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QUANTITATIVE PROTEOMICS ANALYSIS OF SPECIFIC PROTEIN EXPRESSION AND OXIDATIVE MODIFICATION IN AGED SENESCENCE-ACCELERATED-PRONE 8 MICE BRAIN

H. F. POON,[a] A. CASTEGNA,[a] S. A. FARR,[b,c] V. THONGBOONKERD,[d] B. C. LYNN,[a,e,g] W. A. BANKS,[c] J. E. MORLEY,[c] J. B. KLEIN[d] AND D. A. BUTTERFIELD[a,f,g]*

[a]*Department of Chemistry, University of Kentucky, Lexington, KY 40506-0055, USA*

[b]*Geriatric Research Education and Clinical Center, VA Medical Center, St. Louis, MO 63109, USA*

[c]*Department of Internal Medicine, Division of Geriatric Medicine, St. Louis University School of Medicine, St. Louis, MO 63104, USA*

[d]*Kidney Disease Program and Proteomics Core Laboratory, University of Louisville School of Medicine and VAMC, Louisville, KY 40202, USA*

[e]*Mass Spectrometry Facility, University of Kentucky, Lexington, KY 40506, USA*

[f]*Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506, USA*

[g]*Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506, USA*

Abstract—The senescence-accelerated mouse (SAM) is a murine model of accelerated senescence that was established using phenotypic selection. The SAMP series includes nine substrains, each of which exhibits characteristic disorders. SAMP8 is known to exhibit age-dependent learning and memory deficits. In our previous study, we reported that brains from 12-month-old SAMP8 have greater protein oxidation, as well as lipid peroxidation, compared with brains from 4-month-old SAMP8 mice. In order to investigate the relation between age-associated oxidative stress on specific protein oxidation and age-related learning and memory deficits in SAMP8, we used proteomics to identify proteins that are expressed differently and/or modified oxidatively in aged SAMP8 brains. We report here that in 12 month SAMP8 mice brains the expressions of neurofilament triplet L protein, lactate dehydrogenase 2 (LDH-2), heat shock protein 86, and α-spectrin are significantly decreased, while the expression of triosephosphate isomerase (TPI) is increased compared with 4-month-old SAMP8 brains. We also report that the specific protein carbonyl levels of LDH-2, dihydropyrimidinase-like protein 2, α-spectrin and creatine kinase, are significantly increased in the brain of 12-month-old SAMP8 mice when compared with the 4-month-old SAMP8 brain. These findings are discussed in reference to the effect of specific protein oxidation and changes of expression on potential mechanisms of abnormal alterations in metabolism and neurochemicals, as well as to the learning and memory deficits in aged SAMP8 mice. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

The senescence-accelerated mouse (SAM) is a model of accelerated senescence that was established through phenotypic selection from a common genetic pool of AKR/J strain of mice (Takeda et al., 1981). The SAM model consists of senescence-accelerated-prone mice (SAMP), which exhibit accelerated aging with a shortened life span and increased amyloidosis, and senescence-accelerated-resistant mice (SAMR), which exhibit normal aging characteristics (Miyamoto, 1997). The SAMP series includes nine substrains, each of which exhibits characteristic disorders, such as loss of normal behavior, various skin lesions, or increased lordokyphosis. SAMP8 exhibits age-dependent learning and memory deficits (Yagi et al., 1988; Ohta et al., 1989). Therefore, SAMP8 is a model for studying age-related cognitive impairments.

Comparing aged SAMR1 mice to SAMP8, the aged SAMP8 shows impairments in learning tasks, altered emotion, abnormality of circadian rhythm (Miyamoto, 1997), and increased oxidative stress (Butterfield et al., 1997). Preliminary evidence also showed that the dendritic arbor of dentate granule cells in the SAMP8 are reduced in size and complexity when compared with that of SAMR1 (Morley et al., 2002). Evidence strongly suggests that a cause of the cognitive decline in the SAMP8 is an age-related overexpression of the precursor to amyloid-β (Aβ) in the hippocampus and other brain regions (Morley et al., 2000). Unlike transgenic mice that have five to 14 times the normal amount of Aβ in their brains, SAMP8 have only about a 100% increase of Aβ between 4 month and 12 months of age (Kumar et al., 2001), which is much closer to the estimated 50% increase in Aβ seen in AD (Rosenberg, 2000). Established cognitive deficits in 12-month-old SAMP8 mice can be reversed with either Aβ-directed antibody or Aβ-directed antisense (Maekawa et al., 1993; Kumar et al., 2000; Morley et al., 2000; Banks et al., 2001). In addition, SAMP8 mice develop amyloid plaques in the late stage of their life (Morley et al., 2000) after the learning

*Correspondence to: D. A. Butterfield, Department of Chemistry, Center of Membrane Sciences, and Sander-Brown Center on Aging, University of Kentucky, Lexington, KY 40506-0055, USA. Tel: +1-859-257-3184; fax: +1-859-257-5876.
E-mail address: dabcns@uky.edu (D. A. Butterfield).

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CK, creatine kinase; CRMP, collapsin response mediator protein; DNP, 2,4-dinitrophenyl hydrazone; DRP-2, dihydropyrimidinase-like protein 2; DS, Down syndrome; hsp86, heat shock protein 86; LDH, lactate dehydrogenase; LDH-2, lactate dehydrogenase 2; NF, neurofilament; NF-L, neurofilament triplet L protein; NSE, neuron-specific enolase; SAM, senescence-accelerated mouse; SAMP, senescence-accelerated-prone mice; SAMR, senescence-accelerated-resistant mice; TCA, trichloroacetic acid; TPI, triosephosphate isomerase; BCIP/NBT, 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

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and memory deficits have developed (Nomura et al., 1996).

