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Quantitative proteomics analysis of differential protein expression and oxidative modification of specific proteins in the brains of old mice

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Abstract

The brain is susceptible to oxidative stress, which is associated with age-related brain dysfunction, because of its high content of peroxidizable unsaturated fatty acids, high oxygen consumption per unit weight, high content of key components for oxidative damage, and the relative scarcity of antioxidant defense systems. Protein oxidation, which results in functional disruption, is not random but appears to be associated with increased oxidation in specific proteins. By using a proteomics approach, we have compared the protein levels and specific protein carbonyl levels, an index of oxidative damage in the brains of old mice, to these parameters in the brains of young mice and have identified specific proteins that are altered as a function of aging. We show here that the expression levels of dihydropyrimidinase-like 2 (DRP2), α-enolase (ENO1), dynamin-1 (DNM1), and lactate dehydrogenase 2 (LDH2) were significantly increased in the brains of old versus young mice; the expression levels of three unidentified proteins were significantly decreased. The specific carbonyl levels of β-actin (ACTB), glutamine synthase (GS), and neurofilament 66 (NF-66) as well as a novel protein were significantly increased, indicating protein oxidation, in the brains of old versus young mice. These results were validated by immunochemistry. In addition, enzyme activity assays demonstrated that oxidation was associated with decreased GS activity, while the activity of lactate dehydrogenase was unchanged in spite of an up-regulation of LDH2 levels. Several of the up-regulated and oxidized proteins in the brains of old mice identified in this report are known to be oxidized in neurodegenerative diseases as well, suggesting that these proteins may be particularly susceptible to processes associated with neurodegeneration. Our results establish an initial basis for understanding protein alterations that may lead to age-related cellular dysfunction in the brain.

Keywords: Oxidative stress; Dihydropyrimidinase-like 2; Glutamine synthase

1. Introduction

Oxidative stress is one of the most important mediators in the progressive decline of cellular function during aging. In the brain, free radical-mediated oxidative stress plays a critical role in the age-related decline of cellular function as a result of the oxidation of nucleic acids, lipids, and proteins, which alters the structure and function of these macromolecules [5,28]. A number of studies indicate a strong role for increases in protein oxidation as a primary cause of cellular dysfunction observed during aging as well as in age-related neurodegenerative diseases [8,9,37].

The brain is susceptible to oxidative stress because of its high content of peroxidizable unsaturated fatty acids, high oxygen consumption per unit weight, high levels of free radical-inducing iron/ascorbate, and relatively low levels of antioxidant defense systems [18,28,29]. In most cases, the oxidation of proteins, including those involved in biosynthesis, energy production, cytoskeletal dynamics, and signal

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transduction, leads to their dysfunction [9]. Although protein oxidation contributes to this functional decline, not all proteins are oxidized: many enzymes preserve their activity during aging, suggesting that specific proteins are targets of oxidative modification during aging and in age-related neurodegenerative disorders [6,23,30,37].

In this study, we have used proteomics to compare protein expression levels and the oxidation of specific proteins, as assessed by elevated protein carbonyl levels, in the brains of old versus young mice and to identify the differentially expressed and oxidized proteins. Our results provide insight into how these differences may be associated with age-related decline of cellular function.

2. Methods

2.1. Animals

A total of 10 C57BL/6 male mice were obtained from Harlan, USA; five, from the National Institute on aging aged rodent colonies, were 80 weeks old (the "old" cohort), and five were 6 weeks old (the "young" cohort). It should be noted that at 6 weeks of age, mice are sexually mature, so "young adult" could be equally used to describe these mice. All 10 mice were maintained in an animal facility at the Department of Laboratory Animal Research on a 12 h light:dark cycle in Bioclean units with sterile-filtered air and provided food and water ad libitum. All protocols were implemented in accordance with NIH guidelines and approved by the University of Kentucky Institutional Animal Care and Use Committee. The body weights of the old mice ranged from 32 to 35 g and of the young mice from 19 to 24 g. Following euthanasia with CO_2, the brain was removed quickly, weighed and snap frozen in liquid N_2 prior to analysis.

2.2. Sample preparation

The 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 $MgSO_4$) containing protease inhibitor leupeptin (0.5 mg/mL), pepstatin (0.7 μg/mL), trypsin inhibitor (0.5 μg/mL), and PMSF (40 μg/mL). Homogenates were centrifuged at 15,800 × *g* for 10 min to remove debris. The supernatant was extracted to determine the total protein concentration by the BCA method (Pierce, Rockford, IL).

2.3. Two-dimensional gel electrophoresis

Samples of the proteins in the whole brains were prepared as previously described [30]. Briefly, 200 μg of protein from the brains of five old and five young mice were each applied to ten pH 3–10 ReadyStrip™ IPG strips (Bio-Rad, Hercules, CA) for isoelectric focusing (IEF). After focusing, the IEF strips were stored at −80 °C until second dimension electrophoresis was performed.

For second dimension electrophoresis, 10 linear gradient (8–16%) Precast criterion Tris–HCl gels (Bio-Rad) were used to separate proteins according to their molecular weight (MrW) after IEF. Precision Protein™ Standards (Bio-Rad) were run along with the samples. After electrophoresis, the 10 separate gels were incubated in fixing solution for 20 min. The gels were stained with SYPRO Ruby for 2 h, after which the gels were placed in deionized water overnight for destaining.

