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Redox proteomics identification of oxidatively modified brain proteins in inherited Alzheimer's disease: An initial assessment

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Abstract. *Objective:* To identify oxidatively modified proteins in brains of persons with inherited Alzheimer's disease. *Methods*: Redox proteomics was used to identify oxidatively modified brain proteins in persons with mutations in the genes for presenilin-1 (PS-1). *Results*: An initial redox proteomics assessment of oxidatively modified proteins from brains of individuals with PS-1 mutations was performed. These PS1 mutations, Q222H and M233T, are completely penetrant causing early-onset familial AD as previously reported in these Australian families. We show that oxidative modifications of ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), γ-enolase, actin, and dimethylarginine dimethylaminohydrolase 1 (DMDMAH-1) are present in the brain of familial AD subjects. *Conclusions*: These initial results suggest that oxidatively modified proteins are important common features in both familial and sporadic AD.

Keywords: Redox proteomics, familial Alzheimer's disease, oxidatively modified brain proteins

1. Introduction

Proteomics leads to the identification of proteins [1, 2]. Previously we have used redox proteomics approaches [3], including 2-dimensional electrophoresis followed by 2-dimensional Oxyblots, to identify oxidized proteins in Alzheimer's disease (AD) [1,2, 4–10]. In other cases, immunochemical detection of oxidatively modified proteins in AD brain was achieved [11–13]. Oxidized proteins in AD brain included those related to energy metabolism, excitotoxicity, proteasomal-mediated recycling of damaged or aggregated proteins, glutathione metabolism and excretion from neurons of GSH conjugates of the damaging lipid peroxidation product, 4-hydroxy-2-nonenal (HNE), phospholipid asymmetry, cholinergic processes, phosphorylation of tau protein, maintenance of neuronal pH, and dendritic elongation. Each of these

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functions are known to be altered in AD brain [11–16]. In essentially all cases examined thus far, oxidative modification of proteins leads to diminution, if not loss, of their function [11–14,17–20]. Thus, prior redox proteomic studies of oxidatively modified proteins in AD brain suggest that the oxidation and subsequent loss of activity of these proteins contributes to the biochemical and/or pathological alterations in pathways with which the various proteins are normally associated.

Increased insight into whether particular oxidatively modified proteins are fundamental to the processes involved in AD or a downstream consequence of the disease may possibly be gleaned by a similar redox proteomics analysis of inherited AD brain. The latter has the advantage over sporadic AD brain in that there is a single identified cause of the disease, which results in an early onset disease manifestation, thereby excluding the complication of age-associated processes.

Familial AD, largely the result of mutations in the genes for presenilin-1 (PS-1), presenilin-2 (PS-2), or amyloid precursor protein (APP) [21], is rare, accounting for only approximately 5% of AD cases. Accordingly, in the current study we carried out an initial assessment of oxidatively modified proteins from brains of individuals with known PS-1 mutations, Q222H and M233T, which are highly aggressive and fully penetrant. These brains have been carefully evaluated previously for the PS-1 mutations present [Q222H and M233T] [22]. We report that oxidatively modified proteins are central to both familial and sporadic AD.

2. Materials and methods

2.1. Brain samples

The brain tissue samples investigated in the current study were obtained from two male individuals belonging to West Australian pedigrees with familial AD caused by mutations in PS-1. In both cases, informed consent from legally responsible family members was obtained by Prof. Martins and approved by the Hollywood Private Hospital Institutional Review Board. One case, with PS-1 mutation M233T (case 1), died at the age of 43 years and was diagnosed with clinical AD five years prior to death, while the other individual (case 2), with the PS-1 mutation Q222H suffered from dementia for seven years until passing away at the age of 53. The post-mortem intervals for recovery of brain tissue were 42.5 and 19.5 hours for cases 1 and 2, respectively, and neuropathological examination confirmed AD in addition to congophilic angiopathy in both cases. Case 2 also had evidence of early Parkinson's disease (Braak stage 3) with mild depigmentation and α-synuclein positive pale body formation in the substantia nigra and rare cortical Lewy bodies and Lewy neurites in the cingulate, parahippocampal and hippocampal CA2/3 cortices.

2.2. Sample preparation

Frozen samples from frontal cortex were homogenized in lysis buffer (pH 8.8, 10 mM HEPES, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$ and 0.5 mg/mL leupeptin, 0.7μg/mL pepstatin, 0.5 μg/mL trypsin inhibitor, and 40 μg/mL PMSF) (Sigma, St. Louis, MO). Homogenates were centrifuged at 15,800 g for 10 min to remove debris. The supernatant was extracted to determine the protein concentration by the BCA method (Pierce, IL).