Aged SAMP8 mice also show clear age-related impairments in learning assessed by foot shock avoidance (Flood and Morley, 1993; Flood et al., 1995, 1996), which correlate with oxidative stress parameters (Farr et al., 2003). These observations are consistent with the theory of aging that postulates that the oxidative modification by free radicals on biomolecules, such as proteins, are responsible for the functional deterioration related to aging (Harman, 1969). Treating aged SAMP8 mice with antioxidants, such as lipoic acid, *N*-acetylcysteine, L-acetylcarnitine, and melatonin, not only decreases oxidative stress in aged SAMP8 brains, but also improves their learning and memory (Okatani et al., 2002; Yasui et al., 2002; Farr et al., 2003). This indicates that oxidative stress is involved in the impairment of learning and memory as observed in the SAMP8 mouse. Whereas changes in metabolism and neurochemicals were detected in SAMP8 mice (Ikegami et al., 1992; Shimano, 1998), at present, little is known about the mechanism underlying the oxidative stress associated learning and memory deficit observed in aged SAMP8 mice.

In our previous study, we showed that 12-month-old SAMP8 mice have higher protein oxidation, and lipid peroxidation compared with 4-month-old SAMP8 (Farr et al., 2003). In order to investigate the relations among age-related oxidative stress on proteins, physiological alterations and impairments in learning and memory, we used proteomics to identify brain proteins that are expressed differently and oxidatively modified with aging in SAMP8 mice.

EXPERIMENTAL PROCEDURES

Rodent subjects

Experimentally naive, 4- and 12-month-old male SAMP8 were obtained from our breeding colony. The colony is derived from siblings generously provided by Dr. Takeda of Kyoto University, Japan, and has been maintained as an inbred strain for 12 years under clean-room procedures (i.e. use of sterile gloves in handling mice, sterilized cages and bedding, restricted access to breeding area), and housed in microisolator HEPA filter units (Allentown Caging, Allentown, PA, USA). The colony routinely undergoes serological testing for viral and bacterial contamination and has remained free of pathogens for over 5 years. Mice are housed in rooms with a 12-h light/dark cycle (lights on at 06:00 h) at 20–22 °C with water and food (Richmond Laboratory Rodent Diet 5001) available *ad libitum*. All experiments were conducted after institutional approval of the animal use subcommittee, which subscribes to the NIH Guide for Care and Use of Laboratory Animals. Six 4-month-old and six 12-month-old SAMP8 animals were used in this study. This number was the minimum number of animals to test for significance. Methods to kill the animals and harvest the brain were approved and involved no or only transient minimal pain.

Sample preparation

Six 4-month-old and six 12-month-old SAMP8 brains were flash frozen in liquid nitrogen in St. Louis and sent to Lexington on dry ice overnight. Brain samples were homogenized in a lysis buffer (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, 0.7 μg/mL pepstatin, 0.5 μg/mL trypsin inhibitor, and 40 μg/mL PMSF). Homogenates were centrifuged at 15,800×*g* for 10 min to remove debris. The supernatant was extracted to determine the concentration by the BCA method (Pierce, Rockford, IL, USA).

Two-dimensions electrophoresis

Samples of brain proteins were prepared according to the procedure of Levine et al. (1994). Two hundred micrograms of protein were incubated with four 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) to obtain a final concentration of 15% TCA. Samples are placed on ice for 10 min to allow precipitation of proteins. Precipitates were centrifuged at 15,800×*g* for 2 min. 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, Hercules, CA, USA). The samples then were 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 IPG strip (Bio-Rad). The strips were soaked in the sample solution for 1 h to allow uptake of the proteins. The strip is then actively rehydrated in protean IEF cell (Bio-Rad) for 16 h at 50 V. The isoelectric focusing was performed at 300 V for 2 h linearly; 500 V for 2 h linearly; 1000 V for 2 h linearly, 8000 V for 8 h linearly and 8000 V for 10 h rapidly. All the processes above were carried out at 22 °C. The strip was stored at −80 °C until second dimension electrophoresis was performed.

For second dimension electrophoresis, IPG Strips pH 3–10 were equilibrated for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate, 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodoacetamide in place of dithiothreitol. Linear Gradient Precast criterion Tris-HCl gels (8–16%; Bio-Rad) were used to perform second dimension electrophoresis. Precision Protein standards (Bio-Rad) were run along with the sample at 200 V for 65 min.

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

Western blotting

Protein (200 μg) was incubated with four volumes of 20 mM DNPH at room temperature (25 °C) for 20 min. The gels were prepared in the same manner as 2D-electrophoresis. The proteins from the second dimension electrophoresis gels were transferred to a nitrocellulose paper (Bio-Rad) using the Transblot-Blot SD semi-Dry Transfer Cell (Bio-Rad) at 15 V for 4 h. The 2,4-dinitrophenyl hydrazone (DNP) adduct of the carbonyls of the proteins was detected on the nitrocellulose paper using a primary rabbit antibody (Intergen) specific for DNP-protein adducts (1:100) and then a secondary goat anti-rabbit IgG (Sigma) antibody. The resultant stain was developed by application of Sigma-Fast 5-Bromo-4-chloro-3-indolyl-phosphate/Nitrobule tetrazolium (BCIP/NBT) tablets.

