Journal of Neuroscience Research 85:1506–1514 (2007)

An Increase in S-Glutathionylated Proteins in the Alzheimer's Disease Inferior Parietal Lobule, a Proteomics Approach

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by neurofibrillary tangles, senile plaques, and loss of synapses. Many studies support the notion that oxidative stress plays an important role in AD pathogenesis. Previous studies from our laboratory employed redox proteomics to identify oxidatively modified proteins in the AD inferior parietal lobule (IPL) and hippocampus. The proteins were consistent with biochemical or pathological alterations in AD and have been central to further investigations of the disease. The present study focused on the identification of specific targets of protein S-glutathionylation in AD and control IPL by using a redox proteomics approach. For AD IPL, we identified deoxyhemoglobin, α-crystallin B, glyceraldehyde phosphate dehydrogenase (GAPDH), and α-enolase as significantly S-glutathionylated relative to these brain proteins in control IPL. GAPDH and α-enolase were also shown to have reduced activity in the AD IPL. This study demonstrates that specific proteins are sensitive to S-glutathionylation, which most likely is due to their sensitivity to cysteine oxidation initiated by the increase in oxidative stress in the AD brain.

Key words: Alzheimer's disease; redox proteomics; glutathione; GAPDH; α-enolase

Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with a decline in memory and cognition. AD pathology has three significant characteristics: synapse loss, neurofibrillary tangles (NFT), and senile plaques (SP). NFT are intracellular deposits of hyperphosphorylated tau protein, which is responsible for intracellular axonal transport. SP are extracellular, fibrillar protein deposits surrounded by dying neurites and whose major protein component is amyloid-beta peptide. The initial origin of AD pathogenesis has not been determined, although it is becoming evident that oxidative stress is implicated in the development of AD (Butterfield, 1997, 2002; Markesbery, 1997).

Oxidative stress is caused by a shift in the balance of proxidants vs. antioxidants, which is manifested by protein oxidation, lipid peroxidation, DNA oxidation, advanced glycation end products, and reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation (Butterfield and Stadtman, 1997). ROS and RNS can directly modify proteins and activate other pathways that may lead to additional impairment of cellular functions and neuronal loss (Smith et al., 1994; Markesbery, 1997; Koppal et al., 1999; Butterfield et al., 2001; Lovell et al., 2001; Butterfield, 2002). To prevent oxidative damage, cells and tissues have antioxidant systems to scavenge ROS and RNS.

These antioxidant systems include a low-molecular-weight thiol compound, glutathione (GSH), which is present at high concentrations (0.5–10 mM) in brain cells (Halliwell and Gutteridge, 1999; Sies, 1999). GSH is composed of three amino acids (γ-glutamyl-cysteinyl-glycine), in which the thiol on the cysteine plays an important role in maintaining the cellular redox state under oxidative stress. The general mechanism of this protection comes from the ability of GSH to form a disulfide (GSSG) when oxidized, which can then be reduced back to GSH by glutathione reductase. This process makes GSH a recyclable antioxidant molecule.

Contract grant sponsor: NIH (to D.A.B.).

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Received 20 November 2006; Revised 4 January 2007; Accepted 10 January 2007

Published online 23 March 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21275

Previous research shows that moderate oxidation can result in the formation of a mixed disulfide bond between protein cysteine side chains (PSH) and GSH to form S-glutathionylated proteins (PSSG; Dalle-Donne et al., 2003). S-glutathionylation is a reversible posttranslational modification from which the release of GSH can be catalyzed enzymatically by glutaredoxin, a thioltransferase (Chrestensen et al., 2000; Cotgreave et al., 2002; Shenton et al., 2002). The cell could potentially utilize S-glutathionylation as a form of redox regulation of protein function and activity (Klatt and Lamas, 2000; Casagrande et al., 2002; Dalle-Donne et al., 2003).

The purpose of the current study was to use redox proteomics to identify those proteins that have increased S-glutathionylation in AD. These results provide insight into how the proteins are important to the pathology of AD and how are they maybe related to other oxidative modifications.

MATERIALS AND METHODS

The samples used for this study were obtained from the inferior parietal lobule (IPL) of five AD patients and five aged-matched controls. The autopsy samples had average post-mortem intervals (PMIs) of 3.11 hr for AD patients and 3.14 hr for control subjects, as provided by the Rapid Autopsy Program of the University of Kentucky Alzheimer's Disease Clinical Center (UK ADC; Table I). 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, 10-D-5, and α-synuclein immunohistochemistry were used on multiple neocortical, hippocampal, entorhinal, amygdala, brainstem, and cerebellum sections for diagnosis. Some patients were also diagnosed with AD plus dementia with Lewy bodies. Control subjects underwent annual mental status testing and semiannual physical and neurological examinations, as a part of the UK ADC 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. Other characteristics of AD and control patients that were available from medical records are provided in Table I.

TABLE I. Demographic Data of AD and Control Subjects (Mean ± SD)*

Samples (n = 5)	Post-mortem intervals (hr)	Age (years)	Sex	Brain weight (g)
Control	3.15 ± 0.93	83 ± 13	4F/1M	1,149 ± 151
AD	3.11 ± 0.46	85 ± 8	4F/1M	1,056 ± 150

*AD, Alzheimer's disease; SD, standard deviation; n, number of individuals.

Sample Preparation

Brain samples were sonicated and suspended in 10 mM HEPES buffer (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.1 mM EDTA, and 0.6 mM $MgSO_4$ as well as proteinase inhibitors: leupeptin (0.5 mg/ml), pepstatin (0.7 μg/ml), type II S soybean trypsin inhibitor (0.5 μg/ml), and phenylmethylsulfonyl fluoride (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).

