

Forum Original Research Communication

Proteomics Analyses of Specific Protein Oxidation and Protein Expression in Aged Rat Brain and Its Modulation by L-Acetylcarnitine: Insights Into the Mechanisms of Action of This Proposed Therapeutic Agent for CNS Disorders Associated with Oxidative Stress

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ABSTRACT

Impaired function of the central nervous system (CNS) in aged animals is associated with increased susceptibility to the development of many neurodegenerative diseases. Age-related functional deterioration in brain is consistent with the free radical theory of aging that predicts, among other things, that free radical reactions with and damage to biomolecules, such as proteins and membrane lipid bilayers, leads to loss of neurons and subsequently diminished cognition. These oxidatively modified biomolecules are believed to contribute to the decreased antioxidant content, mitochondrial dysfunction, and impaired plasticity in aged brains. Treatment of rodents with L-acetylcarnitine (LAC; γ -trimethyl- β -acetylbutyrobetaine) can improve these functional losses. Although it is well established that administration of LAC can decrease protein oxidation in aged brains, it is not clear which proteins are decreased in their level of oxidation in the brains of aged rats treated with LAC. The current study used a parallel redox proteomics approach to identify the proteins that are oxidized in aged rat cortex and hippocampus of aged rats. Moreover, those proteins that are reduced in oxidation status were identified in aged brains from rats treated *in vivo* with LAC. The findings are discussed in reference to brain aging and age-related cognitive impairment. *Antioxid. Redox Signal.* 8, 381–394.

INTRODUCTION

IMPAIRED FUNCTION of the central nervous system (CNS) in aged animals is associated with increased susceptibility to the development of many neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). Age-related brain functional deterioration is consistent with the free radical theory of aging, postulating that free radical reactions with biomolecules such as proteins and lipids leads to cognitive decline

and neurodegeneration (35). This theory was later refined to include the concept that mitochondria play a key role in aging, acting as the major source and target of oxidants (58). Mitochondrial dysfunction plays a significant role in this increased oxidative damage, since damaged mitochondrial enzymes in aged brains lead to increased generation of free radicals (86). However, mitochondria are also a target of increased oxidative stress in aged brains (75), causing mitochondrial decay in a reinforcing cycle that contributes to accelerated aging (8, 12). Such increased oxidative stress and

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mitochondrial dysfunction cause oxidation of proteins, thereby leading to their dysfunction in most cases (1, 2, 5, 14, 15, 81). However, oxidation of proteins during aging and age-related neurodegenerative disorders is specific (1, 2, 16, 18–20, 42, 69, 82, 83).

L-Acetylcarnitine (LAC; γ -trimethyl- β -acetylbutyrobaine) is the acetyl ester of carnitine, which facilitates the transport of fatty acids and other moieties across the membranes of mitochondria, thereby participating in the production of energy and mitochondrial function within the brain (84). LAC levels are reduced in aged brains, and chronic administration of LAC to aged rats improves age-related cognitive impairment (21). LAC-mediated modulation of cognitive impairment in aged animals is reported to be due to its similar structure as acetylcholine, thereby promote synthesis and release of acetylcholine (21). However, other mechanisms also likely play a role. For example, LAC elevates levels of neurotrophins, which are key factors in the mediation of neural plasticity and are required for memory consolidation (56). LAC can also reduce brain oxidative stress by decreasing mitochondria decay (50). Although it is well established that administration of LAC can decrease protein oxidation in aged brains (50, 51, 80, 93), it is not clear which specific proteins are reduced in oxidative damage in the brains of aged rats treated with LAC. In order to gain insight into the protective mechanism of LAC in aged brains relevant to protein oxidation, we used a parallel proteomics approach to identify the proteins that are oxidized in aged brains and those proteins that are have reduced oxidative damage in aged brains in aged rats treated with LAC.