Image analysis

The gels and nitrocellulose papers were scanned and saved in TIFF format using Scanjet 3300C (Hewlett Packard, Palo Alto, CA, USA). Investigator HT analyzer (Genomic Solutions Inc., Ann Arbor, MI, USA) was used for matching and analysis of 56 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. After completion of spot matching, the normalized intensity of each protein spot from individual gels (or oxyblots) was compared between groups using statistical analysis.

Trypsin digestion

Samples were prepared using the techniques described by Jensen et al. (1999), and as modified by Thongboonkerd et al. (2002). The protein spots that shown different expression and/or expression by image analysis 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. Acetonitrile (200 μl) was added. After a 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, USA) 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 shaking incubator.

Mass spectrometry

All mass spectra reported in this study were acquired from the University of Kentucky Mass Spectrometry Facility. LC/MS/MS spectra were acquired on a Finnigan LCQ 'Classic' quadrupole ion trap mass spectrometer (Finnigan, Bremen, Germany). Separations were performed with an HP 1100 HPLC modified with a custom splitter to deliver 4 μL/min to a custom C18 capillary column (300 μm id×15 cm, packed in-house with Macrophere 300 5 μm C18 (Alltech Associates). Gradient separations consisted of 2 min isocratic at 95% water:5% acetonitrile (both phases contain 0.1% formic acid), the organic phase was increased to 20% acetonitrile over 8 min, then increased to 90% acetonitrile over 25 min, held at 90% acetonitrile for 8 min, then increased to 95% in 2 min, and finally returned to initial conditions in 10 min (total acquisition time 45 min with a 10-min recycle time). Tandem mass spectra were acquired in a data dependent manner. Three microscans were averaged to generate the data-dependent full scan spectrum. The most intense ion was subjected to tandem mass spectrometry and three microscans were averaged to produce the MS/MS spectrum. Masses subjected to the MS/MS scan were placed on an exclusion list for 2 min. The tandem spectra obtained were searched against the NCBI protein databases using the MASCOT search engine (http://www.matrixscience.com). MS/MS spectra, the peptides were also assumed to be monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. A 0.8-Da MS/MS mass tolerance was used for search of the NCBI protein databases.

A Bruker Autoflex MALDI TOF (matrix assisted laser desorption ionization-time of flight) mass spectrometer (Bruker Daltonic, Billerica, MA, USA) operated in the reflectron mode was used to generate peptide mass fingerprints. Peptides resulting from in-gel digestion with trypsin were analyzed on a 384 position, 600 μm AnchorChip Target (Bruker Daltonics, Bremen, Germany) and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2; Bruker Daltonics, Bremen, Germany). Briefly, 1 μl of digestate was mixed with 1 μl of α-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 s. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) using 1 μl of a solution of ethanol: acetone:0.1% TFA (6:3:1 ratio). Reported spectra are a summation of 100 laser shots. External calibration of the mass axis was used for acquisition and internal calibration using either trypsin autolysis ions or matrix clusters was applied post acquisition for accurate mass determination.

The MALDI and tandem spectra used for protein identification from tryptic fragments were searched against the NCBI protein databases using the MASCOT search engine (http://www.matrixscience.com). Peptide mass fingerprinting used the assumption that peptides are monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues (Butterfield and Castegna, 2003; Butterfield et al., 2003; Castegna et al., 2002a,b, 2003). Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. Mass tolerance of 150 ppm was the window of error allowed for matching the peptide mass values.

Statistics

The data of protein level and protein specific carbonyl level were analyzed by Student's *t*-test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Protein expression level

Proteomics was used to study oxidized proteins in AD (Castegna et al., 2002a,b, 2003). The specific carbonyl levels were obtained by dividing the carbonyl level of a protein spot on the nitrocellulose membrane by the protein level of its corresponding protein spot on the gel. Such numbers give the carbonyl level per unit of protein. We found that in comparison to 4-month-old SAMP8 mice, 12-month-old SAMP8 mice brain have five proteins that are expressed significantly differently and five proteins that have significantly higher specific carbonyl levels. All the mass spectra (not shown) of the peptides were matched to the mass spectra in NCBI protein databases. The identified proteins are listed in Table 1. The probability-based Mowse score is assigned for each spectrum to indicate the probability that the match between the database and the spectra is a random event. Scores greater than 66 were considered significant. Thus, if a match has a score higher than 66, the probability of the match being a random event is lower than 0.05. All protein identifications agree with the expected MrW and pI range based on their positions on the gel.