2.4. Western blotting

Western blotting of the 2D gels was performed as previously described [30]. Two hundred micrograms of protein from each of the five young and five old mice were incubated with 10 mM 2,4-dinitrophenyl hydrazine (DNPH) solution (2N HCl) at room temperature for 20 min. The gels were prepared in the same manner as for 2D electrophoresis as described above. The proteins from the 2D electrophoresis gels were transferred onto nitrocellulose paper using a Transblot-Blot® SD semi-dry transfer cell (Bio-Rad) at 15 V for 2 h. The DNP adducts of the carbonyls of the brain proteins were detected immunochemically as described above.

2.5. Trypsin digestion

Samples were digested using the techniques previously described [30]. Briefly, the selected protein spots were excised and washed with ammonium bicarbonate (NH_4HCO_3), then acetonitrile at room temperature. The protein spots were incubated with dithiothreitol, then iodoacetamide solutions. The gel pieces were digested with 20 ng/μl modified trypsin (Promega, Madison, WI) using 25 mM NH_4HCO_3 with the minimum volume to cover the gel pieces. The gel pieces were chopped into smaller pieces and incubated at 37 °C overnight in a shaking incubator.

2.6. Mass spectrometry

Digests (1 μL) were mixed with 1 μL α-cyano-4-hydroxy-*trans*-cinnamic acid (10 mg/mL in 0.1% TFA:ACN, 1:1, v/v). The mixture (1 μL) was deposited onto a fast evaporation nitrocellulose matrix surface, washed twice with 2 μL 5% formic acid, and analyzed with a TofSpec 2E (Micromass, Manchester, UK) MALDI-TOF mass spectrometer in reflectron mode. The mass axis was adjusted with trypsin autohydrolysis peaks (*m/z* 2239.14, 2211.10, or 842.51) as lock masses. The MALDI spectra used for protein identification from tryptic fragments were searched against the NCBI protein databases using the MASCOT search engine (http://www.matrixscience.com). Peptide mass fingerprinting used the assumption that peptides are monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues [6,10,12,13]. Up to 1 missed trypsin cleavage was allowed. Mass tolerance of 150 ppm was the window of error allowed for matching the peptide mass

values. In order to assign a level of confidence to the identification of specific proteins from the mass spectra, we used the probability-based Mowse score, which indicates the probability that the match between the database and a spectrum is a random event. This probability equals $10^{(-\text{Mowse score}/10)}$. Mowse scores greater than 62 were considered significant.

2.7. Immunochemical detection of lactate dehydrogenase (LDH2), glutamine synthase (GS) and dynamin-(DNM1)

The levels of lactate dehydrogenase 2 and glutamine synthase were measured by the Slot Blot® technique described previously [32]. Briefly, 1 μg of protein was loaded into the slots. The proteins were detected on nitrocellulose paper using a primary rabbit anti-LDH antibody (1:100, Chemicon, Temecula, CA) or mouse anti-GS antibody (1:1000, Chemicon,) followed by an alkaline phosphatase-conjugated secondary anti-rabbit or anti-mouse IgG antibody (Sigma, St. Louis, MO), respectively. Antibody binding was visualized by application of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma-Fast) followed by densitometric measurement using the Scion-Image software package (Scion, Frederick, MD).

For quantification of dynamin-1 levels, 50 μg of protein from five individual mice in the young and old cohorts (total of ten) were resolved by SDS–PAGE and transferred onto nitrocellulose paper. DNM1 was detected by a mouse anti-DNM1 primary antibody (Chemicon) and an alkaline phosphatase-conjugated anti-mouse IgG secondary antibody (Sigma). The bands were developed by BCIP/NBT and quantified by densitometric measurement as described above.

2.8. Enzyme activity assay

Lactate dehydrogenase activity was determined by the method previously described [38]. Briefly, the assay was performed in 100 μL Tris buffer (0.2 M Tris–HCl, 30 mM sodium pyruvate, 6.6 mM NADH, pH 7.3). The reaction was initiated by adding 5 μL of the brain protein samples (2 mg/mL). Lactate dehydrogenase activity was measured as the reduction of NADH to NAD^+. A decrease in absorbance at 340 nm was recorded as the change in A_{340} min^{-1} by using a PowerWaveX® microtiter plate reader spectrophotometer (Bio-Tek Instruments, Winooki, VT). GS activity was determined by the method of Rowe et al. [35] as modified by Miller et al. [29]. The absorbance was recorded at 505 nm as described above.

2.9. Immunochemical detection of total protein carbonyl level, β-actin (ACTB) carbonyl level and neurofilament 66 (NF-66) carbonyl level

Slot blots and Western blots were used to detect the level of total protein oxidation in the brains of young and old mice as previously described [32]. Briefly, total protein in the homogenates from the brains of the five young and five old mice was derivatized by 10 mM DNPH. For slot blot detection of carbonyl levels, 250 ng of 2,4-dinitrophenyl hydrazone (DNP)-protein adducts were loaded into each slot. For Western blot carbonyl detection, 30 μg of DNP-protein adducts from each animal were resolved on SDS–PAGE gels. The technique for the immunochemical detection of the DNP-protein adducts was the same for both methods and was described previously [32]. The quantification of the DNP-protein adducts determined by slot blots was as described above. The quantification of the DNP-protein adducts resolved by Western blotting was by densitometric measurement of the immunoreactivity in the entire lane on the nitrocellulose paper.