2.3. Two-dimensional electrophoresis

Samples of brain proteins were prepared according to the procedure of Levine et al. [23]. One volume (200 μg of brain protein) was incubated with 4 volumes of 2 N HCl at room temperature (25°C) for 20 min. Proteins were then precipitated by addition of ice-cold 100% trichloroacetic acid (TCA). Precipitates were centrifuged at 15,800 g for 2 min. This process removed ions that affect the voltage during the isoelectric focusing. The pellets were washed with 1 mL of 1:1 (v/v) ethanol/ethyl acetate solution. After centrifugation and washing with ethanol/ethyl acetate solution three times, the samples were dissolved in 25 μL of 8 M urea (Bio-Rad, CA). The samples were then mixed with 185 μL of rehydration buffer (8 M urea, 20 mM dithiothreitol, 2.0% (w/v) CHAPS, 0.2% Biolytes, 2 M thiourea and bromophenol blue). In first-dimension electrophoresis, 200 μL of sample solution were applied to a ReadyStrip^{TM} IPG strip (Bio-Rad, CA). The strips were soaked in the sample solution for 1 hour to ensure uptake of the proteins. The strips were then actively rehydrated in the protean IEF cell (Bio-Rad, CA) for 16 hours at 50 V. The isoelectric focusing was performed at 300V for 2 h linearly; 500V for 2 h linearly; 1000V for 2 h linearly, 8000V for 8 hr linearly and 8000V for 10 h rapidly. All the processes above were carried out at 22°C. The strip was stored at −80°C until the second dimension electrophoresis was performed.

For the second dimension, IPG® Strips, pH 3–10, were equilibrated for 10 min in 50 mM Tris-HCl (pH

6.8) containing 6M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodacetamide in place of dithiothreitol. 8–16% linear gradient precast criterion Tris-HCl gels (Bio-Rad, CA) were used to perform second dimension electrophoresis. Precision Protein^{TM} Standards (Bio-Rad, CA) were run along with the sample at 200V for 65 min.

After second dimension electrophoresis, gels were incubated in fixing solution (7% acetic acid, 40% methanol) for 20 min. Approximately, 60 mL of Coomassie blue (Bio-Safe) were used to stain the gel for 2 h. The gels were destained in deionized water overnight.

2.4. Oxyblots

Proteins (200 μg) were incubated with 4 volumes of 20 mM 2,4-dinitrophenyl hydrazine (DNPH) at room temperature (25°C) for 20 min. The gels were prepared in the same manner as for 2D-electrophoresis. After the second dimension, the proteins from gels were transferred to nitrocellulose membranes (Bio-Rad, CA) using the Transblot-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, CA) at 15V for 4 h. The 2,4-dinitrophenylhydrazone (DNP) adducts of the carbonyls of the oxidized proteins were detected on the nitrocellulose paper using a primary rabbit antibody (Chemicon, CA) specific for DNP-protein adducts (1:100) applied for 1.5 h, and then a secondary goat anti-rabbit IgG (Sigma) antibody (1:4000) was applied for 1 h. The resultant stain was developed using Sigma-Fast (BCIP/NBT) tablets.

2.5. Image analysis

The gels and nitrocellulose membranes were scanned and saved in TIFF format using Scanjet 3300C (Hewlett Packard, CA). Investigator HT analyzer (Genomic Solutions Inc., MI) was used for matching and analysis of visualized protein spots among differential gels and oxyblots. The principles of measuring intensity values by 2-D analysis software were similar to those of densitometric measurement. Average mode of background subtraction was used to normalize intensity value, which represents the amount of protein (total protein on gel and oxidized protein on oxyblot) per spot.

2.6. Trypsin digestion

Samples were prepared using techniques described by Jensen et al. [24], modified by Thongboonkerd et al. [25]. The protein spots were excised with a clean blade and transferred into clean microcentrifuge tubes. The protein spots were then washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) at room temperature for 15 min. Acetonitrile was added to the gel pieces and incubated at room temperature for 15 min. The solvent was removed, and the gel pieces were dried in a flow hood. The protein spots were incubated with 20 μl of 20 mM DTT in 0.1 M NH_4HCO_3 at 56°C for 45 min. The DTT solution was then removed and replaced with 20 μl of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The solution was incubated at room temperature in the dark for 30 min. The iodoacetamide was removed and replaced with 0.2 ml of 50 mM NH_4HCO_3 and incubated at room temperature for 15 min. 200 μL of acetonitrile was added. After 15 min incubation, the solvent was removed, and the gel spots were dried in a flow hood for 30 min. The gel pieces were rehydrated with 20 ng/μl modified trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3 with the minimal volume to cover the gel pieces. The gel pieces were chopped into smaller pieces and incubated at 37°C overnight in a shaking incubator.