Fig. 1 shows a gel for 2D-electrophoresis after Coomassie Blue staining. The proteins that were expressed differently in SAMP8 brains are summarized in Table 2. The expression of neurofilament triplet L protein (NF-L), lactate dehydrogenase 2 (LDH-2) and heat shock protein 86 (hsp86), α-spectrin are significantly decreased, whereas the expression of triosephosphate isomerase

Table 1. Summary of proteins identified by mass spectrometry

Identified protein	gI accession #	# Peptides match identified	% Coverage matched peptides	pI, MrW	MALDI	HPLC MS/MS	Mowse score	Probability of a random hit
LDH 2	gi|6678674	14	41	5.87, 36.6		X	632	2×10^{-62}
CK	gi|10946574	8	29	5.52, 42.7	X		80	1×10^{-8}
α-Enolase	gi|12963491	17	47	6.69, 47.1		X	947	2×10^{-95}
TPI	gi|1864018	5	21	7.19, 26.7	X		68	1.6×10^{-7}
DRP-2	gi|1351260	14	35	6.16, 62.16		X	776	2.5×10^{-78}
α-Spectrin 2	gi|31543764	30	10	5.28, 156.1	X		231	7.9×10^{-24}
NF-L	gi|417355	18	37	4.40, 61.5		X	992	6.3×10^{-100}
hsp86	gi|26345918	7	9	4.82, 84.7	X		73	5×10^{-8}

(TPI) was significantly increased in brain from12-month-old SAMP8 mice.

Specific protein carbonyl level

Fig. 2 shows an example of a Western blot for detection of the level of protein carbonyl. The summary of the specific protein carbonyl levels is given in Table 3. The specific protein carbonyl levels of LDH-2, dihydropyrimidinase-like protein 2 (DRP-2), α-spectrin and creatine kinase (CK) are significantly increased in the brain of 12-month-old SAMP8. The specific carbonyl level of α-enolase was showed a trend toward being higher in 12-month-old SAMP8 with *P*-value lower than 0.07.

DISCUSSION

Here, we used proteomics to investigate the expression of proteins and their oxidative stress in the brains from aged SAMP8 mice, a potential animal model of Alzheimer's disease (AD). Proteomics analysis previously was used to identify oxidized and nitrated protein in AD patients (Castegna et al., 2002a,b, 2003). Those studies found that α-enolase, CK, and DRP-2 are more oxidized, and α-enolase and TPI are significantly more nitrated in AD brains when compared with age-matched controls (Butterfield et al., 2002; Castegna et al., 2002b, 2003). The current study shows that the specific protein carbonyl levels of LDH-2, α-enolase, α-spectrin and DRP-2 were significantly increased, and the protein expression level of TPI, LDH-2, NF-L, α-spectrin and hsp86 were significantly changed in 12-month-old SAMP8 mouse brains compared with brains for 4-month-old SAMP8 mice.

α-Enolase is a subunit of enolase, the other subunits being β- and γ-enolase. α-Enolase is present during embryonic development and switches to γ or β, concurring with terminal differentiation of the muscle or neuron (Giallongo et al., 1990). Two of the subunits form active enolase isoforms (αα, ββ, γγ, αβ and αγ), which interconvert 2-phosphoglycerate and phosphoenolpyruvate. Since αγ and γγ isoforms are predominantly in the brain, they are called neuron-specific enolases (NSE; Keller et al., 1994). NSE has been used as a neuronal marker for structural damage (el-Mallakh et al., 1992) and as marker for neuronal metabolic properties (Trapp et al., 1981; Marangos and Schmechel, 1987; Hamre et al., 1989). It has also been reported that the expression of NSE coincides with the onset of synaptic connections (Maxwell et al., 1982; Whitehead et al., 1982; Hedgecock et al., 1985). These studies show that enolase is not only involved in metabolism, but also in cell differentiation and normal growth in brains. In primary human fetal mixed brain cell cultures, the NSE level decrease correlates with the treatment of Aβ (1–40) (Hayes et al., 2002). Although the level of NSE is not significantly altered in the aged brain (Kato et al., 1990) or in the AD brain (Kato et al., 1991), α-enolase specific carbonyl level and protein level (Schonberger et al., 2001; Castegna et al., 2002b) are increased in the AD brain when compared with age-matched controls, suggesting that the loss of activity by oxidative modification of α-enolase is compensated for by its increased protein level. It was shown that a decline of enolase activity results in abnormal growth and reduced metabolism in brain (Tholey et al., 1982). The current study showed that the specific carbonyl level of α-enolase is significantly increased, while the protein level of α-enolase is not, suggesting that the activity of α-enolase is reduced in SAMP8 brain. This, conceivably, could reflect ATP levels with consequent deleterious sequelae.

LDH-2 is a subunit of lactate dehydrogenase (LDH). The five isoenzymes of tetrameric LDH are found in various proportions of different somatic tissues in the combination of the A and B subunit in mammals (Sakai et al., 1987). LDH is also a glycolytic protein that catalyzes the reversible NAD-dependent interconversion of pyruvate to L-lactate. A single mutation in LDH-2 lower the activity of LDH (Maekawa et al., 1993; Sudo et al., 1994) suggesting that the LDH-2 subunit is critical to LDH activity. LDH release is a common indicator of damage to plasma membrane integrity. Lactate appears to be the only oxidizable energy substrate available to support neuronal recovery (Schurr et al., 1997a,b). Although the activity of LDH shows no significant difference in AD compared with matched-age controls (Chandrasekaran et al., 1994), many studies show that LDH activity in rat brains declined with increased age (Mizuno and Ohta, 1986; Ferrante and Amenta, 1987; Hrachovina and Mourek, 1990; Agrawal et al., 1996). Total LDH activity in the brains of aged rats that were raised under chronic hypoxia is also diminished (Lai et al., 2003). Since aging and chronic hypoxia are highly associated with oxidative stress (Hensley et al., 1995;