The method used for the detection of β-actin carbonyl levels was similar to that for total protein carbonyl level detection described above. The quantification of the DNP-actin adduct was by densitometric measurement of the bands at 40 kDa where actin is predominately present.

Neurofilament 66 was derivatized by DNPH for carbonyl detection as described above. The carbonyl levels of NF-66 were detected by Western blot after immunoprecipitation (IP). IP was performed as described previously [24]. A mouse anti-NF-66 antibody (5 μL, Chemicon) was added directly to the brain homogenate, and the mixture was incubated on a rotary mixer overnight at 4 °C. The NF-66/antibody complexes were precipitated with protein G-conjugated agarose beads. Protein G beads were added in 50 μL aliquots from a stock of 300 mg/mL in PBS and mixed on a rotary mixer for 1 hour at room temperature. Beads were then centrifuged and washed with the washing buffer (pH 8, 50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) three times. The NF-66 proteins from each animal were resolved by SDS–PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The method used for the detection and quantification of NF-66 carbonyl levels was similar to that for total protein carbonyl level detection described above.

2.10. Image analysis

The gels and nitrocellulose blots were scanned and saved in TIF format using a Storm 860 Scanner (Molecular Dynamics) and a Scanjet 3300C (Hewlett Packard), respectively. PDQuest software (Bio-Rad) was used for matching and analysis of visualized protein spots among different gels and oxyblots. The principles of measuring intensity values by 2D analysis software were similar to those of densitometric measurement. The average mode of background subtraction was used to normalize intensity values, which represent the amount of protein (total protein on gel or oxidized protein on oxyblot) per spot. After completion of spot matching, the average normalized intensity of five individual gels (or oxyblots) from the five young mice was compared to the average normalized intensity of five individual gels (or oxyblots) from the five old mice.

2.11. Statistics

The levels of expression of specific proteins and carbonyl levels in specific proteins, measured by the intensity of the carbonyl level divided by the intensity of protein level of an individual spot, were obtained from five individual 2D gels from each of the animals in each cohort. The data, including those from the enzyme activity assays, were analyzed by Student's *t*-tests. A value of $p < 0.05$ was considered statistically significant. Only those proteins that were expressed at significantly different levels or were significantly oxidized in the brains of the old versus the young mice were selected for identification by mass spectrometry.

3. Results

To assess whether there were any changes in the proteomic profile in the brains of aging mice, we first assayed the differential expression of proteins in the brains of young and old mice. We found that the expression level of seven proteins was significantly altered (four proteins showed increased expression and three proteins showed decreased expression); and the specific carbonyl levels of four proteins were significantly increased in the old mice.

Comparing the densitometric intensities of individual spots on the gels, we determined that four proteins were expressed at significantly higher levels, and three proteins were expressed at significantly lower levels in the brains of the old compared to young mice. Fig. 1 shows representative gels from the brains of a young and old mouse after 2D-electrophoresis. To identify the differentially expressed proteins, the mass spectra of the peptides were matched to the mass spectra in NCBI protein databases. The four proteins that were up-regulated in the brains of the old mice were identified with Mowse scores >62; they were dihydropyrimidinase-like 2 (DRP2), α-enolase (ENO1), dynamin-1 (DNM1), and lactate dehydrogenase 2 (LDH2). An example of the mass spectrum for LDH2, which was significantly up-regulated in the brains of old mice, is shown in Fig. 2A (top), and the results of the database search are shown in Fig. 2A (bottom). The parameters for the identification of these proteins by mass spectrometry are summarized in Table 1; all protein identifications agreed with the expected MrW and pI range based on their positions on the gels. The quantitative details of their relative expression levels in old versus young mice are summarized in Table 2. None of the down-regulated proteins were identified with a Mowse score >62.

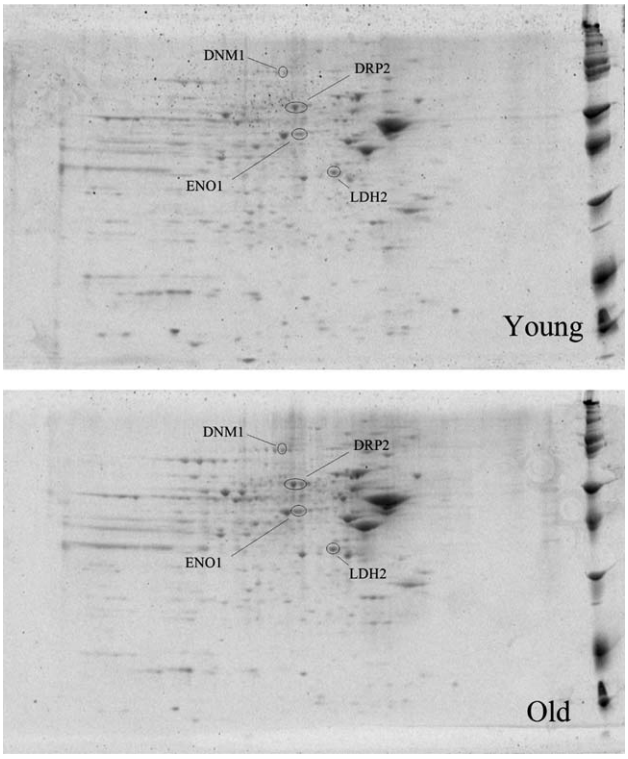

Fig. 1. Representative 2D gels show proteins from the brains of a young mouse (top) and an old mouse (bottom).

