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Proteomic analysis of specific brain proteins in aged SAMP8 mice treated with alpha-lipoic acid: implications for aging and age-related neurodegenerative disorders

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Abstract

Free radical-mediated damage to neuronal membrane components has been implicated in the etiology of Alzheimer's disease (AD) and aging. The senescence accelerated prone mouse strain 8 (SAMP8) exhibits age-related deterioration in memory and learning along with increased oxidative markers. Therefore, SAMP8 is a suitable model to study brain aging and, since aging is the major risk factor for AD and SAMP8 exhibits many of the biochemical findings of AD, perhaps as a model for and the early phase of AD. Our previous studies reported higher oxidative stress markers in brains of 12-month-old SAMP8 mice when compared to that of 4-month-old SAMP8 mice. Further, we have previously shown that injecting the mice with α-lipoic acid (LA) reversed brain lipid peroxidation, protein oxidation, as well as the learning and memory impairments in SAMP8 mice. Recently, we reported the use of proteomics to identify proteins that are expressed differently and/or modified oxidatively in aged SAMP8 brains. In order to understand how LA reverses the learning and memory deficits of aged SAMP8 mice, in the current study, we used proteomics to compare the expression levels and specific carbonyl levels of proteins in brains from 12-month-old SAMP8 mice treated or not treated with LA. We found that the expressions of the three brain proteins (neurofilament triplet L protein, α-enolase, and ubiquitous mitochondrial creatine kinase) were increased significantly and that the specific carbonyl levels of the three brain proteins (lactate dehydrogenase B, dihydropyrimidinase-like protein 2, and α-enolase) were significantly decreased in the aged SAMP8 mice treated with LA. These findings suggest that the improved learning and memory observed in LA-injected SAMP8 mice may be related to the restoration of the normal condition of specific proteins in aged SAMP8 mouse brain. Moreover, our current study implicates neurofilament triplet L protein, α-enolase, ubiquitous mitochondrial creatine kinase, lactate dehydrogenase B, and dihydropyrimidinase-like protein 2 in process associated with learning and memory of SAMP8 mice.

Keywords: Brain protein; Alzheimer's disease; Proteomics; Aging; Oxidative modification effects on learning and memory

1. Introduction

Aging and age-related neurodegenerative disorders are becoming more important as the mean age of the United States' population increases. One of the most compelling theories explaining the diseases of aging is the role of free radical-induced oxidative stress in aging (Harman, 1994; Butterfield et al., 1997; Butterfield and Stadtman, 1997). Free radical-mediated damage to neuronal membrane components also are implicated in aging, as well as the etiology of Alzheimer's disease (AD; Selkoe, 1996).

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Numerous lines of genetic and biochemical evidence suggest that amyloid β-peptide (Aβ) is central to the pathogenesis of AD (Butterfield et al., 2001a,b; Kanski et al., 2002), and Aβ-associated oxidative stress induces damage to neurons in vitro (Yatin et al., 1999; Varadarajan et al., 2000, 2001; Butterfield, 2002; Kanski et al., 2002) and in vivo (Yatin et al., 1999; Drake et al., 2003).

One of the animal models that are used to study AD and aging is the senescence-accelerated mouse (SAM). In 1981, Takeda et al. established several SAM lines using phenotypic selections from a common genetic pool (Takeda et al., 1981). The SAMP8 strain exhibits age-related deterioration in memory and learning (Yagi et al., 1988; Ohta et al., 1989) along with increased oxidative markers (Butterfield et al., 1997; Farr et al., 2003) and expression of amyloid precursor protein (APP) (Nomura et al., 1996; Morley et al., 2000). SAMP8 mice also show decreased glucose metabolism (Shimano, 1998), and also in AD energy metabolism is impaired (Blass et al., 1988). Therefore, SAMP8 is a good model to study brain aging and is used as one mouse model of AD.

Ongoing research is being pursued for development of therapeutics for AD, among which are those that scavenge free radicals by antioxidants (Butterfield et al., 2002a,b). α-Lipoic acid (LA), a coenzyme involved in production of ATP in mitochondria, is a potent antioxidant (Packer et al., 1997a,b; Liu et al., 2002). LA derives its neuroprotective capability from its ability to: (a) act as a scavenger of reactive oxygen species (ROS); (b) chelate metal ions; (c) recycle endogenous antioxidants (Lynch, 2001); and (d) bind to reactive aldehydes, such as 4-hydroxynonenal (HNE) and acrolein (Humphries and Szweda, 1998; Korotchkina et al., 2001) formed by lipid peroxidation (Esterbauer et al., 1991). LA can scavenge singlet oxygen, H_2O_2, $HO^{\bullet}$, NO and $ONOO^{-}$, and the reduced form of LA, dihydrolipoic acid (DHLA), can further scavenge $O_2^{\bullet -}$ and peroxyl radical. LA can also chelate several divalent cations, e.g., Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+} to inhibit Fenton reactions for $HO^{\bullet}$ production, as well as ascorbate-induced H_2O_2 production (Kagan et al., 1992). LA can recycle endogenous antioxidants, e.g., GSH (Kagan et al., 1992) and vitamin C (Sen et al., 1997), which protect the brain from oxidative stress (Butterfield et al., 2001a; Drake et al., 2002). It was shown that LA improves memory in aged female NMR1 and 12-month-old SAMP8 mice (Stoll et al., 1993; Farr et al., 2003). LA can also reverse partial brain mitochondrial decay, RNA/DNA oxidation and memory loss in old rats (Liu et al., 2002; Farr et al., 2003). The beneficial effect of LA in AD was demonstrated when patients were treated with LA for approximately one year, resulting in mild cognitive improvements (Kagan et al., 1992).