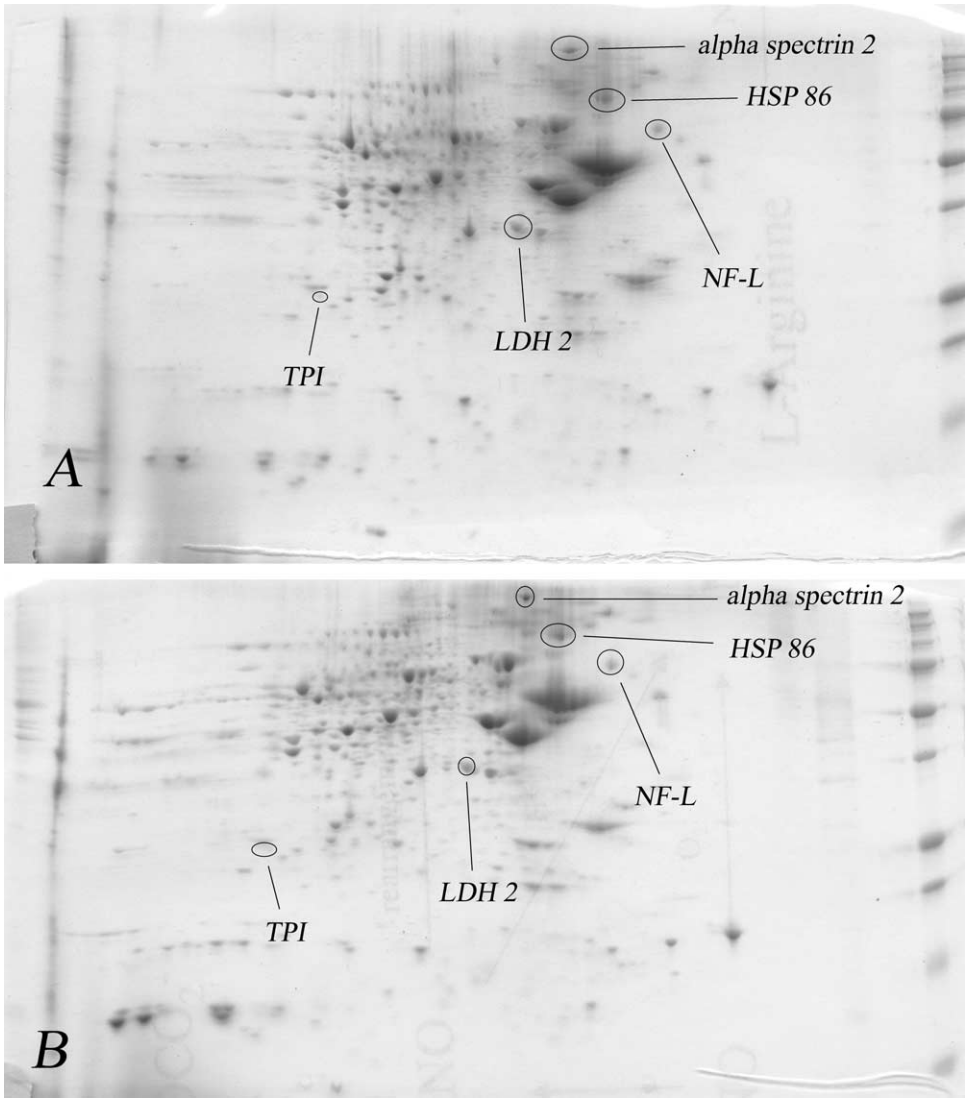

Fig. 1. (A) Proteins from 12-month-old SAMP8 brain. (B) Proteins from 4-month-old SAMP8 brain.

Butterfield and Stadtman, 1997; Butterfield et al., 1999; Fu et al., 2003; Ozaki et al., 2003), the above studies suggest that the observed LDH activity loss may be caused by the oxidative modification of the enzyme. Our current study shows direct evidence that LDH-2 is significantly modified by oxidative insults in aged SAMP8 brains.

TPI catalyzes the reversible interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate during glycolysis. A case study in 1982 reported that a 12-year-old girl who had chronic nonspherocytic hemolytic aemia, due to TPI deficiency, eventually developed cerebellar dysfunction and spasticity with hyperreflexia (Clay et al., 1982). Also, inhibition of TPI by α-monochlorohydrin causes decreased neuronal ATP production followed by progressive neuronal death (Sheline and Choi, 1998). Therefore, if TPI is oxidized or nitrated in AD (Castegna et al., 2003), lower activity of TPI should be observed. However, the activity of TPI is not altered in either AD patients (Meier-Ruge et al., 1984) or demented patients (Iwangoff et al., 1980). The current study provides a possible explanation: since the level of TPI in aged SAMP8 brains is significantly increased, the "non-specific" carbonyl level must also increase to maintain a similar (94%) specific carbonyl level between the 4-month-old and 12-month-old SAMP8. Thus, the increase in TPI levels compensate for the decreased activity of oxidized TPI. As a result, no net decrease in TPI activity is observed. Consistent with this explanation, the oxidative stress associated with hypoxia is accompanied by an increase in the TPI protein level (Lushchak et al., 1998). This is consistent with an upregulation of TPI to compensate for its loss of activity by oxidative modification.