MATERIALS AND METHODS

Animals

All animal protocols were approved by the University of Catania Laboratory Animal Care Advisory Committee. Wistar rats purchased from Harlan (Udine, Italy) were maintained in a temperature and humidity-controlled room with a 12-h light:dark cycle. Rats were fed *ad libitum* a certified diet prepared according to the recommendations of the American Institute of Nutrition. Seven young animals were between 6 and 12 months old and fourteen old animals were 28 months old. The old animals was divided into two subgroups (seven each): one receiving the control diet and the other supplemented, from the 24th month up to 28th month (4 months), with acetylcarnitine (LAC), given orally dissolved in drinking water. The calculated intake of LAC to this subgroup of rats was 150 mg/kg/day. This group of animals was then sacrificed at 28 months of age. After sacrifice, brains were quickly removed and dissected into the cerebral cortex, hippocampus, septal area, and striatum, according to a standardized procedure, in a cold anatomical chamber and following a protocol that allows a maximum of 50 sec time-variability for each sample across animals. Substantia nigra was dissected from the deepest part of the interpeduncular fossa.

Sample preparation and methods employed

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 inhibitors leupeptin (0.5 mg/ml), pepstatin (0.7 $\mu\text{g/ml}$), trypsin inhibitor (0.5 $\mu\text{g/ml}$), and PMSF (40 $\mu\text{g/ml}$). Homogenates were centrifuged at 15,800 g for 10 min to remove debris. The supernatant was extracted to determine the protein concentration by the bicinchoninic acid protein (BCA) method (Pierce, Rockford, IL, USA).

Immunocytochemistry

Levels of 3-nitrotyrosine (3-NT), 4-hydroxynonenal (HNE), and protein carbonyls were determined immunochemically as previously described (70). Protein carbonyl levels were determined as adducts of 2,4-dinitrophenylhydrazine (DNPH) (4, 82). The 2,4-dinitrophenyl hydrazone (DNP) adduct of the carbonyls was detected on nitrocellulose membranes using a primary rabbit antibody (Chemicon, Temecula, CA, USA)(1:1000) specific for DNP-protein adducts. HNE and 3-NT levels were determined in the same manner. The HNE levels were detected using a primary rabbit antibody (Alpha Diagnostics, San Antonio, TX, USA) specific for HNE-modified protein (1:8000) and the 3-NT levels were detected by primary rabbit antibody (Chemicon) specific for 3-NT (1:100). The same secondary goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) antibody was used in all applications. The resultant stain was developed by application of Sigma-Fast [5-bromo-4-chloro-3-indolyl phosphate dipotassium/nitrotetrazolium blue chloride (BCIP/NBT)] tablets; and the line densities were quantified using Scion-Image software (Scion Corporation, Frederick, MD, USA).

Two-dimensional gel electrophoresis

Samples of the proteins in the hippocampus or cortex were prepared as previously described (69). Briefly, 200 μg of protein were applied to a pH 3–10 ReadyStrip™ IPG strip (Bio-Rad, Hercules, CA, USA) for isoelectric focusing, and Linear Gradient (8–16%) Precast criterion Tris-HCl gels (Bio-Rad) were used to separate proteins according to their molecular weight (MrW). Sypro Ruby stain was used to stain the gel for 2 h, after which the gels were placed in deionized water overnight for destaining.

Western blotting

Western blotting for 2D gels was performed as previously described (69). 200 μg of the brain protein were incubated with 10 mM DNPH solution (2 N HCl) at room temperature (25°C) for 20 min. The gels were prepared in the same manner as for 2D-electrophoresis. The proteins from the second dimension electrophoresis gels were transferred onto nitrocellulose paper (Bio-Rad) using a Transblot-Blot® SD semi-Dry Transfer Cell (Bio-Rad) at 15V for 2 h. The adducts of the carbonyls of the brain proteins were detected immunochemically.

Image analysis

The gels and nitrocellulose blots were scanned and saved in TIF format using a Storm 860 Scanner (Molecular Dynamics, Sunnyvale, CA, USA) and Scanjet 3300C (Hewlett Packard, Palo Alto, CA, USA), respectively. PDQuest (Bio-Rad) was the software used for matching and analysis of visu-

alized protein spots among differential gels and oxyblots. 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 digested using the techniques previously described (69). Briefly, the selected protein spots were excised and washed with ammonium bicarbonate (NH_4HCO_3), then acetonitrile at room temperature. The gel pieces were digested with 20 ng/ μl modified trypsin (Promega, Madison, WI, USA) and incubated at 37°C overnight in a shaking incubator.