Increased protein oxidation and lipid peroxidation were demonstrated in 12-month-old SAMP8 mouse brains when compared to 4-month-old SAMP8 and the same age SAMR1 mice (Butterfield et al., 1997; Farr et al., 2003). The increases in protein oxidation and lipid peroxidation seen in aged SAMP8 mouse brains were reversed by injecting the mice with LA (Farr et al., 2003). We rationalized that LA improves the learning and memory in SAMP8 mouse by reducing oxidation and changing the expression of specific proteins in SAMP8 mice brain. To test this hypothesis, we used proteomics to identify the proteins that are expressed differently and/or the proteins that are less oxidized in the 12-month-old SAMP8 mouse brain treated with LA.

2. Materials and methods

2.1. Subjects

Experimentally naive, 12-month-old male SAMP8 mice were obtained from our breeding colony. The colony is derived from siblings generously provided by Dr. Takeda (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, PA). The colony routinely undergoes serological testing for viral and bacterial contamination, and has remained free of pathogens for over 12 years. Mice are housed in rooms with a 12-h light:12-h 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.

2.2. Drug administration

LA as a racemic mixture was a gift from Jarrow Formulas, Inc. (Los Angeles, CA). LA was dissolved in saline at pH 7.0. LA (100 mg/kg) or saline were administered subcutaneously once daily for 4 weeks at 1 p.m.

2.3. Sample preparation

The whole brains of SAMP8 mice were flash frozen in liquid nitrogen in the laboratory of Geriatric Research Education and Clinical Center (GRECC), VA Medical Center, St. Louis and sent to the Chemistry Department, University of Kentucky, Lexington on dry ice overnight. The whole 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, IL).

2.4. Two-dimensional electrophoresis

Samples of brain proteins were prepared according to the procedure of Levine et al. (1994). 200 μg of protein was incubated with four volumes of 2N HCl at room temperature (25 °C) for 20 min. Proteins were then precipitated by the addition of ice-cold 100% trichloroacetic acid (TCA) to obtain a final concentration of 15% TCA final. Samples are placed on ice for 10 min to let protein precipitate. 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 was applied to a ReadyStrip™ IPG strip (Bio-Rad, CA). The strips were soaked in the sample solution for 1 h to ensure uptake of the proteins. The strip was then actively rehydrated in the 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 above processes were carried out at 22 °C. The strip was stored in −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 6 M 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™ Standards (Bio-Rad, CA) 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 was used to stain the gel for 2 h. The gels were destained in deionized water overnight.

2.5. Western blotting

200 μg of protein 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 for 2-D-electrophoresis. After the second dimension, the proteins from gels were transferred to nitrocellulose papers (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 (Chemicon, CA) specific for DNP-protein adducts (1:100), and then a secondary goat anti-rabbit IgG (Sigma, MO) antibody was applied. The resultant stain was developed by application of Sigma-Fast (BCIP/NBT) tablets.

2.6. Image analysis

The gels and nitrocellulose papers 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. After completion of spot matching, the normalized intensity of each protein spot from individual gels (or oxyblots) was compared between groups using statistical analysis.

2.7. Trypsin digestion

Samples were prepared using techniques described by Jensen et al. (1999) and modified by Thongboonkerd et al. (2002). 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.

2.8. Mass spectrometry

All mass spectra reported in this study were acquired from the University of Kentucky Mass Spectrometry Facility (UKMSF). LC/MS/MS spectra were acquired on a Finnigan LCQ 'Classic' quadrupole ion trap mass spectrometer (Finnigan). Separations were performed with an HP 1100

Table 1
Summary of proteins identified by mass spectrometry

Identified protein	GI accession number	Number of peptide matches identified	% coverage matched peptides	pI, MrW	Mowse score	Probability of a random hit
Lactate dehydrogenase 2 (LDH 2)	gi|6678674	14	41	5.87, 36.6	632	2×10^{-62}
α-Enolase	gi|12963491	17	47	6.69, 47.1	947	2×10^{-95}
Dihydropyrimidinase-like 2 (DRP-2)	gi|1351260	14	35	6.16, 62.16	776	2.5×10^{-78}
Ubiquitous mitochondrial creatine kinase (uMiCK)	gi|6753428	8	14	8.65, 47.7	245	3.2×10^{-25}
Neurofilament-L (NF-L)	gi|417355	18	37	4.40, 61.5	922	6.3×10^{-100}

Mowse scores greater than 40 are considered significant ($p < 0.05$).

HPLC modified with a custom splitter to deliver 4 μL/min to a custom C18 capillary column (300 μm i.d. × 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). For the 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 searching.

2.9. Statistics

The data of protein levels and protein specific carbonyl levels of six animals per group (control and LA-treated group) were analyzed by Student's *t*-tests. A value of $p < 0.05$ was considered statistically significant. Only the proteins that are considered significantly different by Student's *t*-test are reported.

3. Results

3.1. Protein expression levels

Proteomics has been used to study oxidized proteins in AD (Castegna et al., 2002a,b, 2003; Butterfield, 2004). We used a similar parallel approach to investigate the effect of LA treatment in aged SAMP8 mice. Fifty-six proteins were screened. We found that expression of three proteins was significantly increased and that the specific carbonyl levels of three proteins were significantly decreased in aged 12-month-old SAMP8 mouse brains when compared to that in 12-month-old SAMP8 mice not treated with LA ($n = 6$ in each group). All the mass spectra of the identified proteins were matched to the mass spectra in NCBI protein databases. All identified proteins (Table 1) appeared in the expected MrW and pI range on the gels.

Fig. 1 shows the 2-D-electrophoresis gels after Coomassie blue staining. The levels of the proteins that are expressed differently are summarized in Table 2. We report here that the expressions of brain resident neurofilament triplet L protein (NF-L), α-enolase, and ubiquitous mitochondrial creatine kinase (uMiCK) are significantly increased in aged SAMP8 mice injected with LA compared to aged, non-treated SAMP8 mice.