Table 1
Mass spectrometry identification of proteins up-regulated in the brains of old vs. young mice

Protein	GI accession no.	No. of peptide matches identified	% coverage matched peptides	pI, MrW	Mowse score[a]
Dihydropyrimidinase-like 2 (DRP2)	gi|40254595	14	35	6.16, 62.16	776
α-Enolase (ENO1)	gi|19353272	17	47	6.37, 47.5	166
Dynamin-1 (DNM1)	gi|21961254	22	24	7.61, 98.1	155
Lactate dehydrogenase 2 (LDH2)	gi|28386162	13	40	5.87, 36.6	120

[a] Mowse scores greater than 62 are considered significant.

Table 2
Identified proteins up-regulated in the brains of old vs. young mice

Protein	Young (A.U. ± S.E.M.) ($n = 5$)	Old (A.U. ± S.E.M.) ($n = 5$)	Fold increase in old	*p*-value
DRP2	545 ± 175	1327 ± 221	2.4	0.024
ENO1	1761 ± 202	2589 ± 259	1.5	0.036
DNM1	740 ± 142	1135 ± 92	1.5	0.048
LDH2	1489 ± 372	3770 ± 286	2.5	0.0012

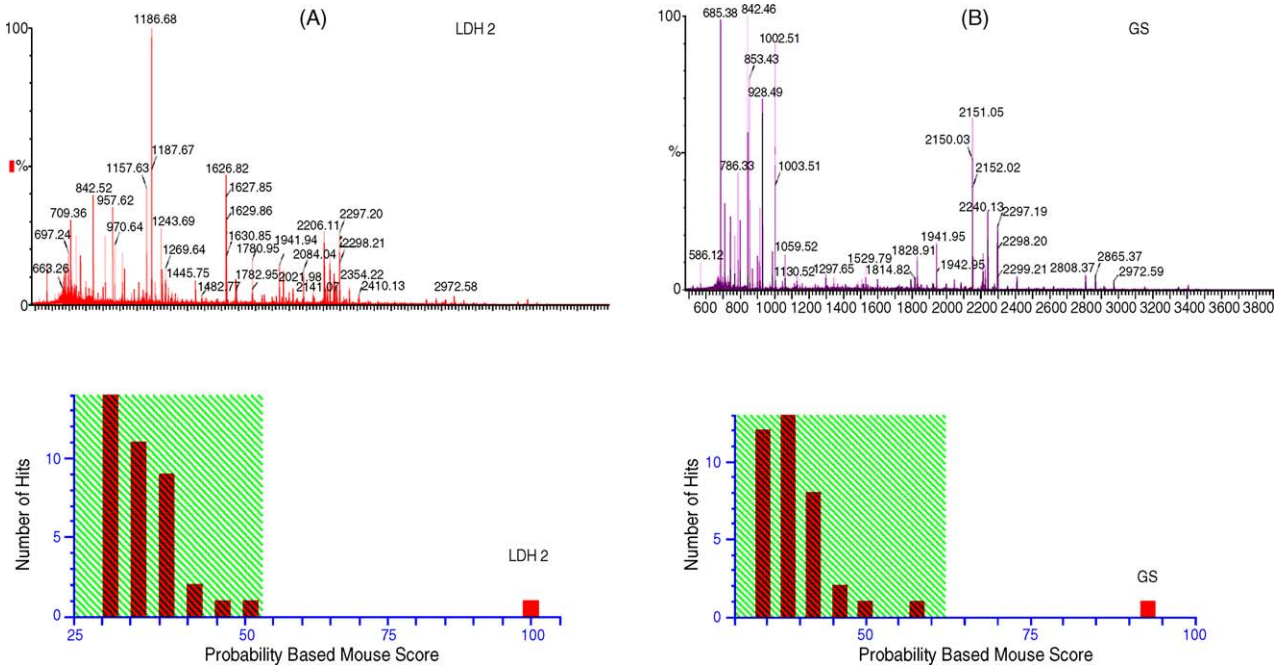

Fig. 2. Mass spectrometry and peptide mass fingerprinting. (A) Top: spectral masses (in mass per charge unit, *m*/*z*) of lactate dehydrogenase 2 (LDH2) obtained by MALDI-TOF mass spectrometry. Bottom: possible matched proteins to the spectral masses of LDH2 are presented as multiple bars with differential probability-based MOWSE scores (*x*-axis). (B) Top: mass spectrum of glutamine synthase (GS). Bottom: possible matched proteins to the spectral masses of GS were presented as multiple bars with differential probability-based MOWSE scores. Only proteins with MOWSE scores greater than 62 (outside shaded area) were considered significantly matched.