Two-Dimensional Electrophoresis

From each sample, 150 μg of protein was aliquoted and dissolved in rehydration buffer containing 8 M urea, 2 M thiourea, 2% CHAPS, 0.2% (v/v) biolytes, and bromophenol blue. The samples were sonicated three times for 20 sec each on ice and then applied to the IPG Readystrip (pH 3–10) from Bio-Rad (Hercules, CA). Proteins were loaded onto the strip via active rehydration, which was carried out for 16 hr at 50 V and 20°C by using the protean IEF cell (Bio-Rad). Isoelectric focusing was then performed at 20°C as follows: 800 V for 2 hr linear gradient, 1,200 V for 4 hr of slow gradient, 8,000 V for 8 hr of linear gradient, 8,000 V for 10 hr of rapid gradient. The strips were stored at −80°C until second-dimension separation was performed. Gel strips were equilibrated before second dimension for 15 min in 50 mM Tris-HCl (pH 6.8) containing 6 M urea, 1% (m/v) sodium dodecyl sulfate (SDS), and 30% (v/v) glycerol. The gel strips were then placed in the linear gradient precast Criterion Tris-HCl gels (8–16%; Bio-Rad) to perform the second-dimension electrophoresis. Precision protein standards (Bio-Rad) were run along 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 then stained overnight at room temperature with agitation in 50 ml Sypro Ruby gel stain (Bio-Rad).

Oxyblot Immunochemical Detection

For immunoblotting analysis, electrophoresis was carried out as previously stated. The gels were transferred to a nitrocellulose membrane using the Transblot-BlotSD Semi-Dry Transfer Cell at 45 mA per gel for 2 hr. The membranes were blocked with 2% bovine serum albumin (BSA) in PBS containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST) overnight at 4°C. The membranes were incubated with rabbit monocolonal anti-GSH (Virogen, Watertown, MA; 1:10) in PBST for 2 hr with gentle rocking at room temperature. The membranes were then washed three times for 5 min with PBST followed by incubation with anti-rabbit alkaline phosphatase secondary antibody (1:3,000) in PBST for 2 hr at room temperature. The membranes were then washed three times for 5 min with PBST and developed with Sigma-Fast tablets [5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT)]. Blots were dried and scanned with Adobe Photoshop.

Image Analysis

The nitrocellulose membranes were scanned with a Scanjet 3300C Hewlett Packard, Palo Alto, CA), and the gels were visualized with a UV transilluminator (λ_{ex} = 470 nm, λ_{em} = 618 nm; Molecular Dynamics, Sunnyvale, CA), and images were saved in TIFF format. These images were used to compare the protein expression by 2D gels and immunoreactivity by 2D blots with the assistance of PDQuest software (Bio-Rad). Average mode of background subtraction was used to normalize intensity value, which represents the amount of protein (total protein on gel and oxidized protein on oxyblot) per spot. After spots had been matched, the data set was obtained from the normalized intensity of each protein spot from the control and AD gels and oxyblots. To allow for protein concentration changes, the spot intensity values obtained on the blots are divided by the spot intensity value on the gels to get the level of specific protein S-glutathionylation. These, in turn, are analyzed for significance by Student's *t*-test.

Trypsin In-Gel Digestion

Based on the data obtained from image analysis, the protein spots that showed a significant increase in S-glutathionylation in AD IPL were excised from the gel with a clean razor blade and transferred into clean 1.5-ml microcentrifuge tubes. According to Thongboonkerd et al. (2002), the gel pieces were washed with 0.1 M ammonium bicarbonate (NH_4CO_3) for 15 min at room temperature under a flow hood. This was 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 underwent subsequent incubation with 20 μl of 20 mM dithiothreitol (DTT) in 0.1 M NH_4HCO_3 for 45 min at 56°C. The DTT solution was removed, and 20 μl of 55 mM iodoacetamide (IA) in 0.1 M NH_4HCO_3 was added and incubated for 30 min in the dark at room temperature. The remaining fluid was removed, and the gel pieces were incubated with 200 μl of 50 mM NH_4HCO_3 at room temperature for 15 min. Acetonitrile was then 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) in 50 mM NH_4HCO_3, which were incubated while shaking overnight (~18 hr) at 37°C.

Mass Spectrometry

The mass peptide fingerprint (PMF) for each trypsin digested protein spot determined by a Tof-Spec 2E (Micromass, United Kingdom) MALDI-TOF mass spectrometer operated in reflectron mode. One microliter of tryptic digested protein was mixed with 1 μl α-cyano-4-hydroxy-trans-cinnamic acid (10 mg/ml in 0.1% TFA:ACN, 1:1, v/v) directly on the target and allowed to dry at room temperature. The sample spot was then washed with 1 μl of 1% TFA solution for approximately 60 sec. The TFA droplet was gently removed from the sample spot with compressed air. The resulting diffuse sample spot was refocused using 1 μl of ethanol:acetone:0.1% TFA (6:3:1) solution. The reported spectra are a summation of 100 laser shots. External calibration of the mass axis, used for acquisition and internal calibration with either trypsin autolysis ions or matrix clusters, was applied postacquisition for accurate mass determination.

Peptide Sequence Analysis

The PMF from the tryptic fragments was used to identify the protein by searching against the entire NCBI and SwisProt protein databases with the MASCOT search engine (http://www.matrixscience.com). Peptide mass fingerprinting assumes that peptides are monoisotopic and oxidized at methionine residues, while allowing for up to one missed trypsin cleavage. Mass tolerance of 150 ppm was the window of error allowed for matching the peptide mass values. Probability-based MOWSE scores were estimated by the comparison of search results against estimated random-match population and were reported as $-10 \log_{10}(p)$, where p is the probability that the identification of the protein is not correct. MOWSE scores greater than 59 were considered to be significant ($P < 0.05$). Protein identifications were determined to be in the expected size and pI range based on position in the gel. Previous proteomics analysis of AD brain proteins from our laboratory was verified in some cases by immunochemical methods. Thus, we are confident in the correct identity of the proteins identified in this paper.