3.2. Specific protein carbonyl levels

Fig. 2 shows the resultant nitrocellulose membranes for protein carbonyl detection. The summary of the proteins that have significantly increased specific carbonyl levels is given in Table 3. We show that the specific protein carbonyl levels of lactate dehyrogenase 2 (LDH-2), dihydropyrimidinase-like protein 2 (DRP-2), and α-enolase are significantly decreased in the brains of aged SAMP8 mice treated with LA.

4. Discussion

LA is able to partially reverse memory loss in normal aging rats by delaying mitochondrial dysfunction and RNA/DNA oxidation (Liu et al., 2002). In SAMP8 mice, LA treatment markedly improved learning and memory of even older rodents (Farr et al., 2003). Mitochondrial dysfunction is accompanied by a leakage of $O_2^{\bullet}$ and H_2O_2. Since LA is readily taken up into mitochondria, it is suggested that LA may be a useful therapeutic agent in diseases that are characterized by mitochondria dysfunction or oxidative stress (Packer et al., 1997a,b; Lynch, 2001). We report here that in brains of aged SAMP8 mice treated with LA, the specific carbonyl levels of LDH-2, DRP-2, and α-enolase

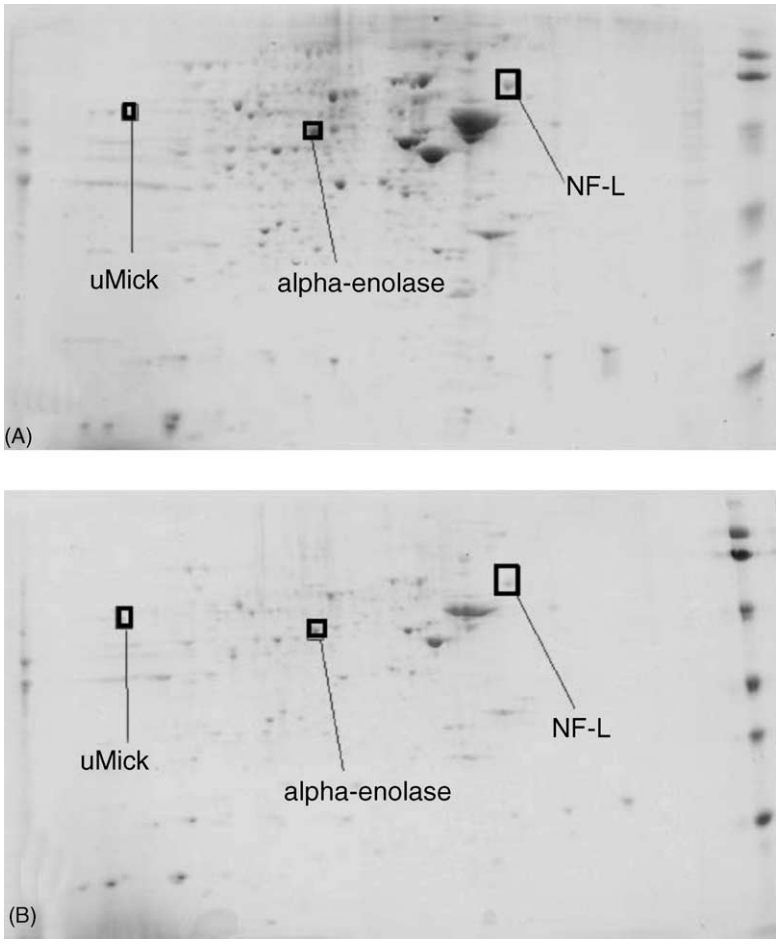

Fig. 1. (A) Representative gel of proteins from 12-month-old SAMP8 mice brain treated with LA. (B) Representative proteins from 12-month-old SAMP8 mice brain not treated with LA.

are significantly decreased and the expression levels of the α-enolase, NF-L, and uMiCK, are increased in comparison to aged SAMP8 mice not treated with LA.

α-Enolase and DRP-2 is known to be more oxidized in AD (Castegna et al., 2002a,b). Our previous study showed that the specific carbonyl levels of α-enolase, DRP-2, LDH-2, creatine kinase (CK), and α-spectrin are increased in aged SAMP8 brains (Poon et al., 2004). We report here that injecting LA can decrease the specific carbonyl levels of α-enolase, DRP-2, and LDH-2 and can increase the protein levels of uMiCK, α-enolase, and LDH-2 in aged SAMP8 mice brains. These alterations of proteins may be responsible for the improvement of learning and memory in LA-injected SAMP8.

DRP-2 is involved in axonal outgrowth and path finding through transmission and modulation of intracellular signals. (Goshima et al., 1995; Minturn et al., 1995; Byk et al., 1996). DRP-2 can induce growth cone collapse (Goshima et al., 1995; Wang and Strittmatter, 1996). Decreased expression or increased oxidation of DRP-2 in AD (Castegna et al., 2002b), adult DS (Lubec et al., 1999), fetal DS (Weitzdoerfer et al., 2001), schizophrenia and affective disorders suggest that the DRP-2 activity is decreased in these disorders. An increased specific carbonyl level of DRP-2 is also observed in aged SAMP8 brains (Poon et al., 2004), suggesting the oxidative deactivation of DRP-2 may be responsible for the impairment of learning and memory in aged SAMP8. We show here that the

Table 2
Proteins expressed differently when 12-month-old SAMP8 mice are treated with LA compared to non-treated 12-month-old SAMP8 mice

Identified proteins	Protein levels of 12-month-old SAMP8 injected with LA[a] (n = 6) (%)	p-value
Neurofilament triplet L protein (NF-L)	625 ± 58	<0.005
α-Enolase	143 ± 47	<0.05
Ubiquitous mitochondrial creatine kinase (uMiCK)	183 ± 28	<0.05

[a] Percent of the level found in brain from 12-month old, not-treated SAMP8 mice.