2.7. Mass spectrometry

A TOF Spec 2E (Micromass, UK) MALDI-TOF mass spectrometer operated in the reflectron mode was used to generate peptide mass fingerprints. Briefly, 1 μL of digestate was mixed with 1 μL of alpha-cyano-4-hydoxycinnamic 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 seconds. 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 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

Table 1
Summary of FAD PS-1 brain proteins identified by redox proteomics

Most Likely Candidate of the Protein Spots Indicated in Fig. 1C	Score	pI	kDa	gi Number
$\gamma-$Enolase	247	4.91	47.58	5803011
Actin	135	5.55	40.54	15277503
Dimethylarginine dimethylaminohydrolase 1	125	5.53	31.44	6912328
Ubiquitin carboxyl-terminal hydrolase L1	42	5.53	23.35	4185720

Table 2
Summary of Oxidatively Modified Brain Proteins Identified by Redox Proteomics in Subjects with Alzheimer's Disease from the Butterfield Laboratory

Energy-related enzymes	CK, Enolase, TPI, PGM1, GAPDH, LDH
Excitatory Neurotrasmitter-related proteins	EATT2*, GS
Proteasome-related proteins	UCH L-1, HSPs
Cholinergic system	Neuropolypeptide h3
pH regulation protein	CA2 II
Synaptic abnormalities and LTP	Gamma-SNAP
Mitochondrial abnormalities	ATP synthase alpha chain, VDAC-1
Structural proteins	DRP2, β-actin
Regulation of Cell cycle entry, tau dephosphorylation, and Aβ production	PIN1
HNE handling proteins	GST*, MRP1*

*Oxidatively modified protein detected using immunochemistry.

fingerprinting used the assumption that peptides are monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues [1,5,8,19,26]. 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.

3. Results

Following these procedures that have been employed previously for the identification of oxidatively modified brain proteins in sporadic AD [1,2,4–10], four proteins were identified as carbonylated in the familial AD cases with PS-1 mutations (Fig. 1 and Table 1). These proteins were identified as γ-enolase, actin, and dimethylarginine dimethylaminohydrolase 1 [DMDMAH-1], and ubiquitin carboxyl-terminal hydrolase L1 [UCH-L1].

4. Discussion

Mutations in PS-1 induce neuronal calcium- dyshom eostasis, promote elevated production of amyloid-β-peptide (Aβ), and lead to increased vulnerability to oxidative stress, synaptic dysfunction, excitotoxicity, and apoptosis [27,28]. Elevated protein oxidation and lipid peroxidation are found in brain from mice with PS-1 mutations [29,30]. Mutant PS-1 leads to increased sensitivity of glia to NO-mediated inflammatory processes [31]. As seen in our familial AD cases with PS-1 mutations and reported earlier in our sporadic AD cases, enolase, UCH L-1 and actin are proteins in common that are oxidatively modified in both forms of AD [2,6–10], suggesting that oxidative stress is an important characteristic of AD rather than a consequence of the disease [2,6–10,32,33].

Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis and therefore its oxidation would lead to decreased ATP production. PET studies demonstrate that energy utilization is highly diminished in AD brain [34], and decreased enolase activity occurs in AD brain [35]. This is consistent with our findings of oxidatively modified γ-enolase in AD and a resultant loss of enzymatic function and ATP production. UCH L1 removes ubiquitin from proteins prior to proteasomal-facilitated degradation to maintain the level of ubiquitin. Oxidative modification of UCH-L1 is suggested to account for increased ubiquitination, decreased proteasomal activity, and increased accumulation of damaged or aggregated proteins in AD brain [1,2,4,6]. Moreover, altered UCH-L1 can itself lead to brain protein oxidation [26]. Therefore, oxidative modification of UCH L-1 in AD [6,7,9] likely depletes the availability of free ubiquitin, consequently