CK, which is highly sensitive to oxidation, is found in the cytoplasm and mitochondria of cells which catalyze the reversible transfer of high energy phosphoryl groups between ATP and creatine phosphate (Schlegel et al., 1990;

Table 2. Change of protein expression in 12-month-old SAMP8 mice brains compared to 4-month-old SAMP8 mice brains (n=6 for each group)

Identified protein	Protein levels (% control±S.E.M.)	*P*-value
TPI	122±6	<0.01
LDH-2	22±4	<0.05
α-Spectrin 2	66±6	<0.005
NF-L	74±4	<0.05
hsp86	52±7	<0.005

Wallimann et al., 1992; Wyss et al., 1992; Kaldis et al., 1994). It is proposed that three CK genes exist (Eppenberger et al., 1967; Sobel et al., 1972; Roberts, 1980) to encode the three protein subunits, designated M (muscle), B (brain), and mitochondrial (Mi). These subunits form three dimeric cytosolic (MM, BB and MB) and one distinct mitochondrial (Mi-CKs) isoenzymes. In AD brains, the expression of CK-BB is decreased compared with age-matched controls (David et al., 1998). It is also well established that oxidative modification of CK-BB decreases its activity in aging, AD and other neurodegenerative diseases (Aksenova et al., 1998, 1999, 2000; Yatin et al., 1999; Castegna et al., 2002a). Our current study also shows that CK in aged SAMP8 brain is oxidized significantly, which therefore affects its activity to produce ATP.

Glucose metabolism has been reported to be reduced in aged SAMP8 mice (Shimano, 1998). Our current study indicates that the reduced ATP production is possibly caused by a loss of activity of specific glycolytic enzymes and of CK by oxidative modification. Since 20% of ATP in brain is produced from glycolysis, loss of activity of the glycolytic enzymes by oxidative modification would significantly decrease the energy availability to neurons and synaptic elements, and ATP is needed to defend against oxidative stress. It may be that this loss of glycolytic enzyme function is related to the abnormally low levels of acetylcholine found in aged SAMP8 brains and hippocampi (Ikegami et al., 1992). Choline is derived from serine, an endogenous product from glycolysis. The loss of activity of glycolytic enzymes and CK by oxidative modification could reduce the production of the substrate, glycerate-3-phosphate, needed to generate serine endogenously, therefore potentially accounting for the lowered the concentration of choline for acetylcholine synthesis. Our current study is consistent with the notion that oxidative modification of CK and glycolytic enzymes may be responsible for the altered level of acetylcholine reported in SAMP8 mice. In addition, ATP is needed for LTP and other

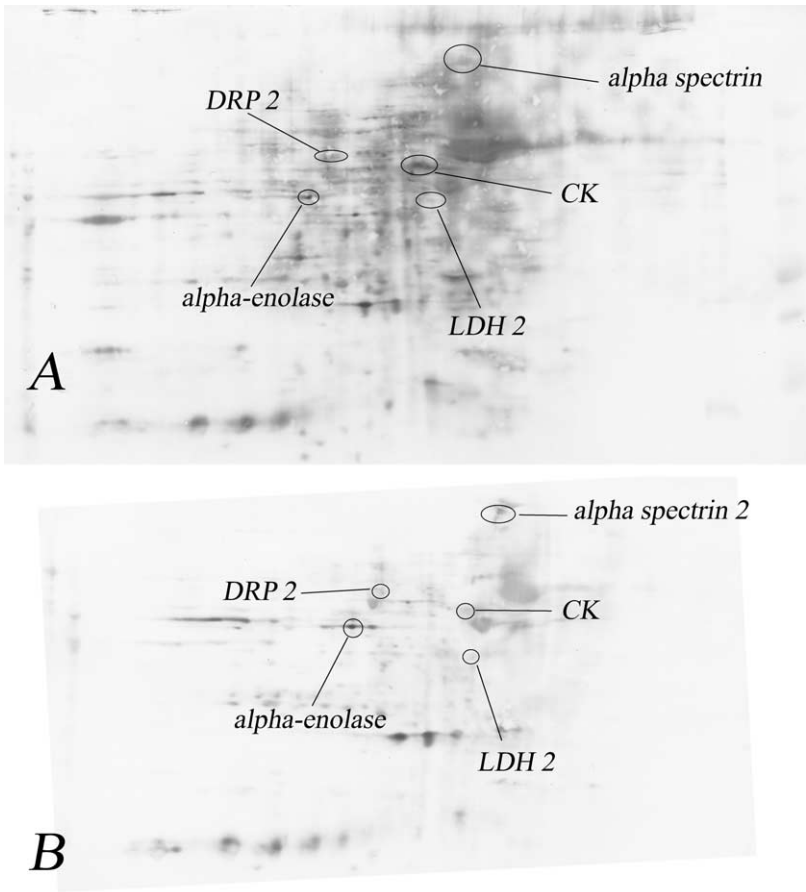

Fig. 2. (A) Carbonyl immuno-blot from 12-month-old SAMP8. (B) Carbonyl immuno-blot from 4-month-old SAMP8.

Table 3. Brain proteins oxidized in 12-month-old SAMP8 mouse compared to 4-month-old SAMP8 mice brains (n=6 for each group)

Identified protein	Specific carbonyl levels (% control±S.E.M.)	P-value
α-Enolase	3920±1879	<0.07
LDH-2	4224±1853	<0.005
CK	322±72	=0.05
DRP-2	443±130	<0.05
α-Spectrin 2	195±34	<0.05

modes of learning and memory (Wieraszko and Ehrlich, 1994; Fujii et al., 1995; Chen et al., 1996; Hoyer, 2003; Yamazaki et al., 2003). If ATP levels are decreased due in part to oxidized α-enolase in aged SAMP8 mice, learning and memory could be affected.