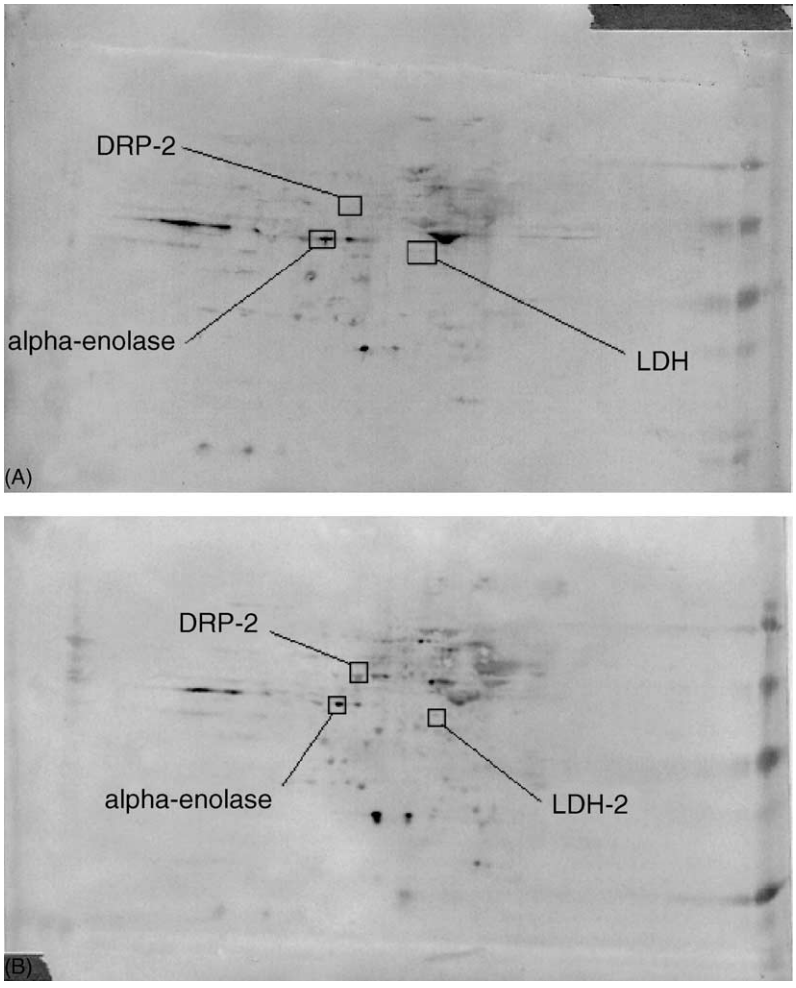

Fig. 2. (A) Representative carbonyl Western blot from 12-month-old SAMP8 mice treated with LA. (B) Representative carbonyl Western blot from 12-month-old SAMP8 mice not treated with LA.

oxidative modification of DRP2 is significantly reduced by treating the aged SAMP8 mice with LA, possibly resulting in restoration of DRP-2 activity, and thus, the normal neurogenesis of axons.

α-Enolase, a subunit of enolase, is also found oxidized in aged SAMP8 mouse brains (Poon et al., 2004). Enolase is involved not only in metabolism but also in cell differentiation and normal brain growth. Although the levels of neuronal specific enolase (NSE) are not significantly altered in aged brains (Kato et al., 1990) and in AD brains (Kato et al., 1991), the specific carbonyl level and protein level of α-enolase are increased in AD (Schonberger et al., 2001; Castegna et al., 2002b), suggesting the reduced enolase activity caused by oxidation is compensated for by its increased expression. The decline of enolase activity results in abnormal growth and reduced metabolism in brains (Tholey et al., 1982), therefore, oxidation of α-enolase observed in aged SAMP8 brains (Poon et al., 2004) may be responsible for the reduced ATP production (Shimano) and acetylcholine concentration (Ikegami et al., 1992) seen in the brains of aged SAMP8 mice. Our current study shows that injecting the SAMP8 mice with LA is able to reduce the oxidative modification and increase the protein level of α-enolase, suggesting the possibility that the

Table 3
Proteins that have specific carbonyl level decreased by LA

Identified proteins	Specific protein carbonyl levels of 12-month-old SAMP8 injected with LA[a] (n = 6) (%)	p-value
Lactate dehyrogenase 2 (LDH-2)	11.5 ± 10.2	<0.01
Dihydropyrimidinase-like 2 (DRP-2)	16.4 ± 16.4	<0.05
α-Enolase	0.17 ± 0.17	0.05

[a] Percent of the level found in brain from 12-month old, not-treated SAMP8 mice.

activity of α-enolase may be restored. Therefore, the reduced glucose metabolism and neurochemical alterations in SAMP8 mouse brains (Ikegami et al., 1992; Shimano) are possibly reversed by the LA injections as well.