Enzymatic Activity

The specific activities of GAPDH and α-enolase were determined by using a protocol outlined by Sigma Aldrich (St. Louis, MO). The same age-match control (n = 5) and AD (n = 5) samples were used in this analysis as were used in proteomics study. Proteins were precipitated with the addition of cold 100% trichloroacetic acid (TCA) for a final concentration of 15%. The samples were then centrifuged at 14,000*g* and 4°C for 2 min. The resulting pellet was then washed with 500 μl of 1:1 (v/v) ethyl acetate/ethanol. Centrifugation and washing were repeated three times to remove any remaining lipids. The protein sample was then dissolved in100 mM triethylamine buffer (pH 7.4) for a final concentration of 0.750 μg/μl.

To determine the activity of GAPDH, 5 μg of protein was added to an assay mixture (100 mM 3-phosphoglyceric acid, 200 U/ml 3-phosphoglyceric phosphokinase, 200 mM cysteine, 100 mM $MgSO_4$, 34 mM ATP, 7.0 mM β-NADH) in a UV-transparent microtiter plate (Corning). The change in absorbance at 340 nm was monitored during a 5-min period with a powerwave X plate reader (Bio-Tek Instrument Inc., Winooshi, VT).

To analyze the activity of α-enolase 5 μg of protein sample was added to an assay mixture (56 mM 2-phosphoglycerate, 20 mM ADP, 700 U/ml pyruvate kinase, 7 mM β-NADH, 1000 U/ml L-lactic dehydrogenase) in a UV-transparent microtiter plate (Corning). The decrease in absorbance 340 nm was recorded over 5 min with a powerwave X plate reader (Bio-Tek Instrument Inc.).

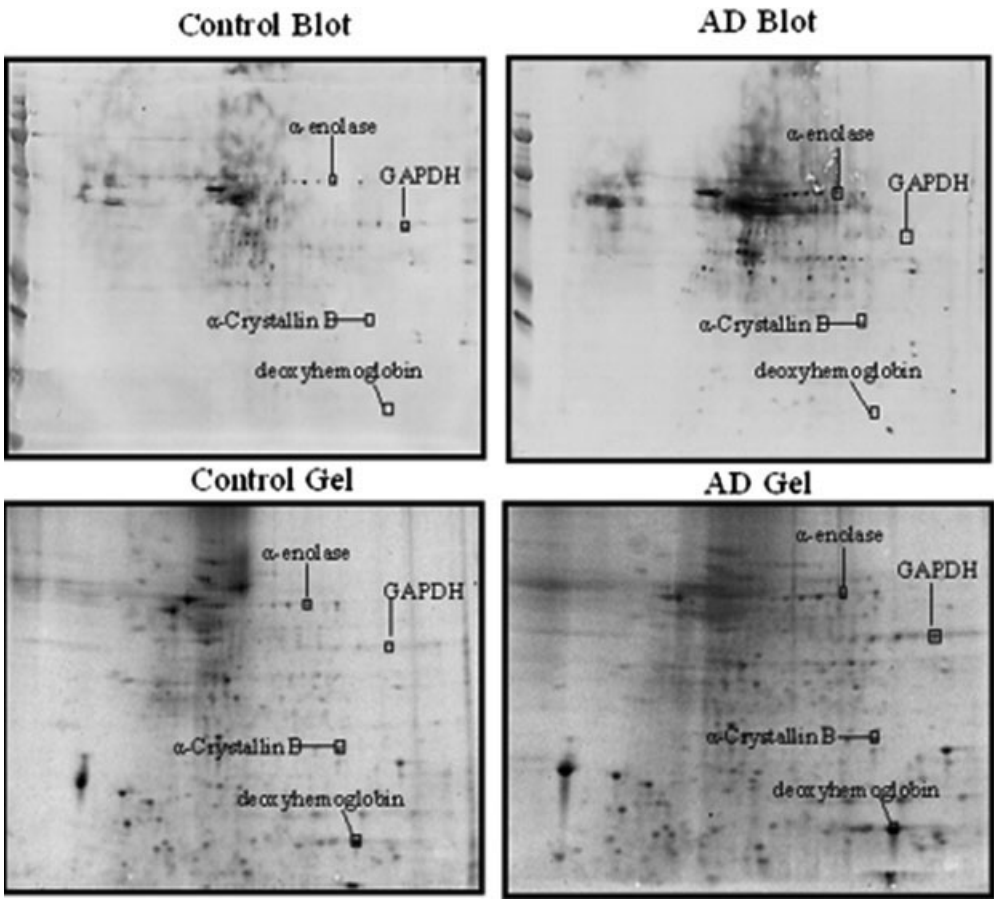

Fig. 1. The proteins from AD and age-matched controls were separated by 2D electrophoresis. The gels are then stained with Sypro Ruby stain. An example control and AD gel are displayed at the bottom. 2D oxyblots demonstrate the immunochemical detection of glutathionylated proteins. An example control and AD blot are presented at the top. The protein spots are labeled with the proteins identified in this study.

Statistical Analysis

The data concerning the enzymatic activities of GAPDH and α-enolase were analyzed via Student's *t*-test. The significance of the change in S-glutathionylation of specific proteins in the proteomics study was evaluated via nonparametric Mann-Whitney-Wilcoxon test. $P < 0.05$ was considered statistically significant. As discussed extensively by Maurer et al. (2005), the proteome set of excessively glutathionylated proteins with only several protein spots is much smaller than microarray data sets with at least several thousand genes. Consequently, with this low number of proteins, microarray algorithms and statistical approaches are not applicable for proteomics, so we relied on the nonparametric Mann-Whitney-Wilcoxon for the small sample size.