We then investigated total protein oxidation levels and the oxidation of specific proteins in the brains of the old versus young mice. The total level of oxidized proteins as determined by slot blots and Western blots was significantly higher (by approximately 30–40%) in the brains of the old versus young mice (Fig. 3). Comparing the densitometric intensities of individual spots on the oxyblots, we determined that four proteins had significantly higher specific carbonyl levels in the brains of old mice compared to young. Fig. 4 shows representative oxyblots from the brains of a young and an old mouse. The significantly oxidized proteins were identified by matching their mass spectra to those in the NCBI protein databases as described above. The four oxidized proteins were identified; they are β-actin (ACTB), glutamine synthase (GS), neurofilament 66 (NF-66), and an unnamed protein. An example of the mass spectrum for GS is shown in Fig. 2B (top), and the results of the database search for GS are shown in Fig. 2B (bottom). The parameters for the identification of the oxidized proteins by mass spectrometry are summarized in Table 3; these protein identifications agreed with the expected MrW and pI range based on their positions on the blots. The quantitative details of their relative specific carbonyl levels in old versus young mice are summarized in Table 4.

Table 3
Mass spectrometry identification of oxidized proteins in the brains of old vs. young mice

Protein	GI accession no.	No. of peptide matches identified	% coverage matched peptides	pI, MrW	Mowse Score[a]
β-Actin (ACTB)	gi|49868	13	49	5.78, 39.4	121
Glutamine synthase (GS)	gi|15929291	11	26	6.64, 42.8,	93
Neurofilament 66 (NF-66)	gi|609535	18	37	5.49, 55.5	71
Unnamed protein	gi|38089221	9	12	N/A	66

[a] Mowse scores greater than 62 are considered significant.

Table 4
Identified proteins oxidized in the brains of old vs. young mice

Protein	Young (A.U ± S.E.M.)	Old (A.U. ± S.E.M.)	Fold increase in old	*p*-value
ACTB	1.25 ± 0.20	3.03 ± 0.68	3.4	0.035
GS	2.4 ± 0.56	12.0 ± 2.89	5.2	0.011
NF-66	0.70 ± 0.21	3.52 ± 1.18	7.2	0.046
Unnamed protein	0.59 ± 0.196	31.7 ± 13.3	68	0.048

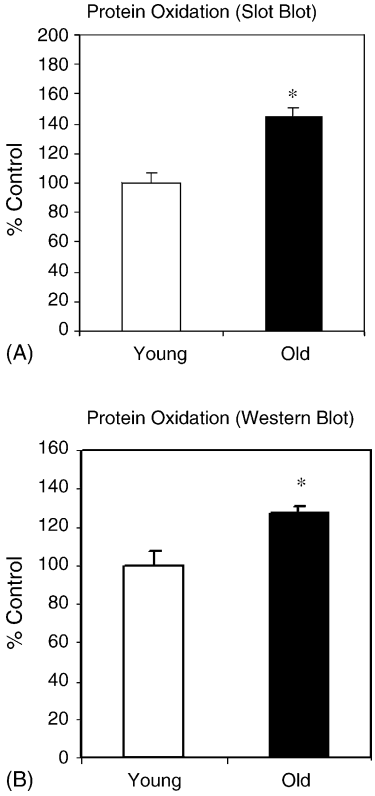

Fig. 3. (A) Total protein carbonyl level in brains of young and old mice determined by slot blot analysis. (B) Total protein carbonyl level in brains of young and old mice determined by Western blot analysis. The total carbonyl level is significantly increased in the brains of old mice when compared to young. Bars represent mean ± S.E.M. $^{*}p < 0.05$, $n = 5$ samples from young and five samples from old cohorts.

We hypothesized that oxidative modification of specific enzymes would decrease their activity but that the activity of enzymes whose expression level was up-regulated would not necessarily be changed. To test this, we measured the activities of GS, which was oxidized in the brains of the old mice, and lactate dehydrogenase, the LDH2 subunit of which was expressed at a higher level in the brains of the old mice. First, using immunochemical analysis (Fig. 5A), we validated the proteomic results that indicated that the level of expression of GS was unchanged and that of LDH2 was up-regulated by about 20%. In support of our hypothesis, Fig. 5B shows that the activity of GS in the brains of old mice was significantly lower (by about 20%) than in the brains of young mice. In contrast, lactate dehydrogenase activity in the brains of old mice showed no significant difference relative to that in the brains of the young mice. Because the expression level of LDH2 increased, this suggests that there is a relatively lower activity per unit of lactate dehydrogenase enzymatic activity in the brains of the old mice.

We validated our proteomics results for three additional proteins. With immunochemical detection, we demonstrated that the level of expression of DNM1 in the brains of old mice was significantly increased by 57% (Fig. 6), which is in close agreement with the results of the proteomics analysis