LDH-B is a subunit of lactate dehydrogenase (LDH), which catalyzes the reversible NAD-dependent interconversion of pyruvate to L-lactate. This may be an important step for neuroprotection as lactate is considered the only oxidizible energy substrate available to support neuronal recovery (Schurr et al., 1997a,b). Although the activity of LDH shows no significant alteration in AD (Chandrasekaran et al., 1994), many studies showed that LDH activity in rat brains declined in advanced age (Mizuno and Ohta, 1986; Ferrante and Amenta, 1987; Hrachovina and Mourek, 1990; Agrawal et al., 1996), suggesting the LDH activity loss may be caused by the oxidative modification of the enzyme. Oxidation and depleted levels of LDH were observed in aged SAMP8 mouse brain, suggesting LDH activity is also impaired in aged SAMP8 brain (Poon et al., 2004). Our current study shows that the carbonyl level of LDH decreases when SAMP8 mice are injected with LA, suggesting that treatment of LA can possibly restore the ability of LDH to produce lactate in aged SAMP8. Therefore, recovered LDH activity can possibly facilitate neuronal recovery, and thus improvement in learning and memory, in aged SAMP8 mice.

uMiCK, one of the isoenzymes of creatine kinase (CK), is considered as the counterpart of cytoplasmic brain-type creatine kinase (CK-BB) in brain (Kanemitsu et al., 2000). uMiCK is responsible for the transfer of a high-energy phosphoryl group (Pi) from mitochondria to the cytosolic carrier and creatine (Payne and Strauss, 1994). In neurons, a coordinated pattern in expression of CKBB and uMiCK was demonstrated (Friedman and Roberts, 1994), suggesting metabolic energy was transferred via a creatine phosphate energy shuttle between cytosol and mitochondria. Recently, it was proposed that uMiCK couples with CK to regulate ATP concentration in cerebral gray matter (Kekelidze et al., 2001). Moreover, the mitochondrial synthesis of creatine phosphate is restricted to uMiCK expressing neurons, suggesting uMiCK protects neurons from energy shortage during periods of increased energy demands (Boero et al., 2003). It was suggested that the increased oxidation of CKBB (Poon et al., 2004) could be the cause of the decreased production of ATP observed in aged SAMP8 mice brains (Shimano, 1998). Increasing uMiCK expression by LA injection conceivably can compensate for the decreased ATP synthesis due to the loss of CKBB activity in aged SAMP8 brains. This would result in restoration of ATP production for antioxidant defensive systems in neurons and synaptic elements.

Neurofilament-L is a subunit of neurofilaments (NFs), which give axons their structure and diameter (Hoffman et al., 1987; Brady, 1993). Depletion of NF-L level in AD, DS, and ALS brains (Bergeron et al., 1994; Bajo et al., 2001) indicates that the normal NF-L expression is critical to central nervous system (CNS) functions. It was suggested that the decreased NF-L expression in SAMP8 brains causes the increased axonal dystrophy in the gracile nucleus (Poon et al., 2004). We report here that treating SAMP8 mice with LA can raise the level of NF-L in aged SAMP8 brains and possibly decrease the axonal dystrophy in the gracile nucleus, which consequently improves the learning and memory in aged SAMP8.

Our previous study showed that LA reverses learning and memory impairment by decreasing protein oxidation and lipid peroxidation of SAMP8 mice brains (Farr et al., 2003). We now identify the specific proteins (DRP-2, α-enolase, LDH, CK, and NF-L) that are protected by LA injection. LA is a disulfide compound that is found naturally in mitochondria as a coenzyme for pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGD). Therefore, LA protection in aged SAMP8 mouse brains is possibly owed to its free radical scavenging ability and its accessibility to mitochondria. Since LDH and enolase are involved in the oxidoreduction-phoshphorylation stage of glycolytic pathways, they locate near the mitochondria for substrate accessibility and become the immediate targets for free radicals produced by mitochondria. Since LA has easy access to mitochondria, LA can scavenge the free radicals produced by mitochondria, or may even reduce the oxidation on the LDH and α-enolase. As a result, a significant decrease of specific carbonyl levels in α-enolase and LDH-2 are observed in SAMP8 brains. The increased level of α-enolase is possibly in response to the recovered activity of LDH, which, in turn, is caused by a reduction of oxidative damage following LA treatment. Demand for pyruvate is increased as the restored LDH activity speeds up the conversion of pyruvates to lactate. In order to meet this demand, enolase levels are increased to produce sufficient phosphophenol pyruvate (PEP) for pyruvate production. The standard free energy of conversation from 2-phospho-D-glycerate to PEP is −14.8 kcal/mol. This suggests that increasing the level of the catalytic enolase, which mediates this high-energy and rate limiting reaction, is the most efficient way to control the substrate concentration for its downstream reactions. Increased uMiCK is possibly controlled by similar mechanisms. Since a Pi is eliminated from PEP to form pyruvate, extra creatine kinase activity will be needed to transfer Pi to creatine. Therefore, the level of uMiCK is increased to reduce the Pi level in SAMP8 mice brain. This indicates that LA can rescue the energy depletion and neurochemical changes observed in aged SAMP8 mouse brains (Ikegami et al., 1992; Shimano, 1998) by improving the function of the glycolytic pathway and increasing NADPH production.

γ-Enolase, encoded by a single gene as α-enolase, is associated with DRP-2 in the complex of NADH-dichlorophenol-indophenol (DCIP) reductase (Bulliard et al., 1997). DCIP reductase, one of the trans-plasma-membrane oxidoreductases (PMOs), is involved in redox control of receptor function through the receptor-mediated signal-transduction pathway (Fuhrmann et al., 1989; Toole-Simms et al., 1991). More importantly, it is involved in

control of cell growth and development in response to external oxidative stress or anti-oxidants (Bulliard et al., 1997). Therefore, the restored enolase activity may trigger the decrease in the carbonyl level of DRP-2 in the DCIP reductase complex, which in turn signals within the cells that the oxidative stress is relieved by the LA treatment. The cells, consequently, increase NF-L production to restore the normal structure of the neuron for recovery. However, more experiments are needed to investigate the putative relationship between enolase and DRP-2, as well as their roles in the signaling of DCIP reductase complex and oxidative stress.