RESULTS

Identification of Specific S-Glutathionylated Proteins

To identify S-glutathionylated proteins, images of the blots and gels of the samples were compared in PD Quest software, and individual protein spots from the 2D blot were normalized to the amount of protein in the 2D gel (Fig. 1). This analysis verified that not all of the protein spots with increased immunoreactivity show an increase in S-glutathionylation, but the increase correlates with an increase in protein expression in AD brain (Castegna et al., 2002a,b; Poon et al., 2004). The 2D blot of AD IPL revealed four significantly increased S-glutathionylated protein spots compared with those of age-matched control IPL (Fig. 1). The identified protein spots were subjected to mass analysis using MALDI mass spectrometry for protein identification after in-gel trypsin digestion. The Mascot search engine successfully identified the four modified proteins. Table II shows the proteins identified with the peptides matched, percentage coverage, and pI and Mr values. Deoxyhemoglobin, α-crystallin B, GAPDH, and α-enolase were identified by quantitative redox proteomics to be S-glutathionylated in the AD IPL compared with control brain. The percentage increases in protein S-glutathionylation for the identified protein spots in AD are shown in Table III.

TABLE II. Summary of Glutathionylated Proteins in the AD IPL Identified With Mass Spectrometry*

Identification	No. of peptides matched of identified protein	Coverage of matched peptides (%)	pI, M_r (kDa)	Mowse score
α-Enolase	15/23	39	7.01, 47.5	182
GAPDH	8/25	36	8.57, 36.1	89
Deoxyhemoglobin	14/31	95	6.76, 15.8	204
α-Crystallin B	10/43	56	6.76, 20.1	116

*GAPDH, glyceraldehyde-3-phosphate dehydrogenase; pI, isoelectric point; M_r, relative mobility.

TABLE III. Summary of Identified S-Glutathionylated Proteins in AD IPL*

Protein	Protein oxidation (% control ± SEM)	*P* value
α-Enolase	549 ± 196	<0.05
GAPDH	740 ± 260	<0.05
Deoxyhemoglobin	459 ± 94	<0.03
α-Crystallin B	435 ± 121	<0.03

*GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SEM, standard deviation of the mean.

Enzymatic Activity of GAPDH and α-Enolase

The measurement of enzymatic activity of GAPDH (Fig. 2) and enolase (Fig. 3) from AD IPL revealed decreased activity compared with the age-matched control. A comparison between the previously reported oxidized proteins in AD IPL (Castegna et al., 2002a,b) and the currently identified S-glutathionylated proteins in AD IPL revealed α-enolase as oxidatively modified in both studies. GAPDH has not been previously identified as oxidized in the IPL region of the AD brain; however, previous studies do show that it is oxidized in the AD hippocampus (Sultana et al., 2006d).

DISCUSSION

The increase of oxidative stress in the AD brain results in an increase in oxidized proteins. Oxidatively modified proteins demonstrate impaired protein function, as observed in the previous and present studies, altering cell function, resulting in neuronal death (Hensley et al., 1995; Lauderback et al., 2003; Butterfield, 2004). Specific proteins appear to be more prone to oxidation, so proteomics is used to determine which proteins have an increase in oxidative modifications (Castegna et al., 2002a,b; Butterfield et al., 2003; Poon et al., 2006; Tangpong et al., 2006).

Some proteins that have been identified by redox proteomics as oxidized in the AD brain were involved in energy metabolism, mitochondrial function, cell cycle, synaptic plasticity, excitotoxicity, proteosomal dysfunction, lipid abnormalities, neuritic abnormalities, tau hyperphosphorylation, etc. (Castegna et al., 2002a,b; Butterfield and Boyd-Kimball, 2004b; Sultana et al., 2006a,b,d). The loss of protein function resulting from oxidative modification could be a plausible mechanism for neurodegeneration found in the AD brain.

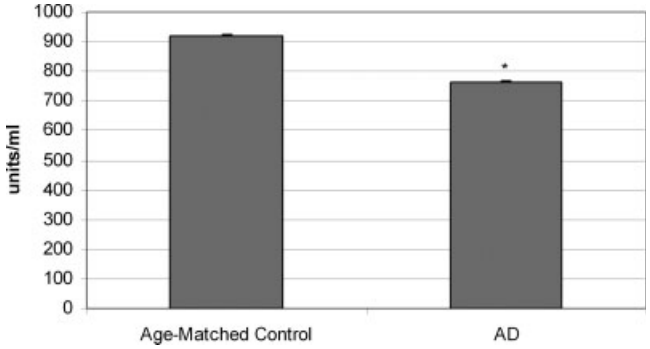

Fig. 2. The measurement of GAPDH enzyme assay was performed as described in Materials and Methods. The age-matched control and AD IPL samples were the same that were used for redox proteomics. The data are the mean ± SEM expressed as units per milliliter. Statistical comparison was by two-tailed Student's *t*-tests (n = 5). *$P < 0.05$ AD vs. age-matched control.

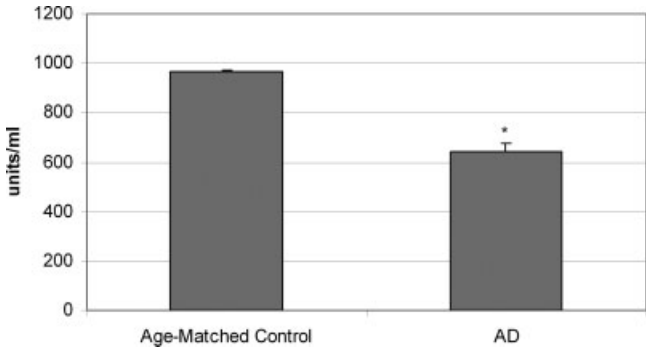

Fig. 3. The measurement of enolase enzyme assay was performed as described in Materials and Methods. The age-matched control and AD IPL samples were the same that were used for redox proteomics. The data are the mean ± SEM expressed as units per milliliter. Statistical comparison was by two-tailed Student's *t*-tests (n = 5). *$P < 0.02$ AD vs. age-matched control.