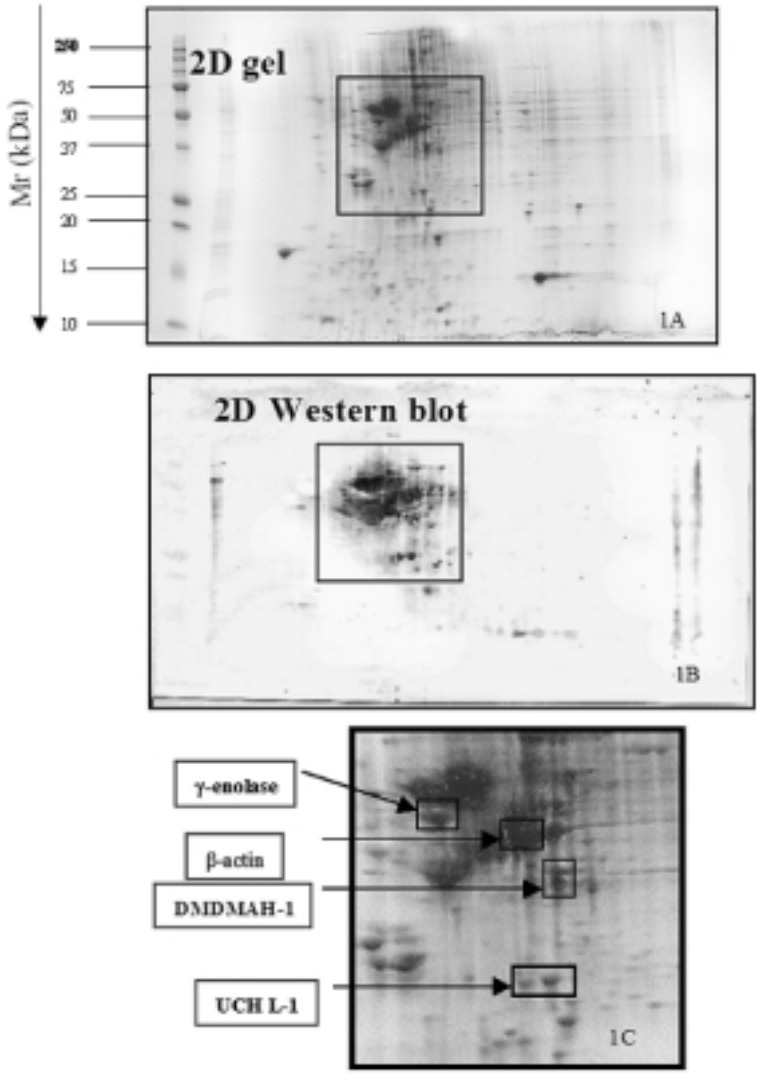

Fig. 1. (**A**) Representative 2D gel of the brain from a familial AD patient. (**B**). Representative 2D Oxyblot of the brain from the same familial AD patient indicating oxidatively modified proteins. (**C**) Expansion of the box outlined in (A) showing the identification of oxidatively modified proteins and their corresponding location on the 2D gels. Molecular weight markers and corresponding molecular weights are shown in Fig. 1A.

impairing proteasomal-mediated protein degradation in neurons. Actin is a core subunit of microfilaments found in both neurons and glial cells and is a target of Aβ(1–42)-mediated protein oxidation [36]. Actin microfilaments play a role in the neuronal cytoskeleton by maintaining the distribution of membrane proteins, by segregating axonal and dendritic proteins, and in learning and memory processes associated with synaptic remodeling [37]. Therefore, oxidation of actin can lead to alteration of membrane cytoskeletal structure, decreased membrane fluidity, and abnormal trafficking of synaptic proteins in axons.

One of the oxidized proteins identified, DMDMAH-1, appears to be found only in the familial AD cases analyzed, suggesting that this abnormality may occur due to the mutatations in PS-1. A function of this protein is to control the availability of NO [38,39] from microglial cells. Microglia are known to contain PS-1 and mutations in PS-1 lead to an increased sensitivity of glia to NO-mediated inflammatory processes [31]. Thus, it is interesting to speculate that the known susceptibility of microglia to NO in PS-1 mutations [31, 39] conceivably may be related to DMDMAH-1 being oxidized.

Table 2 summarizes the oxidatively modified proteins from the inferior parietal lobule and hippocam-

pus in AD previously identified by redox proteomics analyses from our laboratory [5–10]. Clearly, not all oxidatively modified proteins in aged, late-stage AD are found in brains of relatively young subjects with mutant PS-1.

In this initial study, we show that the brain proteins, DMDMAH-1, γ-enolase, actin and UCH-L1, are oxidatively modified in relatively rare familial AD. These findings are consistent with those observed in sporadic AD subjects, suggesting that oxidative modification of these proteins plays an important role in both familial and sporadic AD.

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