Although no direct evidence is provided in the current study that injection of LA can reverse neurochemical changes, increase ATP production, and improve learning and memory in aged SAMP8 mice, we suggest here that the improvement of learning and memory in LA treated SAMP8 mice is associated with the reduced specific carbonyl levels and increased the protein levels of specific proteins. The latter are involved in metabolism (LDH-2, α-enolase, and uMiCK), cell signaling (DRP-2), and axonal structure (NF-L) in cells. The possibly improved glycolytic pathway induced by reduction of enzyme oxidation may provide sufficient energy for neuronal recovery from oxidative stress that may be consequently responsible for the improvement of learning and memory in aged SAMP8 mice treated with LA. Therefore, improvement in energy metabolism may lead to, at least partially, neuronal recovery, such as a restored cytoskeletal network and enhancement of inter-neuronal communication in aged SAMP8 mice. However, further studies will be needed to investigate the relation among enolase, DRP-2, and NF-L in the recovery of neuronal function (Farr et al., 2003). Here, we used proteomics to gain insight of the mechanism of cognitive improvement by LA injection in SAMP8 mice. To our knowledge, this is the first reported study using proteomics to better understand cognitive improvement following lipoic acid administration.

Our current study suggests that the improved learning and memory observed in LA-injected SAMP8 is associated with the restoration to the normal condition of oxidized DRP-2, α-enolase, LDH, uMiCK, and NF-L in aged SAMP8 mouse brains.

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References

Agrawal, A., Shukla, R., Tripathi, L.M., Pandey, V.C., Srimal, R.C., 1996. Permeability function related to cerebral microvessel enzymes during ageing in rats. Int. J. Dev. Neurosci. 14, 87–91.

Bajo, M., Yoo, B.C., Cairns, N., Gratzer, M., Lubec, G., 2001. Neurofilament proteins NF-L, NF-M and NF-H in brain of patients with Down syndrome and Alzheimer's disease. Amino Acids 21, 293–301.

Bergeron, C., Beric-Maskarel, K., Muntasser, S., Weyer, L., Somerville, M.J., Percy, M.E., 1994. Neurofilament light and polyadenylated mRNA levels are decreased in amyotrophic lateral sclerosis motor neurons. J. Neuropathol. Exp. Neurol. 53, 221–230.

Blass, J.P., Sheu, R.K., Cedarbaum, J.M., 1988. Energy metabolism in disorders of the nervous system. Rev. Neurol. (Paris) 144, 543–563.

Boero, J., Qin, W., Cheng, J., Woolsey, T.A., Strauss, A.W., Khuchua, Z., 2003. Restricted neuronal expression of ubiquitous mitochondrial creatine kinase: changing patterns in development and with increased activity. Mol. Cell. Biochem. 244, 69–76.

Brady, S.T., 1993. Motor neurons and neurofilaments in sickness and in health. Cell 73, 1–3.

Bulliard, C., Zurbriggen, R., Tornare, J., Faty, M., Dastoor, Z., Dreyer, J.L., 1997. Purification of a dichlorophenol-indophenol oxidoreductase from rat and bovine synaptic membranes: tight complex association of a glyceraldehyde-3-phosphate dehydrogenase isoform, TOAD64, enolase-gamma and aldolase C. Biochem. J. 324 (Pt 2), 555–563.

Butterfield, D., Castegna, A., Pocernich, C., Drake, J., Scapagnini, G., Calabrese, V., 2002a. Nutritional approaches to combat oxidative stress in Alzheimer's disease. J. Nutr. Biochem. 13, 444.

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

Butterfield, D.A., Castegna, A., Drake, J., Scapagnini, G., Calabrese, V., 2002b. Vitamin E and neurodegenerative disorders associated with oxidative stress. Nutr. Neurosci. 5, 229–239.

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

Butterfield, D.A., Howard, B.J., LaFontaine, M.A., 2001b. Brain oxidative stress in animal models of accelerated aging and the age-related neurodegenerative disorders, Alzheimer's disease and Huntington's disease. Curr. Med. Chem. 8, 815–828.

Butterfield, D.A., Howard, B.J., Yatin, S., Allen, K.L., Carney, J.M., 1997. Free radical oxidation of brain proteins in accelerated senescence and its modulation by *N-tert*-butyl-alpha-phenylnitrone. Proc. Natl. Acad. Sci. U.S.A. 94, 674–678.

Butterfield, D.A., Stadtman, E.R., 1997. Protein oxidation processes in aging brain. Adv. Cell Aging Gerontol. 2, 161–191.

Byk, T., Dobransky, T., Cifuentes-Diaz, C., Sobel, A., 1996. Identification and molecular characterization of Unc-33-like phosphoprotein (Ulip), a putative mammalian homolog of the axonal guidance-associated unc-33 gene product. J. Neurosci. 16, 688–701.

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

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

Chandrasekaran, K., Giordano, T., Brady, D.R., Stoll, J., Martin, L.J., Rapoport, S.I., 1994. Impairment in mitochondrial cytochrome oxidase gene expression in Alzheimer disease. Brain Res. Mol. Brain Res. 24, 336–340.

Drake, J., Kanski, J., Varadarajan, S., Tsoras, M., Butterfield, D.A., 2002. Elevation of brain glutathione by gamma-glutamylcysteine ethyl ester protects against peroxynitrite-induced oxidative stress. J. Neurosci. Res. 68, 776–784.

Drake, J., Link, C.D., Butterfield, D.A., 2003. Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid beta-peptide (1–42) in a transgenic Caenorhabditis elegans model. Neurobiol. Aging 24, 415–420.

Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11, 81–128.

Farr, S.A., Poon, H.F., Dogrukol-Ak, D., Drake, J., Banks, W.A., Eyerman, E., Butterfield, D.A., Morley, J.E., 2003. The antioxidants alpha-lipoic acid and *N*-acetylcysteine reverse memory impairment and brain oxidative stress in aged SAMP8 mice. J. Neurochem. 84, 1173–1183.