Glutathione is a natural antioxidant recycled by neurons to protect cell function from oxidative stress (Klatt and Lamas, 2000). During oxidative stress, GSH reacts with oxidized cysteine residues of proteins as a transient modification, S-glutathionylation. This could potentially serve as protection from irreversible, more dangerous oxidation of cysteine residues (Souza and

Radi, 1998; Baty et al., 2005). A previous study of S-glutathionylated actin showed that there is a reduction in function, which can then be reversed with GSH or DTT (Dalle-Donne et al., 2003). In the present study, we identified the specific targets of S-glutathionylation in AD inferior parietal lobule compared with age-matched controls. The identifications of both α-enolase and GAPDH confirm previous studies showing that these proteins contain oxidative modifications in the AD brain (Sultana et al., 2006b,d). Deoxyhemoglobin and α-crystallin B were also identified as showing an increase in S-glutathionylation, although these proteins have not been previously identified as oxidized in the AD brain. The role of S-glutathionylation has not been completely elucidated, but some studies report S-glutathionylation as a protective mechanism to divert from permanent protein oxidation; other studies show the potential of S-glutathionylation as a redox regulation of protein function reactions (Thomas et al., 1995; Yang et al., 2002; Dalle-Donne et al., 2003).

GAPDH has been connected with two of the major components of AD. Oxidized GAPDH was previously isolated from neurofibrillary tangles in the AD hippocampus (Wang et al., 2005; Sultana et al., 2006d). Studies have also shown that GAPDH is bound to the C-terminal end of APP and Aβ(1–42) (Schulze et al., 1993; Oyama et al., 2000). The effects of this binding are not completely known, although it potentially regulates the clearance of Aβ peptide (Oyama et al., 2000). This could also directly explain the oxidation of GAPDH in the AD brain.

Reduced activity of GAPDH in the AD IPL (Fig. 2) resulting from oxidation could cause alterations in glucose metabolism, which have been reported in the AD brain (Vanhanen and Soininen, 1998; Messier and Gagnon, 2000; Scheltens and Korf, 2000). GAPDH is a cytosolic protein that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-phosphoglycerate, which is the first oxidation-reduction reaction in the glycolytic pathway. The breakdown of glucose is the most prevalent mechanism by which the brain produces ATP, and consequently energy, making the glycolytic pathway crucial for neuron survival. The oxidation and decrease in activity of GAPDH were previously reported in the AD hippocampus and now in the IPL region, consistent with the altered glucose tolerance and metabolism confirmed by positron emission tomography (PET) scanning studies of AD patients (Blass and Gibson, 1991; Vanhanen and Soininen, 1998; Messier and Gagnon, 2000; Scheltens and Korf, 2000; Sultana et al., 2006c,d). This information combined with recent data suggests that altered function of GAPDH resulting from oxidative conditions could lead to neuronal death (Hernandez-Fonseca et al., 2005; Hara et al., 2006; Opii et al., 2006).

How S-glutathionylation alters the function of GAPDH is not clear, but studies confirm that the modification reduces activity and is reversible with available reduced GSH (Mohr et al., 1999; Dalle-Donne et al., 2003). Other studies show that S-glutathionylation does not specifically decrease the activity, but the oxidation of the cysteine residue reduces GAPDH activity (Lind et al., 1998; Eaton et al., 2002). An increase of GAPDH disulfide bonds in the AD brain, which can also be induced by the introduction of proxidants in neuronal cells, has been shown in previous work. This supports the theory that increased oxidative stress in the AD brain can increase S-glutathionylation of GAPDH (Cumming et al., 2004; Cumming and Schubert, 2005). Recent work shows that GAPDH acts as an NO sensor, showing drastic reduction activity when nitrosylated, which is also confirmed in the AD brain (Castegna et al., 2003; Hara et al., 2006; Sultana et al., 2006e). This activity can also be recovered with the addition of GSH (Batthyany et al., 2006). This knowledge leads to the theory that GAPDH is sensitive to oxidation and that S-glutathionylation serves as a temporary shield, allowing GAPDH to continue to function once the redox state of the cell returns to normal.

Alterations in another glycolytic enzyme, α-enolase, were identified, which continues to implicate the loss of ATP production as a viable mechanism for neurodegeneration. Currently, three isoforms of enolase are known, α-, β-, and γ-enolase. Enolase exists as homodimers or heterodimers. Previous studies recognized α-enolase as oxidatively modified with diminished activity in AD hippocampus compared with age-matched controls (Meier-Ruge et al., 1984; Schonberger et al., 2001; Castegna et al., 2002a). Results from this inquiry confirm that α-enolase is also S-glutathionylated in the IPL region of the AD brain and that the activity continues to be depleted. S-glutathionylated α-enolase performs at a reduced rate (Fig. 3); this activity can be recovered if the cell returns to a normal redox state (Beer et al., 2004). α-Enolase is a highly conserved cytoplasmic glycolytic enzyme that catalyzes the formation of phosphoenolpyruvate from 2-phosphoglycerate. This high-energy intermediate propels the generation of ATP in glycolysis (Harris et al., 1976). This study is consistent with the decrease in glucose metabolism in AD pathology with α-enolase oxidation and activity decline.

Oxygen is used in the breakdown of glucose; i.e., oxygen is critical to energy production in the brain via glucose and consequent neuron survival. Deoxyhemoglobin is the counterpart to oxyhemoglobin that carries the blood back to the heart once the blood has been deoxygenated. Previous studies showed that patients with AD dementia have decreased cerebral blood flow, indicating that neurons could be receiving less oxygen and therefore producing less energy (Haxby et al., 1988). Our studies are consistent with these findings in distinguishing the protein deoxyhemoglobin as oxidatively modified in the AD IPL compared with age-matched controls. This alteration could change the way in which hemoglobin functions, decreasing its ability to supply oxygen to neurons in the brain. Irregularity in energy metabolism, which could be initiated by a decrease in oxygen consumption, is characterized in the AD brain (Butterfield and Boyd-Kimball, 2004a).