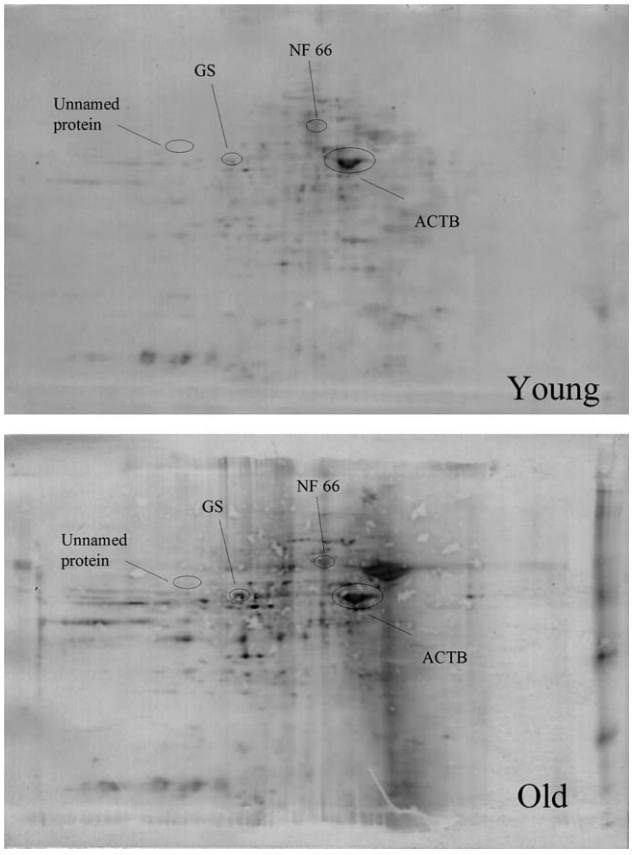

Fig. 4. Representative 2D oxyblots show oxidized proteins from the brains of a young mouse (top) and an old mouse (bottom).

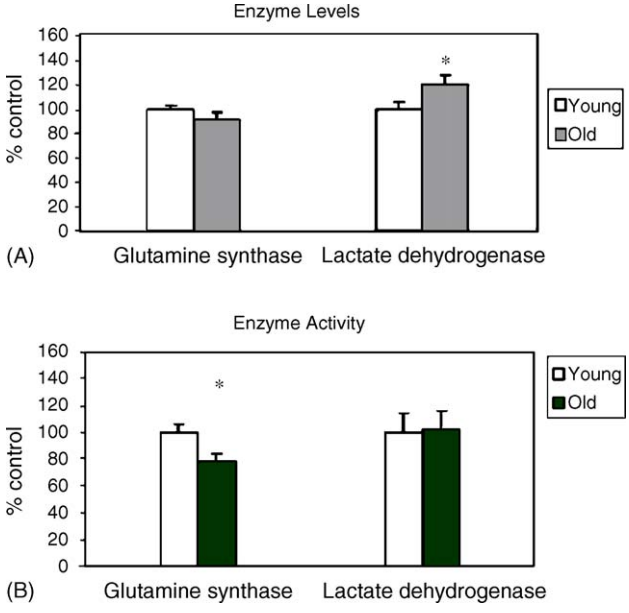

Fig. 5. (A) Levels of glutamine synthase (GS, left) and lactate dehydrogenase 2 (LDH2, right) determined by immunochemistry show that GS levels are unchanged and LDH2 levels are significantly up-regulated in the brains of old vs. young mice. (B) Activities of GS (left) and lactate dehydrogenase (right) determined by spectrometry show significantly decreased levels of GS activity and unchanged levels of lactate dehydrogenase activity in the brains of old vs. young mice.

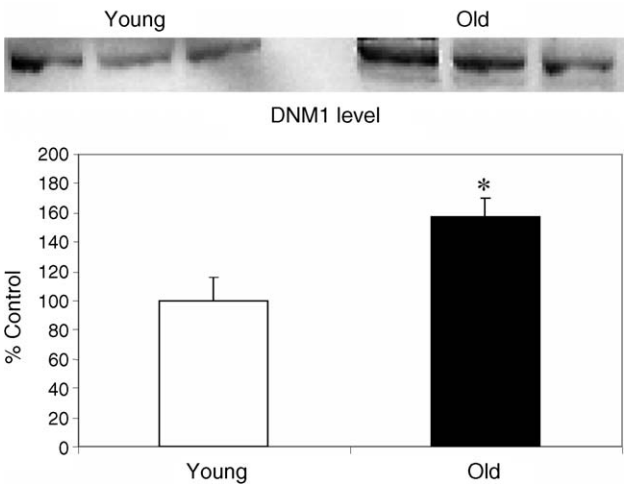

Fig. 6. Dynamin-1 (DNM1) levels in brains of young and old mice determined by Western blot analysis. The DNM1 level is significantly increased in brains of old mice compared to young. Bars represent mean ± S.E.M. $^{*}p < 0.05$, $n = 5$ samples from young and five samples from old cohorts.

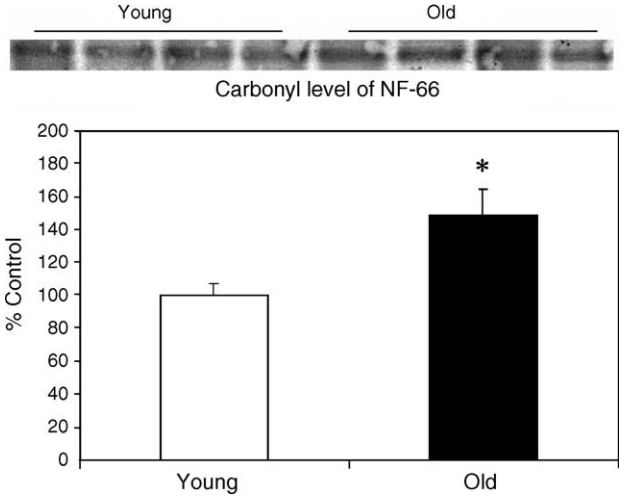

Fig. 8. Carbonyl levels of neurofilament 66 (NF-66) in brains of young and old mice determined by Western blot after immunoprecipitation. The carbonyl level of NF-66 is significantly increased in the brains of old mice compared to young. Bars represent mean ± S.E.M. $^{*}p < 0.05$, $n = 5$ samples from young and five samples from old cohorts.