Ferrante, F., Amenta, F., 1987. Enzyme histochemistry of the choroid plexus in old rats. Mech. Ageing Dev. 41, 65–72.

Friedman, D.L., Roberts, R., 1994. Compartmentation of brain-type creatine kinase and ubiquitous mitochondrial creatine kinase in neurons: evidence for a creatine phosphate energy shuttle in adult rat brain. J. Comp. Neurol. 343, 500–511.

Fuhrmann, G.F., Fehlau, R., Schneider, H., Knauf, P.A., 1989. The effect of ferricyanide with iodoacetate in calcium-free solution on passive cation permeability in human red blood cells: comparison with the Gardos-effect and with the influence of PCMBS on passive cation permeability. Biochim. Biophys. Acta 983, 179–185.

Goshima, Y., Nakamura, F., Strittmatter, P., Strittmatter, S.M., 1995. Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. Nature 376, 509–514.

Harman, D., 1994. Free-radical theory of aging. Increasing the functional life span. Ann. N.Y. Acad. Sci. 717, 1–15.

Hoffman, P.N., Cleveland, D.W., Griffin, J.W., Landes, P.W., Cowan, N.J., Price, D.L., 1987. Neurofilament gene expression: a major determinant of axonal caliber. Proc. Natl. Acad. Sci. U.S.A. 84, 3472–3476.

Hrachovina, V., Mourek, J., 1990. Lactate dehydrogenase and malate dehydrogenase activity in the glial and neuronal fractions of the brain tissue in rats of various ages. Sb Lek. 92, 39–44.

Humphries, K.M., Szweda, L.I., 1998. Selective inactivation of alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase: reaction of lipoic acid with 4-hydroxy-2-nonenal. Biochemistry 37, 15835–15841.

Ikegami, S., Shumiya, S., Kawamura, H., 1992. Age-related changes in radial-arm maze learning and basal forebrain cholinergic systems in senescence accelerated mice (SAM). Behav. Brain Res. 51, 15–22.

Jensen, O.N., Wilm, M., Shevchenko, A., Mann, M., 1999. Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. Meth. Mol. Biol. 112, 513–530.

Kagan, V.E., Shvedova, A., Serbinova, E., Khan, S., Swanson, C., Powell, R., Packer, L., 1992. Dihydrolipoic acid — a universal antioxidant both in the membrane and in the aqueous phase. Reduction of peroxyl, ascorbyl and chromanoxyl radicals. Biochem. Pharmacol. 44, 1637–1649.

Kanemitsu, F., Mizushima, J., Kageoka, T., Okigaki, T., Taketa, K., Kira, S., 2000. Characterization of two types of mitochondrial creatine kinase isolated from normal human cardiac muscle and brain tissue. Electrophoresis 21, 266–270.

Kanski, J., Aksenova, M., Schoneich, C., Butterfield, D.A., 2002. Substitution of isoleucine-31 by helical-breaking proline abolishes oxidative stress and neurotoxic properties of Alzheimer's amyloid beta-peptide. Free Radic. Biol. Med. 32, 1205–1211.

Kato, K., Kurobe, N., Suzuki, F., Morishita, R., Asano, T., Sato, T., Inagaki, T., 1991. Concentrations of several proteins characteristic of nervous tissue in cerebral cortex of patients with Alzheimer's disease. J. Mol. Neurosci. 3, 95–99.

Kato, K., Suzuki, F., Morishita, R., Asano, T., Sato, T., 1990. Selective increase in S-100 beta protein by aging in rat cerebral cortex. J. Neurochem. 54, 1269–1274.

Kekelidze, T., Khait, I., Togliatti, A., Benzecry, J.M., Wieringa, B., Holtzman, D., 2001. Altered brain phosphocreatine and ATP regulation when mitochondrial creatine kinase is absent. J. Neurosci. Res. 66, 866–872.

Korotchkina, L.G., Yang, H., Tirosh, O., Packer, L., Patel, M.S., 2001. Protection by thiols of the mitochondrial complexes from 4-hydroxy-2-nonenal. Free Radic. Biol. Med. 30, 992–999.

Levine, R.L., Williams, J.A., Stadtman, E.R., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Meth. Enzymol. 233, 346–357.

Liu, J., Head, E., Gharib, A.M., Yuan, W., Ingersoll, R.T., Hagen, T.M., Cotman, C.W., Ames, B.N., 2002. Memory loss in old rats is associated with brain mitochondrial decay and RNA/DNA oxidation: partial reversal by feeding acetyl-L-carnitine and/or R-alpha-lipoic acid. Proc. Natl. Acad. Sci. U.S.A. 99, 2356–2361.

Lubec, G., Nonaka, M., Krapfenbauer, K., Gratzer, M., Cairns, N., Fountoulakis, M., 1999. Expression of the dihydropyrimidinase related protein 2 (DRP-2) in Down syndrome and Alzheimer's disease brain is downregulated at the mRNA and dysregulated at the protein level. J. Neural Transm. Suppl. 57, 161–177.

Lynch, M.A., 2001. Lipoic acid confers protection against oxidative injury in non-neuronal and neuronal tissue. Nutr. Neurosci. 4, 419–438.

Minturn, J.E., Fryer, H.J., Geschwind, D.H., Hockfield, S., 1995. TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a C. elegans gene involved in axon outgrowth. J. Neurosci. 15, 6757–6766.

Mizuno, Y., Ohta, K., 1986. Regional distributions of thiobarbituric acid-reactive products, activities of enzymes regulating the metabolism of oxygen-free radicals, and some of the related enzymes in adult and aged rat brains. J. Neurochem. 46, 1344–1352.

Morley, J.E., Kumar, V.B., Bernardo, A.E., Farr, S.A., Uezu, K., Tumosa, N., Flood, J.F., 2000. Beta-amyloid precursor polypeptide in SAMP8 mice affects learning and memory. Peptides 21, 1761–1767.