The specific modification to deoxyhemoglobin reported in this study is S-glutathionylation. The effects on the attachment of glutathione have been previously investigated, resulting in an increase in affinity for oxygen, a reduction in the Bohr effect, and oxidation of the protein (Wodak et al., 1986). The increase in affinity for oxygen consequently reduces the oxygen release and contributes to a decline in ATP production. The alkaline Bohr effect was also reduced by 38% in AD brain, which demonstrated a decrease in pH dependence on oxygen affinity (Craescu et al., 1986). The increase in S-glutathionylation of deoxyhemoglobin likely decreases energy production and mechanistically contributes to the pathology of AD.

α-Crystallin B is a small heat shock protein (sHSP) that functions as a heat-inducible chaperone. α-Crystallin B has been found to be elevated in AD brain and codeposited with Aβ in SP (Ingvar and Schwartz, 1974; Hoshi and Tamura, 1993). SP-resident Aβ(1–42) has demonstrated toxic characteristics with the ability to induce oxidative damage to neuronal proteins (Boyd-Kimball et al., 2004). If α-crystallin B is codeposited with Aβ(1–42), then it is conceivable that this protein would be oxidatively modified in the AD brain, consistent with this study. It can also be predicted that there would be an increase in expression, in that α-crystallin B is a stress-inducible chaperone. Protein oxidation leads to dysfunction and decreased performance of α-crystallin B that could cause an increase in misfolded proteins in AD pathology (Raman et al., 2005). Previous studies have shown that α-crystallin B actually protects antioxidant enzymes from oxidation during acute inflammation (Masilamoni et al., 2005). Other studies also link α-crystallin B to reduced thiol oxidation of other lens crystallins during oxidative stress, which could be an explanation for its own thiol oxidation. Often in thiol oxidation-reduction reactions, to reduce one thiol group, another thiol group is oxidized. Once oxidized, this would make α-crystallin B an S-glutathionylation target.

In conclusion, this study reports that specific proteins have an increased S-glutathionylation in the AD brain compared with age-matched controls. The exact function of this reversible oxidative modification is unknown. The activity of S-glutathionylated enzymes is diminished, but studies show that the activity can be recovered under reducing conditions. Further studies investigating the specific in vivo effects of S-glutathionylation in oxidative stress are important to determining the role of S-glutathionylation in the AD brain.

REFERENCES

Batthyany C, Schopfer FJ, Baker PR, Duran R, Baker LM, Huang Y, Cervenansky C, Branchaud BP, Freeman BA. 2006. Reversible post-translational modification of proteins by nitrated fatty acids in vivo. J Biol Chem 281:20450–20463.

Baty JW, Hampton MB, Winterbourn CC. 2005. Proteomic detection of hydrogen peroxide-sensitive thiol proteins in Jurkat cells. Biochem J 389:785–795.

Beer SM, Taylor ER, Brown SE, Dahm CC, Costa NJ, Runswick MJ, Murphy MP. 2004. Glutaredoxin 2 catalyzes the reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins: implications for mitochondrial redox regulation and antioxidant defense. J Biol Chem 279:47939–47951.

Blass JP, Gibson GE. 1991. The role of oxidative abnormalities in the pathophysiology of Alzheimer's disease. Rev Neurol 147:513–525.

Boyd-Kimball D, Sultana R, Mohmmad-Abdul H, Butterfield DA. 2004. Rodent Abeta(1–42) exhibits oxidative stress properties similar to those of human Abeta(1–42): implications for proposed mechanisms of toxicity. J Alzheimers Dis 6:515–525.

Butterfield DA. 1997. Beta-amyloid-associated free radical oxidative stress and neurotoxicity: implications for Alzheimer's disease. Chem Res Toxicol 10:495–506.

Butterfield DA. 2002. Amyloid beta-peptide (1–42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. A review. Free Radic Res 36:1307–1313.

Butterfield DA. 2004. Proteomics: a new approach to investigate oxidative stress in Alzheimer's disease brain. Brain Res 1000:1–7.

Butterfield DA, Boyd-Kimball D. 2004a. Amyloid beta-peptide(1–42) contributes to the oxidative stress and neurodegeneration found in Alzheimer disease brain. Brain Pathol 14:426–432.

Butterfield DA, Boyd-Kimball D. 2004b. Proteomics analysis in Alzheimer's disease: new insights into mechanisms of neurodegeneration. Int Rev Neurobiol 61:159–188.

Butterfield DA, Stadtman ER. 1997. Protein oxidation processes in aging brain. Adv Cell Aging Gerontol 2:161–191.

Butterfield DA, Drake J, Pocernich C, Castegna A. 2001. Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide. Trends Mol Med 7:548–554.

Butterfield DA, Boyd-Kimball D, Castegna A. 2003. Proteomics in Alzheimer's disease: insights into potential mechanisms of neurodegeneration. J Neurochem 86:1313–1327.

Casagrande S, Bonetto V, Fratelli M, Gianazza E, Eberini I, Massignan T, Salmona M, Chang G, Holmgren A, Ghezzi P. 2002. Glutathionylation of human thioredoxin: a possible crosstalk between the glutathione and thioredoxin systems. Proc Natl Acad Sci U S A 99:9745–9749.

Castegna A, Aksenov M, Aksenova M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA. 2002a. Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. Free Radic Biol Med 33:562–571.

Castegna A, Aksenov M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA. 2002b. Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrimidinase-related protein 2, α-enolase and heat shock cognate 71. J Neurochem 82:1524–1532.