DRP-2 is one of the four members of the dihydropyrimidinase-related protein family (DRP-1, -2, -3 and -4), which were originally identified in humans by their homology to dihydropyrimidinase (Hamajima et al., 1996; Wang and Strittmatter, 1996; Kato et al., 1998). Other non-human counterparts of the human DRPs are chicken collapsin response mediator protein (CRMP-62; Goshima et al., 1995), rat turned on after division (TOAD)-64 (Minturn et al., 1995), and mouse unc-33-like phosphoprotein (Ulip). The DRP family is involved in axonal outgrowth and path finding through transmission and modulation of extracellular signals (Goshima et al., 1995; Minturn et al., 1995; Byk et al., 1996). It was reported that CRMP-2 can induce growth cone collapse (Goshima et al., 1995; Wang and Strittmatter, 1996) by Rho-kinase phosphorylation (Arimura et al., 2000), and by binding to tubulin heterodimers and bundled microtubules as carriers to promote microtubule assembly and dynamics (Gu and Ihara, 2000; Fukata et al., 2002). Many neurodegenerative diseases are associated with DRP-2. The mutation in the unc-33 gene results in uncoordinated movements and abnormal swelling of axonal endings with premature termination (Pasterkamp et al., 1998). Decreased expression of DRP-2 protein has been observed in AD, adult Down syndrome (DS; Lubec et al., 1999), fetal DS (Weitzdoerfer et al., 2001), schizophrenia, and affective disorders (Johnston-Wilson et al., 2000). The deranged DRP-2 mRNA level in DS (Lubec et al., 1999) and the increased specific carbonyl level of DRP2 in AD (Castegna et al., 2002b) were reported previously. These studies suggested that the loss of DRP-2 activity, resulting from either reduced expression or oxidative modification, disturbs neural development and plasticity in the CNS, resulting in mental retardation and impairment in learning and memory. Our study here found that the oxidative modification of DRP2 is significantly increased in the 12-month-old SAMP8 mouse brain. This suggests that oxidative modification of DRP2 plays an important role in the memory and learning deficit observed in aged SAMP8. For example, one can conceive scenarios by which shortened dendrite lengths, due in part to oxidative modification of DRP2, would lead to decreased interneuronal communication, thereby, affecting learning and memory.

Neurofilaments (NFs) are axonal proteins that give axons their structure and define axonal diameter (Hoffman et al., 1987; Brady, 1993). NFs are composed of light (NF-L), medium (NF-M) and heavy (NF-H) subunits and assemble to form long macromolecular filaments in a 6:2:1 ratio. Since the nature of NFs is dynamic, the individual NF proteins are turned over or exchanged within NFs in the axon (Okabe et al., 1993; Takeda et al., 1994). Modification of the NFs structure results in the destabilization of the interactions between the NF proteins. Such destabilization is particularly damaging to motor neurons, which possess elongated axonal length and high axonal constitution, since motor neurons contain more NFs than other neurons (Crow et al., 1997). Transgenic mice expressing point mutation in NF-L and mice overexpressing either NF-L or NF-H display accumulations of disarrayed filaments in motorneuronal perikarya and proximal axons, and such mice developed motor neuron disease (Cote et al., 1993; Lee et al., 1993). Oxidation and nitration of NF proteins will transform the α-helix to β-sheet and random coil conformations (Gelinas et al., 2000), and these oxidized proteins will then be degraded by proteases (Grune et al., 1996; Davies, 2001; Inai and Nishikimi, 2002; Grune et al., 2003). Consequently, oxidative modification could be responsible for the NF abnormalities observed in several oxidative-stress-related neurodegenerative diseases notably AD, Parkinson's disease, and amyotrophic lateral sclerosis (ALS; Goldman et al., 1983; Ulrich et al., 1987a,b; Manetto et al., 1988; Munoz et al., 1988; Toyoshima et al., 1989; Zhang et al., 1989; Cammarata et al., 1990; Schmidt et al., 1991; Troost et al., 1992). The level of NF-L was also reported as decreased in AD, DS, ALS brains (Bergeron et al., 1994; Bajo et al., 2001). However, in the cerebrospinal fluid of AD and vascular dementia patients and aged human, the level of NF-L is increased (Hu et al., 2002). This increase could be caused by the discharge of abnormal NF-L, possibly by oxidative modifications, from the brain. It is known that there is a decrement in the transcription rate and mRNA level of NF-L in aged male Fischer 344 rat brains (Krekoski et al., 1996). Consistent with this result, the expression NF-L in SAMP8 brains is significantly decreased in aged SAMP8 brain, suggesting the decreased level of NF-L in brain caused the increased axonal dystrophy in the gracile nucleus observed in aged SAMP8 mice (Kawamata et al., 1998). Similarly, in the brain from gracile axonal dystrophy mice, NF-L is oxidized (Castegna et al., 2004).