Nomura, Y., Yamanaka, Y., Kitamura, Y., Arima, T., Ohnuki, T., Oomura, Y., Sasaki, K., Nagashima, K., Ihara, Y., 1996. Senescence-accelerated mouse. Neurochemical studies on aging. Ann. N.Y. Acad. Sci. 786, 410–418.

Ohta, A., Hirano, T., Yagi, H., Tanaka, S., Hosokawa, M., Takeda, T., 1989. Behavioral characteristics of the SAM-P/8 strain in Sidman active avoidance task. Brain Res. 498, 195–198.

Packer, L., Roy, S., Sen, C.K., 1997a. Alpha-lipoic acid: a metabolic antioxidant and potential redox modulator of transcription. Adv. Pharmacol. 38, 79–101.

Packer, L., Tritschler, H.J., Wessel, K., 1997b. Neuroprotection by the metabolic antioxidant alpha-lipoic acid. Free Radic. Biol. Med. 22, 359–378.

Payne, R.M., Strauss, A.W., 1994. Developmental expression of sarcomeric and ubiquitous mitochondrial creatine kinase is tissue-specific. Biochim. Biophys. Acta 1219, 33–38.

Poon, H.F., Farr, S.A., Thongboonkerd, V., Lynn, B., Banks, W.A., Moreley, J.E., Klein, J.B., Butterfeld, D.A., 2004. Quantitative Proteomics Analysis of Specific Proteins Expression and Oxidative Modification in Aged SAMP8 Mice Brain. Neurosci. 126, 915–926.

Schonberger, S.J., Edgar, P.F., Kydd, R., Faull, R.L., Cooper, G.J., 2001. Proteomic analysis of the brain in Alzheimer's disease: molecular phenotype of a complex disease process. Proteomics 1, 1519–1528.

Schurr, A., Payne, R.S., Miller, J.J., Rigor, B.M., 1997a. Brain lactate is an obligatory aerobic energy substrate for functional recovery after hypoxia: further in vitro validation. J. Neurochem. 69, 423–426.

Schurr, A., Payne, R.S., Miller, J.J., Rigor, B.M., 1997b. Brain lactate, not glucose, fuels the recovery of synaptic function from hypoxia upon reoxygenation: an in vitro study. Brain Res. 744, 105–111.

Selkoe, D.J., 1996. Amyloid beta-protein and the genetics of Alzheimer's disease. J. Biol. Chem. 271, 18295–18298.

Sen, C.K., Roy, S., Han, D., Packer, L., 1997. Regulation of cellular thiols in human lymphocytes by alpha-lipoic acid: a flow cytometric analysis. Free Radic. Biol. Med. 22, 1241–1257.

Shimano, Y., 1998. Studies on aging through analysis of the glucose metabolism related to the ATP–production of the senescence accelerated mouse (SAM). Hokkaido Igaku Zasshi 73, 557–569.

Stoll, S., Hartmann, H., Cohen, S.A., Muller, W.E., 1993. The potent free radical scavenger alpha-lipoic acid improves memory in aged mice: putative relationship to NMDA receptor deficits. Pharmacol. Biochem. Behav. 46, 799–805.

Takeda, T., Hosokawa, M., Takeshita, S., Irino, M., Higuchi, K., Matsushita, T., Tomita, Y., Yasuhira, K., Hamamoto, H., Shimizu, K., Ishii, M., Yamamuro, T., 1981. A new murine model of accelerated senescence. Mech. Ageing Dev. 17, 183–194.

Tholey, G., Ledig, M., Mandel, P., 1982. Modifications in energy metabolism during the development of chick glial cells and neurons in culture. Neurochem. Res. 7, 27–36.

Thongboonkerd, V., Luengpailin, J., Cao, J., Pierce, W.M., Cai, J., Klein, J.B., Doyle, R.J., 2002. Fluoride exposure attenuates expression of Streptococcus pyogenes virulence factors. J. Biol. Chem. 277, 16599–16605.

Toole-Simms, W., Sun, I.L., Faulk, W.P., Low, H., Lindgren, A., Crane, F.L., Morre, D.J., 1991. Inhibition of transplasma membrane electron transport by monoclonal antibodies to the transferrin receptor. Biochem. Biophys. Res. Commun. 176, 1437–1442.

Varadarajan, S., Kanski, J., Aksenova, M., Lauderback, C., Butterfield, D.A., 2001. Different mechanisms of oxidative stress and neurotoxicity for Alzheimer's A beta(1–42) and A beta(25–35). J. Am. Chem. Soc. 123, 5625–5631.

Varadarajan, S., Yatin, S., Aksenova, M., Butterfield, D.A., 2000. Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. J. Struct. Biol. 130, 184–208.

Wang, L.H., Strittmatter, S.M., 1996. A family of rat CRMP genes is differentially expressed in the nervous system. J. Neurosci. 16, 6197–6207.

Weitzdoerfer, R., Fountoulakis, M., Lubec, G., 2001. Aberrant expression of dihydropyrimidinase related proteins-2,-3 and -4 in fetal Down syndrome brain. J. Neural Transm. Suppl. 61, 95–107.

Yagi, H., Katoh, S., Akiguchi, I., Takeda, T., 1988. Age-related deterioration of ability of acquisition in memory and learning in senescence accelerated mouse: SAM-P/8 as an animal model of disturbances in recent memory. Brain Res. 474, 86–93.

Yatin, S.M., Varadarajan, S., Link, C.D., Butterfield, D.A., 1999. In vitro and in vivo oxidative stress associated with Alzheimer's amyloid beta-peptide (1–42). Neurobiol. Aging 20, 325–330 (discussion 339–342).