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 and analyzed with a ToFSpec 2E (Micromass, UK) matrix-assisted laser desorption ionization-time of flight (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>).

Statistics

The data were analyzed by two-tailed Student's *t* tests. A value of $p < 0.05$ was considered statistically significant. Only the proteins in hippocampus and cortex of old rats that were significantly different from those of young brains or from old rats treated with LAC assessed by the Student's *t* test were selected for identification.

RESULTS

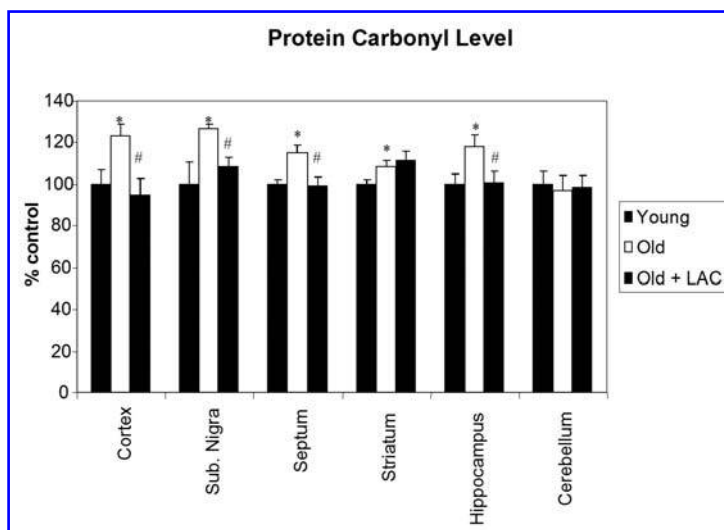
In order to identify the brain regions that show age-associated protein oxidation and the reversal of such protein oxidation

by LAC treatment, we measured the bulk protein 3-NT, HNE, and carbonyl levels of cortex (CX), substantia nigra (SN), septum pellucidum (SP), striatum (ST), hippocampus (HP), and cerebellum (CB). We observed that protein oxidation increased generally in most brain regions of aged rats. However, only certain brain regions of the aged rats show statistically significant increased oxidative modification of proteins, and administration of LAC reduced such age-associated protein oxidation. Old rats show significant increases in protein carbonyl levels in CX, SN, SP, ST, and HP compared to that of young rats (Fig. 1). Administration of LAC reduced the carbonyl level of all of these regions except ST (Fig. 1). Similar to the findings in AD (36), no change in protein oxidation was brain aging was found in CB.

With regard to protein-bound HNE levels, significantly increased values are observed in CX, SP, and HP of old rats, whereas administration of LAC reduced the HNE levels in all of these regions (Fig. 2). We also demonstrated a significant increase of protein 3-NT levels in SN and HP of aged rats, which is reduced by administration of LAC (Fig. 3).

To begin to gain insight into which oxidatively modified proteins are implicated in brain aging relevant to age-related learning and memory deficits, we performed a redox proteomics analysis (14) on the HP and CX, brain regions in which all indices of oxidative modification are elevated in brain aging (Figs. 1–3) and brain regions that are involved in learning and memory. The specific carbonyl levels of three proteins, hemoglobin (HMG), cofilin 1 (COF 1), and β -actin (ACT), are significantly increased in HP of aged rats (Table 1). All of the specific carbonyl levels of these proteins are reduced by LAC administration in old rats brains, although the reduction of HMG and ACT are not statistically significant (Table 1). Figure 4 shows these proteins on the representative 2D Western blots of the hippocampus for young rats, old rats, and old rats treated with LAC. Moreover, expression levels of five proteins were significantly altered in the HP of old rats. Protein levels of mitochondrial aconitase (ACO 2), inositol monophosphatase (IMP), and α -enolase (ENO1) are significantly increased in HP of old rats, while the expression level of creatine kinase B chain (CK-B), and tubulin α -1 chain (TUB 1) were decreased (Table 2). Although LAC adminis-

FIG. 1. Protein carbonyl level. Results represent the average protein carbonyl levels in cortex, substantia nigra, septum pellucidum, striatum, hippocampus, and cerebellum of young rats, old rats, as well as old rats treated with LAC. Error bars indicate the SEM for 5–7 animals in each group. Measured values are normalized to the appropriate age-matched control values. *, $p < 0.05$ when compared to young rats. #, $p < 0.05$ when compared to the old rats.