The spectrins are a family of widely distributed filamentous proteins. α-Spectrin, a component of the membrane-associated cytoskeleton, forms a supporting and organized scaffold for intracellular cohesion with the association of actins (Leto et al., 1988). In rats, the mRNA level of α-spectrin increases gradually during the first postnatal days and reach a plateau between the second and third week of life. This is followed by a decline in levels throughout the brain (Gelot et al., 1994). This temporal expression suggests that α-spectrin is important during CNS development and normal function. The breakdown products of α-spectrin from calcium-activated proteolysis are commonly used as markers of apoptosis (Vanderklish and

Bahr, 2000). Aβ can also induce these α-spectrin breakdown products in cultured rat cortical neurons by activating caspases (Harada and Sugimoto, 1999). Similar increases of α-spectrin breakdown products are observed in some regions of the aged Balb/c mice brain (Bahr et al., 1991), indicating the level of α-spectrin may decreases as function of age (Bahr et al., 1994). Consistent with these studies, our results here show a decreased level of α-spectrin in aged SAMP8 mouse brain, as well as an increased specific carbonyl level, suggesting that the proteolytic mechanism in apoptosis involves oxidative modification and degradation of α-spectrin. This suggests that loss of α-spectrin by oxidation or degradation would disrupt the cytoskeleton and the structure of cells in brain, thereby affecting intercellular and intracellular communications, and consequently causing the learning and memory deficits observed in SAMP8 mice. Moreover, degradation of the intact cytoskeleton by Ca^{2+}-sensitive proteinase may be involved in memory recall (Lynch and Baudry, 1984). Therefore, the findings represented here are consistent with the hypothesis of decreased learning and memory in aged SAMP8 mice.

DRP-2, α -spectrin, and NF-L are involved in signaling, intracellular trafficking and maintaining structure of dendrites and axons in neurons. Increased oxidation or reduced expression of these proteins may account for the neuronal atrophy and loss in the posterior cholinergic column, reduction of dendritic spines in the hippocampal pyramidal neurons, and increased axonal dystrophy in the gracile nucleus all observed in aged SAMP8 (Kawamata et al., 1998). We hypothesize that these physiological alterations may further disrupt the communication between neurons, resulting in learning and memory impairments in aged SAMP8 mice.

Heat shock proteins are a group of proteins whose syntheses are induced when cells in culture are exposed to heat and/or chemical stresses (Welch, 1992; Calabrese et al., 2004; Poon et al., 2004). The most highly expressed heat shock protein in unstressed cells is the 90 kDa heat shock protein (hsp90) (Perdew et al., 1993). While most studies examine hsp90 as a single protein, there are two separate structural genes, hsp86 and hsp84 in the mouse (Moore et al., 1989, 1990), or hsp89α and hsp89β in the human (Rebbe et al., 1989). The sequences of hsp90-related proteins are highly conserved among vertebates (Perdew et al., 1993). Although hsp84 and hsp86 are highly conserved, examination of the level of expression of hsp86/hsp84 in murine tissues revealed that hsp86 is expressed in brain, testes, and placenta, whereas hsp84 is highly expressed in liver, thymus, kidney, and other tissues (Lee, 1990). It was shown that hsp90 has the ability to bind to actin (Koyasu et al., 1989) in a Ca^{2+}-calmodulin-dependent manner (Koyasu et al., 1986, 1989) and interact with many receptors and kinases (Brugge, 1986; Koyasu et al., 1986; Ziemiecki et al., 1986; Perdew, 1988; Matts and Hurst, 1989; Rose et al., 1989; Pratt, 1990; Miyata and Yahara, 1992, 1995). These studies suggest that hsp90 plays a critical role in cell signaling for calcium homeostasis, apoptosis, and cell cycle processes. Hsp90 is also involved in protecting protease activity from oxidative modification (Conconi et al., 1996; Conconi and Friguet, 1997), indicating hsp90 possesses antioxidant activity. Hsp90 (hsp84) level is also decreased in livers of aged rats (Nardai et al., 2002). Consistent with this study, we found here that levels of hsp86 are decreased in aged SAMP8 brains, suggesting that the weakened antioxidant defense in aged SAMP8 may contribute to the increased oxidative modification of proteins (Butterfield et al., 1997; Farr et al., 2003). Moreover, decreased chaperone function might result, leading to increase damaged or aggregated proteins that in turn could affect learning and memory. These conditions could contribute to the neurochemical and behavioral changes observed in SAMP8 mice.

In our current study, we have identified the proteins that are oxidatively modified, and/or differently expressed, in SAMP8 mouse brain as result of senescence. These proteins are critical to energy utilization and metabolism, structure, interneuronal communications, and antioxidant defense of the brain. The oxidative inactivation of LDH, α-enolase and TPI may be responsible for abnormal metabolism (Shimano, 1998) and neurochemical changes (Nardai et al., 2002) in SAMP8 mice brain. The abnormality of DRP2, α-spectrin and NF-L may be responsible for the axonal dystrophy (Kawamata et al., 1998) observed in SAMP8; and decreased hsp86 level may contribute to the increased oxidative parameters in SAMP8 mice brains (Butterfield et al., 1997). Therefore it is possible that the loss of the activities of these proteins by oxidative modification, or by decreased expression, may contribute to the abnormal metabolism (Shimano, 1998) and neurochemical changes (Nardai et al., 2002) seen in SAMP8 mice and might ultimately contribute to their deficits in learning and memory. Therefore, assay of these proteins will be needed to investigate their putative inactivation by oxidative modification. How our findings in aged SAMP8 mice related to normal aging remains to be elucidated. Conceivably, the change of protein specific carbonyl and expression levels reported in our study may be found in normal aging as well. Proteomics comparison between young and normal aging mice is in progress. Nevertheless, our current study forms a framework for subsequent experiments and provides evidence that oxidative stress affects specific proteins in ways that could result in deficits in learning and memory.

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