subset of identified nitrated proteins for IPL of EAD subjects were determined. ATP synthase activity was measured in EAD IPL and was 30% lower in EAD IPL compared with age-matched controls (Fig. 4). ATP synthase also is lower in brains from subjects with MCI, and these results are consistent with the notion of loss of enzymatic activity of oxidatively modified proteins [30, 32]. Glutamate dehydrogenase activity also was reduced by 40% in EAD IPL compared with age-matched controls (Fig. 5).

Discussion

Several proteins were found to be significantly nitrated in EAD IPL. They are involved in antioxidant defence, energy metabolism, lipid
asymmetry, and ATP production. This discussion has been written by categorizing these oxidatively modified proteins by function. In this manner, it is easier to envisage how oxidative modification can greatly impair protein function and how this can correlate with AD pathology.

Antioxidant defence

Peroxiredoxins are peroxidases that serve in an antioxidant capacity by reducing hydrogen peroxide. There are six forms of peroxiredoxin. Prx 1, 2, 3, 4 and 5 use thioredoxin as an electron donor, whereas Prx-6 uses glutathione. Prx2 (or thio-specific antioxidant), only expressed in neurons, blocks apoptosis by preventing the opening of the mitochondrial permeability pore [37] and thereby promotes neuronal cell survival [38, 39]. Overexpression of Prx2 in cell lines ameliorates apoptotic effects induced by oxidants [40]. Prx2, which can be neuroprotective, is increased in PD [41] as well as AD and Down syndrome [42, 43]. Prx2 also reduces peroxynitrite to detoxify RNS [44]. Loss of protein function hampers peroxidase activity and would be predicted to yield an increase in cytotoxic RNS.

We hypothesize that these findings have relevance to the emerging concepts of redox regulation of cellular stress response (vitagenes) in response to oxidative and nitrosative stress [45, 46]. In particular, the family of peroxiredoxins, coupled to thiol homeostasis, could exert a neuroprotective role in age-related neurodegenerative disorders such as AD, and may be a promising, novel therapeutic target for this and other age-related neurodegenerative disorders.

Metabolic enzymes

As noted above, TPI is a glycolytic enzyme that catalyses production of glyceraldehyde-3-phosphate (G3P), which is required to continue glycolysis to produce ATP. In AD brain, TPI is oxidatively modified [2, 3], which can affect ATPases, ion motive pumps and potential gradients.

Alpha enolase hydrolyses 2-phosphoglycerate to phosphoenolpyruvate in glycolysis and impairment of this enzyme can greatly affect ATP production. Reduced enzyme activity of α-enolase has been previously established in brain from subjects with...
MCI [47] and late AD [2, 48]. Altered energy metabolism is a common motif in neurodegenerative diseases as α-enolase also is oxidatively modified in MCI [47], late AD [31, 49], PD [50], Huntington’s disease [33] and amyotrophic lateral sclerosis [51]. Notably, α-enolase is the only nitrated protein to overlap MCI, EAD and late AD [2, 31, 47]. This observation supports the notion that changes in energy metabolism need to be investigated further to gain more knowledge about AD pathogenesis and potentially offer targets for therapeutic intervention of AD.

PGM1 catalyses the interconversion of 3-phosphoglycerate to 2-phosphoglycerate, leading to a second equivalent of ATP produced in glycolysis. Enzyme activity is decreased in AD [52], which could result in protein dysfunction and increased glycolytic intermediates, reduced pyruvate production and decreased ATP production. PGM1 is oxidatively modified in rat brain treated with Aβ(1–42) and AD hippocampus. In the gracile axonal dystrophy mouse brain in which Aβ is deposited, PGM1 is significantly increased, which contributes to the notion of reduced energy metabolism in neurodegenerative diseases [53, 54].

ALDO1 cleaves fructose1,6-bisphosphate into dihydroxy-acetone phosphate (DHAP) and G3P in glycolysis. Levels of ALDO1 are significantly decreased in AD hippocampus [4] and this enzyme is oxidatively modified in the olfactory bulb of aged mice [55]. Getchell et al. reported increased 3-NT immunoreactivity in olfactory bulbs of late AD patients compared with age-matched controls [56]. A diminished sense of smell (olfaction) is observed in some AD patients, as in the case of aging and several age-related neurodegenerative diseases [57, 58]. ALDO1 and TPI deficiencies are associated with hemolytic anaemia resulting in mitochondrial myopathy [59–61]. Enzyme activity is reduced and impairment can cause increased levels of fructose 1,6-bisphosphate, inhibition of complete glycolysis, and ATP depletion.