(Table 2). We also measured the carbonyl levels of NF-66 and ACTB by IP (NF-66) and Western blotting. Consistent with the proteomics results, the carbonyl levels of ACTB (Fig. 7) and NF-66 (Fig. 8) were significantly increased by about 40 and 50%, respectively, in the brains of the old mice as compared to young. The increased carbonyl level of ACTB and NF-66 in the brains of old mice was more robust when detected by proteomics method. The differences in the magnitude of fold changes of carbonyl levels between the two techniques is likely due to the fact that proteomics measures the carbonyl level per unit of protein while Western blotting measures the carbonyl level of total protein. Clearly, both techniques show that ACTB and NF-66 are significantly oxidatively modified in the brains of old mice, thus validating the proteomics results.

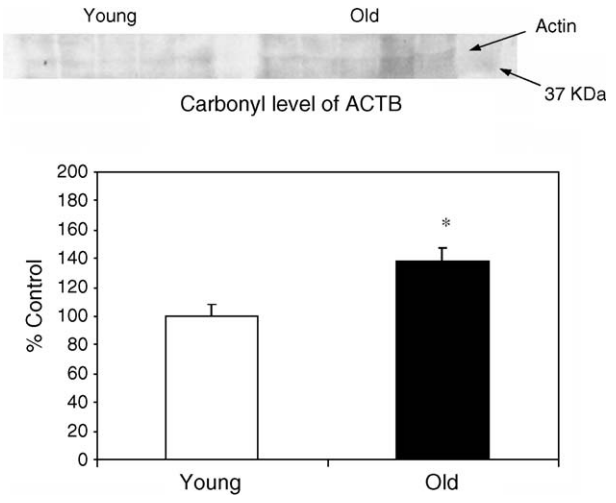

Fig. 7. Carbonyl levels of β-actin (ACTB) in brains of young and old mice determined by Western blot analysis. The carbonyl level of ACTB is significantly increased in the brains of old mice compared to young. Bars represent mean ± S.E.M. $^{*}p < 0.05$, $n = 5$ samples from young and five samples from old cohorts.

4. Discussion

Our aim, in this study, was to identify differentially expressed and oxidized proteins in the normally aging murine brain. Using the proteomics approach previously utilized in our laboratories [10,11,30,31,33], we determined that the expression levels of DRP2, ENO1, DNM1 and LDH2 were significantly increased in the brains of old mice when compared to the brains of young mice. Additionally, the expression levels of three proteins were significantly decreased, but these proteins could not be identified because their mass spectra did not match any in the databases with a significant Mowse score. Further, we show that the total level of protein oxidation increased in the brains of old mice when compared to young, and that the specific carbonyl levels of ACTB, GS, NF-66 and an unnamed protein were significantly increased in the brains of old mice. Selected results were validated using immunochemistry. Additionally, we demonstrated that for GS, which was oxidized but not expressed at significantly different levels in the brains of old versus young mice, oxidation reduced enzyme activity; in contrast, for LDH, whose expression level was up-regulated in the brains of old mice, enzyme activity was unchanged.

DRP2, one of the four proteins whose expression was up-regulated in the brains of old versus young mice, is a member of the dihydropyrimidinase-related protein family. These proteins are involved in axonal outgrowth and path-finding through the transmission and modulation of extracellular signals. It was reported that DRP2 induced growth cone collapse by Rho-kinase phosphorylation [4]. and by binding to tubu-

lin [19]. Decreased expression of DRP2 has been observed in Alzheimer's disease (AD), Down syndrome [25], schizophrenia, and affective disorders [22], and DRP2 is oxidized in brains from AD patients [11]. The increased expression of DRP2 in brains from old versus young animals may indicate that neuronal sprouting is being positively regulated as a compensatory response to neuronal dysfunction in the aged brain.

Another of the up-regulated proteins, ENO1, is the α-subunit of enolase; the αγ isoform is a neuron-specific enolase. We recently reported that ENO1 is up-regulated in the olfactory bulbs (OBs) of old mice as well [31]. Enolase is a cytosolic enzyme involved in metabolism, cell differentiation, and normal growth; a decline of enolase activity results in abnormal growth and reduced metabolism in brains [39]. Increased ENO1 oxidation in the brains of AD patients suggests that the loss of activity by oxidative modification of ENO1 may lead to neurodegeneration [11,30], emphasizing the importance of this glycolytic enzyme in brain metabolism. The increased levels of ENO1 in the brains of old mice may indicate a compensatory response to decreased activity in other metabolic and mitochondrial pathways in the brains of old mice and a protective response against neurodegeneration.