Castegna A, Thongboonkerd V, Klein JB, Lynn B, Markesbery WR, Butterfield DA. 2003. Proteomic identification of nitrated proteins in Alzheimer's disease brain. J Neurochem 85:1394–1401.

Chrestensen CA, Starke DW, Mieyal JJ. 2000. Acute cadmium exposure inactivates thioltransferase (glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis. J Biol Chem 275:26556–26565.

Cotgreave IA, Gerdes R, Schuppe-Koistinen I, Lind C. 2002. S-glutathionylation of glyceraldehyde-3-phosphate dehydrogenase: role of thiol oxidation and catalysis by glutaredoxin. Methods Enzymol 348:175–182.

Craescu CT, Poyart C, Schaeffer C, Garel MC, Kister J, Beuzard Y. 1986. Covalent binding of glutathione to hemoglobin. II. Functional consequences and structural changes reflected in NMR spectra. J Biol Chem 261:14710–14716.

Cumming RC, Schubert D. 2005. Amyloid-beta induces disulfide bonding and aggregation of GAPDH in Alzheimer's disease. FASEB J 19: 2060–2062.

Cumming RC, Andon NL, Haynes PA, Park M, Fischer WH, Schubert D. 2004. Protein disulfide bond formation in the cytoplasm during oxidative stress. J Biol Chem 279:21749–21758.

Dalle-Donne I, Rossi R, Giustarini D, Colombo R, Milzani A. 2003. Actin S-glutathionylation: evidence against a thiol-disulphide exchange mechanism. Free Radic Biol Med 35:1185–1193.

Halliwell B, Gutteridge J. 1999. Free radicals in biology and medicine. New York: Oxford University Press.

Hara MR, Cascio MB, Sawa A. 2006. GAPDH as a sensor of NO stress. Biochim Biophys Acta 1762:502–509.

Harris RC, Essen B, Hultman E. 1976. Glycogen phosphorylase activity in biopsy samples and single muscle fibres of musculus quadriceps femoris of man at rest. Scand J Clin Lab Invest 36:521–526.

Haxby JV, Grady CL, Koss E, Horwitz B, Schapiro M, Friedland RP, Rapoport SI. 1988. Heterogeneous anterior-posterior metabolic patterns in dementia of the Alzheimer type. Neurology 38:1853–1863.

Hensley K, Hall N, Subramaniam R, Cole P, Harris M, Aksenov M, Aksenova M, Gabbita SP, Wu JF, Carney JM, et al. 1995. Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. J Neurochem 65: 2146–2156.

Hernandez-Fonseca K, Montiel T, Massieu L. 2005. Inhibition of glyceraldehydes-3-phoshate dehydrogenase by oxidative stress during metabolic inhibition leads to neuronal death in cultured hippocampal neurons. Soc Neurosci Abstr 35.

Hoshi Y, Tamura M. 1993. Detection of dynamic changes in cerebral oxygenation coupled to neuronal function during mental work in man. Neurosci Lett 150:5–8.

Ingvar DH, Schwartz MS. 1974. Blood flow patterns induced in the dominant hemisphere by speech and reading. Brain 97:273–278.

Klatt P, Lamas S. 2000. Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. Eur J Biochem 267:4928–4944.

Koppal T, Drake J, Yatin S, Jordan B, Varadarajan S, Bettenhausen L, Butterfield DA. 1999. Peroxynitrite-induced alterations in synaptosomal membrane proteins: insight into oxidative stress in Alzheimer's disease. J Neurochem 72:310–317.

Lauderback CM, Drake J, Zhou D, Hackett JM, Castegna A, Kanski J, Tsoras M, Varadarajan S, Butterfield DA. 2003. Derivatives of xanthic acid are novel antioxidants: application to synaptosomes. Free Radic Res 37:355–365.

Lind C, Gerdes R, Schuppe-Koistinen I, Cotgreave IA. 1998. Studies on the mechanism of oxidative modification of human glyceraldehyde-3-phosphate dehydrogenase by glutathione: catalysis by glutaredoxin. Biochem Biophys Res Commun 247:481–486.

Lovell MA, Xie C, Markesbery WR. 2001. Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures. Neurobiol Aging 22:187–194.

Markesbery WR. 1997. Oxidative stress hypothesis in Alzheimer's disease. Free Radic Biol Med 23:134–147.

Masilamoni JG, Jesudason EP, Bharathi SN, Jayakumar R. 2005. The protective effect of α-crystallin against acute inflammation in mice. Biochim Biophys Acta 1740:411–420.

Maurer MH, Feldmann RE Jr, Bromme JO, Kalenka A. 2005. Comparison of statistical approaches for the analysis of proteome expression data of differentiating neural stem cells. J Proteome Res 4:96–100.

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. 1984. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 34:939–944.

Meier-Ruge W, Iwangoff P, Reichlmeier K. 1984. Neurochemical enzyme changes in Alzheimer's and Pick's disease. Arch Gerontol Geriatr 3:161–165.

Messier C, Gagnon M. 2000. Glucose regulation and brain aging. J Nutr Health Aging 4:208–213.

Mohr S, Hallak H, de Boitte A, Lapetina EG, Brune B. 1999. Nitric oxide-induced S-glutathionylation and inactivation of glyceraldehyde-3-phosphate dehydrogenase. J Biol Chem 274:9427–9430.

Opii WO, Joshi G, Head E, Milgram NW, Muggenburg BA, Klein JB, Pierce WM, Cotman CW, Butterfield DA. 2006. Proteomic identification of brain proteins in the canine model of human aging following a long-term treatment with antioxidants and a program of behavioral enrichment: relevance to Alzheimer's disease. Neurobiol Aging.

Oyama R, Yamamoto H, Titani K. 2000. Glutamine synthetase, hemoglobin α-chain, and macrophage migration inhibitory factor binding to amyloid beta-protein: their identification in rat brain by a novel affinity chromatography and in Alzheimer's disease brain by immunoprecipitation. Biochim Biophys Acta 1479:91–102.