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Fig. 3 Representative 2D Western blot of inferior parietal lobule probed with anti-3-nitrotyrosine for control (A) and early Alzheimer’s disease (EAD) (B).
Lipid asymmetry

Neuropolypeptide h3, also known as hippocampal cholinergic neurostimulating peptide, phosphatidylethanolamine binding protein (PEBP), or Raf kinase inhibitor protein, plays a role in cholinergic process by upregulating the enzyme, choline acetyltransferase. As a phosphatidylethanolamine binding protein, PEBP is important in phospholipid asymmetry. A signal for initiation of apoptosis is phosphatidylserine exposure on the outer leaflet of the membrane lipid bilayer. Neuropolypeptide h3 is nitrated in late AD [2] and modified by the lipid peroxidation product, HNE, in synaptosomes treated with Aβ(1–42) [62]. Loss of PEBP may affect lipid asymmetry as loss of activity is observed in AD [63].

ATP production

Glutamate dehydrogenase (GDH), located in the mitochondrial matrix, catalyses the reversible deamination of glutamate to

Table 2 Proteomic identification of nitrated proteins found in EAD IPL

| gi accessioning number | Name                                | Mowse score | pI    | Apparent molecular weight (Mr) | # peptides matched | Protein nitration (% control) | Probability |
|------------------------|-------------------------------------|-------------|-------|-------------------------------|--------------------|-------------------------------|-------------|
| gi|32189392 | Peroxiredoxin 2 (Prx2)               | 135         | 5.67  | 21,918                        | 11/34              | 187 ± 0.15                    | <0.05       |
| gi|4507645  | Triose phosphate isomerase (TPI)     | 74          | 6.51  | 26,807                        | 6/18               | 463 ± 63.5                    | <0.02       |
| gi|4885281  | Glutamate dehydrogenase (GDH)       | 112         | 7.66  | 61,701                        | 14/40              | 376 ± 29.1                    | <0.05       |
| gi|913519   | Neuropolypeptide h3                  | 104         | 7.42  | 21,027                        | 9/36               | 23.5 ± 0.04*                  | <0.02       |
| gi|4505753  | Phosphoglycerate mutase 1 (PGM)     | 91          | 6.75  | 28,769                        | 9/32               | 230 ± 4.20                    | <0.05       |
| gi|4502317  | H+ transporting ATPase              | 83          | 7.71  | 26,186                        | 7/13               | 84.8 ± 0.05*                  | <0.04       |
| gi|4503571  | α-Enolase                            | 191         | 6.99  | 47,350                        | 20/48              | 516 ± 25.1                    | <0.01       |
| gi|4930167  | Fructose 1,6-bisphosphate aldolase   | 80          | 8.39  | 39,720                        | 7/18               | 199 ± 21.5                    | <0.04       |

*With an n = 4, if one blot has a particularly less intense protein spot for a statistically significant protein spot, this can cause an overall lower level of protein nitration. The overall % protein nitration is based on the average of protein nitration levels of all statistically significant protein spots. Ideally, one should have a greater n to avoid this potential problem, but samples from EAD are extremely rare, so we are fortunate just to have n = 4.
α-ketoglutarate, ammonium and NAD^+. This α-ketoglutarate can be channeled into the Kreb’s cycle to stimulate ATP production. GDH is important in preventing accumulation of extracellular glutamate. Indeed, loss of function of GDH can cause excess glutamate, which in the brain leads to excitotoxicity [64]. NMDA receptors are stimulated by excess glutamate causing an increase in Ca^{2+} influx. Disruption of Ca^{2+} homeostasis can lead to changes in long-term potentiation and consequently, diminished learning and memory. Altered homeostasis of Ca^{2+} also leads to cytochrome c release, as well as activation of calpain, a calcium-sensitive protease. Such alterations would initiate neuronal death and may be important in AD [65–67].

As complex V in the mitochondrial electron transport chain, H^+ transporting two-sector ATPase (ATP synthase) catalyses ADP phosphorylation in ATP generation. Nitration of this protein can cause mitochondrial complex disturbance. As noted above, ATP synthase expression [68] and activity are decreased in AD [69–72]. H^+ transporting two-sector ATPase is oxidized in synaptosomes treated with the peptide Aβ(1–42) [62]. Reduction in enzyme activity in brain of subjects with EAD (Fig. 4), coupled with changes in the mitochondrial complex, can lead to electron leakage and reactive oxygen species production, potentiating oxidative stress in AD via mitochondrial dysfunction [73–75].

To our knowledge, this is the first report of elevated 3-NT in brain of subjects with EAD. In conclusion, the identified nitrated proteins in EAD IPL play major roles in antioxidant defence and energy metabolism directly or indirectly linked to AD pathology. Comparative analysis of nitrated proteins between MCI, EAD, and late AD showed α-enolase was the only protein nitrated in all three disease stages [2, 3, 31, 35, 47]. Aldolase, phosphoglycerate mutase 1, neuropolypeptide h3 and TPI are oxidatively modified in EAD and late AD brain [2, 31, 35, 47]. Our data suggest that protein nitration of these specific brain proteins could be a mechanism to trigger the further conversion of EAD to more advanced AD. A Venn diagram (Fig. 6) shows the relationship between nitrated proteins in MCI, EAD and late AD. Future studies using animal models of AD at different ages and different Aβ pathologies should help to further delineate mechanisms of AD pathogenesis and to perhaps lead to development of effective therapies to combat disease progression.

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