Our proteomics analysis, validated with immunochemistry, demonstrated that DNM1 increased in abundance in the brains of old versus young mice. Among its functions, DNM1 is known to inhibit phosphatidylinositol 3-kinase (PI3K), a survival signaling molecule that acts via its effector, Akt [20]. Thus, through its inhibition of PI3K, DNM1 up-regulation may cause increased cell death in the brains of old mice. Alternatively, the formation of complexes between DNM1 and the actin-binding protein profilin at sites of synaptic vesicle recycling has been well-characterized [40]; the significant decrease in DNM1 mRNA and protein levels in AD brains was interpreted to reflect its role in synaptic vesicle endocytosis [41]. We recently reported that DNM1 protein is less abundant in the OBs of old versus young mice [33]; however, because the OB is a site of on-going synaptic remodeling, DNM1 may be constitutively expressed at high levels, and its down-regulation may reflect this regional specialization. Thus, the increased expression of DNM1 in the brains of old mice may indicate increased synaptic vesicle recycling associated with increased synaptic plasticity as a compensatory response to age-related synaptic loss such as that proposed to occur in neurodegenerative diseases [21].

LDH2, which was also up-regulated in the brains of old mice, is a subunit of the enzyme lactate dehydrogenase that catalyzes the reversible NAD-dependent interconversion of pyruvate and lactate. In astrocytes, lactate dehydrogenase favors the formation of lactate over that of pyruvate; the lactate is secreted by astrocytes, taken up by neurons, and converted to pyruvate, which enters the Kreb's cycle for ATP production [15]. Lactate appears to be the main energetic compound delivered by astrocytes and is the only oxidizable energy substrate available to support neuronal recovery in the CNS [36]. The increased expression of LDH2 in the brains of old mice may compensate for metabolic down-regulation in other enzyme systems to provide sufficient lactate and ATP for cellular processes and neuronal survival.

The proteins that were identified as being up-regulated in the brains of old mice in this study have also been shown to be oxidized in the brains of SAMs with cognitive deficit and in the brains of patients with neurodegenerative diseases and in models thereof [6,10–12,30]. Taken together, one can speculate that the up-regulation of these proteins may play critical roles in the cognitive stability of aged mice without cognitive deficit. Results from our laboratory, as well as others, have demonstrated that total protein oxidation in the brain increases as a function of age [9]. Our laboratory has previously used this proteomics approach to identify oxidized proteins in the brains of senescence-accelerated mice and humans in order to gain insights into the mechanism of accelerated aging and age-related neurodegenerative diseases [10,11,30].

ACTB, which was oxidized in the brains of old versus young mice, is a component of the cytoskeletal network responsible for cell structure and motility. Actin polymerization/depolymerization plays an important role in synaptic plasticity in dendritic spines [17,26], and disruption of actin polymerization results in growth cone collapse [27]. Decreased levels of actin in cultured neurons as a function of increasing age indicates that the oxidation of actin may accelerate its degradation [3]. Such an effect is also observed in the brains of patients with Alzheimer's disease [1]. The oxidative modification of ACTB in the brains of old mice may affect actin filament architecture and lead to disarrangement of the cytoskeleton, thus increasing the susceptibility of neurons to age-related neurodegenerative diseases.

It is well documented that GS activity declines as a function of age [2,16]. The decline in enzyme activity is caused by the alteration of protein structure induced by oxidative modification [7,9,10]. GS catalyzes the rapid amidation of glutamate to form the non-neurotoxic amino acid, glutamine. This reaction maintains the optimal level of glutamate and ammonia in neurons and modulates excitotoxicity. The results presented here confirm and extend earlier studies showing that GS is specifically oxidized and its activity reduced in the brains of old mice, suggesting that the glutamate–glutamine cycle in these aged brains may be impaired (reviewed in [9]). Such an impairment would contribute to the cellular functional decline in aging brain. Because both GS and ACTB are also oxidized in AD brains [1,10], the specific oxidation of these proteins may be involved in the increased susceptibility of aged individuals to age-related neurodegenerative diseases.

NF-66 (α-internexin), which was oxidized in the brains of old versus young mice, is an intermediate filament protein that contributes to cytoskeletal organization, neurogenesis and neuronal architecture in the brain. Oxidation or nitration of neurofilament (NF) proteins transform the α-helix secondary structure to β-sheet and random coil conforma-

tions, destabilizing the interactions between the NF proteins and resulting in axonal damage [14]. Binding of NF-66 by viral proteins results in neurological disorders, indicating that NF-66 is critical to the proper functioning of the CNS [34].

A novel unnamed protein was also oxidized in the brains of old mice. Further experiments will be needed to identify this protein and determine it how its oxidation may impact brain function.

In this study, we have shown that there is an altered proteomic profile in the brains of old mice, and we identified the proteins that were differentially expressed or oxidized in the brains of old versus young mice. Our results are consistent with the free radical theory of aging, which proposes that increased protein oxidation occurs as a function of age, and that the oxidation of proteins causes cellular functional decline, thus increasing the susceptibility of aging brains to neurodegeneration. Interestingly, several of the oxidized proteins in the brains of old mice are the same as those that have been identified in the brains of patients with and in animal models of neurodegenerative diseases. Our results also support the possibility that the expression levels of certain proteins may increase as a compensatory response to oxidative stress. This compensation would allow for the maintenance of proper molecular functions in aging brains and protection against neurodegeneration. This report is our initial study of age-related changes in brains of mice, and as such, forms a framework for future studies, including the testing of potential novel therapeutic molecules that may modulate the effects of oxidative stress in brain aging. Such studies are currently in progress.

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