Poon HF, Castegna A, Farr SA, Thongboonkerd V, Lynn BC, Banks WA, Morley JE, Klein JB, Butterfield DA. 2004. Quantitative proteomics analysis of specific protein expression and oxidative modification in aged senescence-accelerated-prone 8 mice brain. Neuroscience 126:915–926.

Poon HF, Shepherd HM, Reed TT, Calabrese V, Stella AM, Pennisi G, Cai J, Pierce WM, Klein JB, Butterfield DA. 2006. Proteomics analysis provides insight into caloric restriction mediated oxidation and expression of brain proteins associated with age-related impaired cellular processes: Mitochondrial dysfunction, glutamate dysregulation and impaired protein synthesis. Neurobiol Aging 27: 1020–1034.

Raman B, Ban T, Sakai M, Pasta SY, Ramakrishna T, Naiki H, Goto Y, Rao Ch M. 2005. AlphaB-crystallin, a small heat-shock protein, prevents the amyloid fibril growth of an amyloid beta-peptide and beta2-microglobulin. Biochem J 392:573–581.

Scheltens P, Korf ES. 2000. Contribution of neuroimaging in the diagnosis of Alzheimer's disease and other dementias. Curr Opin Neurol 13:391–396.

Schonberger SJ, Edgar PF, Kydd R, Faull RL, Cooper GJ. 2001. Proteomic analysis of the brain in Alzheimer's disease: molecular phenotype of a complex disease process. Proteomics 1:1519–1528.

Schulze H, Schuler A, Stuber D, Dobeli H, Langen H, Huber G. 1993. Rat brain glyceraldehyde-3-phosphate dehydrogenase interacts with the recombinant cytoplasmic domain of Alzheimer's beta-amyloid precursor protein. J Neurochem 60:1915–1922.

Shenton D, Perrone G, Quinn KA, Dawes IW, Grant CM. 2002. Regulation of protein S-thiolation by glutaredoxin 5 in the yeast Saccharomyces cerevisiae. J Biol Chem 277:16853–16859.

Sies H. 1999. Glutathione and its role in cellular functions. Free Radic Biol Med 27:916–921.

Smith MA, Taneda S, Richey PL, Miyata S, Yan SD, Stern D, Sayre LM, Monnier VM, Perry G. 1994. Advanced Maillard reaction end products are associated with Alzheimer disease pathology. Proc Natl Acad Sci U S A 91:5710–5714.

Souza JM, Radi R. 1998. Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite. Arch Biochem Biophys 360:187–194.

Sultana R, Boyd-Kimball D, Poon HF, Cai J, Pierce WM, Klein JB, Markesbery WR, Zhou XZ, Lu KP, Butterfield DA. 2006a. Oxidative modification and down-regulation of Pin1 in Alzheimer's disease hippocampus: a redox proteomics analysis. Neurobiol Aging 27:918–925.

Sultana R, Boyd-Kimball D, Poon HF, Cai J, Pierce WM, Klein JB, Merchant M, Markesbery WR, Butterfield DA. 2006b. Redox proteomics identification of oxidized proteins in Alzheimer's disease

hippocampus and cerebellum: an approach to understand pathological and biochemical alterations in AD. Neurobiol Aging 27:1564–1576.

Sultana R, Boyd-Kimball D, Poon HF, Cai J, Pierce WM, Klein JB, Merchant M, Markesbery WR, Butterfield DA. 2006c. Redox proteomics identification of oxidized proteins in Alzheimer's disease hippocampus and cerebellum: an approach to understand pathological and biochemical alterations in AD. Neurobiol Aging 27:1564–1576.

Sultana R, Perluigi M, Butterfield DA. 2006d. Redox proteomics identification of oxidatively modified proteins in Alzheimer's disease brain and in vivo and in vitro models of AD centered around Abeta(1–42). J Chromatogr B Anal Technol Biomed Life Sci 833: 3–11.

Sultana R, Poon HF, Cai J, Pierce WM, Merchant M, Klein JB, Markesbery WR, Butterfield DA. 2006e. Identification of nitrated proteins in Alzheimer's disease brain using a redox proteomics approach. Neurobiol Dis 22:76–87.

Tangpong J, Cole MP, Sultana R, Joshi G, Estus S, Vore M, St Clair W, Ratanachaiyavong S, St. Clair DK, Butterfield DA. 2006. Adriamycin-induced, TNF-α-mediated central nervous system toxicity. Neurobiol Dis 23:127–139.

Thomas JA, Poland B, Honzatko R. 1995. Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. Arch Biochem Biophys 319:1–9.

Thongboonkerd V, Luengpailin J, Cao J, Pierce WM, Cai J, Klein JB, Doyle RJ. 2002. Fluoride exposure attenuates expression of Streptococcus pyogenes virulence factors. J Biol Chem 277:16599–16605.

Vanhanen M, Soininen H. 1998. Glucose intolerance, cognitive impairment and Alzheimer's disease. Curr Opin Neurol 11:673–677.

Wang Q, Woltjer RL, Cimino PJ, Pan C, Montine KS, Zhang J, Montine TJ. 2005. Proteomic analysis of neurofibrillary tangles in Alzheimer disease identifies GAPDH as a detergent-insoluble paired helical filament tau binding protein. FASEB J 19:869–871.

Wodak SJ, De Coen JL, Edelstein SJ, Demarne H, Beuzard Y. 1986. Modification of human hemoglobin by glutathione. III. Perturbations of hemoglobin conformation analyzed by computer modeling. J Biol Chem 261:14717–14724.

Yang KS, Kang SW, Woo HA, Hwang SC, Chae HZ, Kim K, Rhee SG. 2002. Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. J Biol Chem 277:38029–38036.