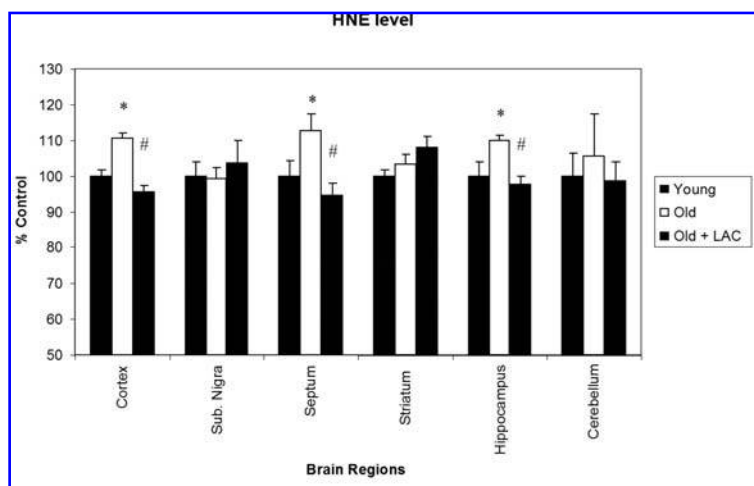


FIG. 2. Protein HNE levels. Results represent the average protein-bound HNE levels in cortex, substantia nigra, septum pellucidum, Striatum, hippocampus, and cerebellum of young rats, old rats, as well as the old rats treated with LAC. Error bars indicate the SEM for 5–7 animals in each group. Measured values are normalized to the appropriate age-matched control values. *, $p < 0.05$ when compared to young rats. #, $p < 0.05$ when compared to the old rats.

tration reversed all these alterations to a level that is similar to that of young rats' HP, only the reduction of IMP level is statistically significant. Figure 5 shows these proteins on the representative 2D electrophoresis gels of the hippocampus of young rats, old rats and old rats treated with LAC.

In the CX of old rats, the specific carbonyl levels of eight proteins were increased. These proteins are heat shock cognate protein 70 (HSC 70), glyoxylase 1 (GOL 1), β -actin (ACT), 3-mercaptopyruvate sulfurtransferase (MPST), peroxiredoxin 1 (PDX), phosphoribosyl pyrophosphate synthetase 1 (PPRPS1), and fumarase (FUM) (Table 3). LAC administration reduced the specific carbonyl levels of all these protein in the CX of old rats though such changes did not reach statistical significance. Figure 6 indicates these proteins on representative 2D Western blots of the CX of young rats, old rats and old rats treated with LAC. Expression levels of F-actin capping protein beta subunit (CAPZ) and Rab GDP dissociation inhibitor β (GDI 2) were decreased in the CX of old rats, although these changes were not statistically significant. In contrast, ubiquitin (UBQ) is increased in CX of old rats when compared to that of young rats. Administration of LAC alters the levels of each of these CX proteins in aged

rats to the levels that are close to those in young (Table 4). Figure 7 indicates these proteins on representative 2D electrophoresis gels of the CX of young rats and old rats and old rats treated with LAC.

In order to determine if any of the changes observed in CX and HP, which do show increased oxidative damage in brain aging, might be artifactual, similar proteomics analysis was performed in aged CB, which did not show any elevated oxidative stress indices in brain aging. Consistent with the idea that our proteomics results reflect reality, all of the alterations mentioned in HP and CX were not observed in the cerebellum.

The identification of the proteins was performed by matching the obtained mass spectrum to the spectrum in the NCBI database. The Mowse score indicates the probability of the match being a random event (i.e., high Mowse score indicates the match is unlikely to be a random event). Prior results suggest that the accuracy of protein identification by mass spectrometry is equivalent to immunochemical identification (18). The data base characteristics and protein properties of proteins identified by mass spectrometry in hippocampus (Table 5) and cortex (Table 6) are summarized.

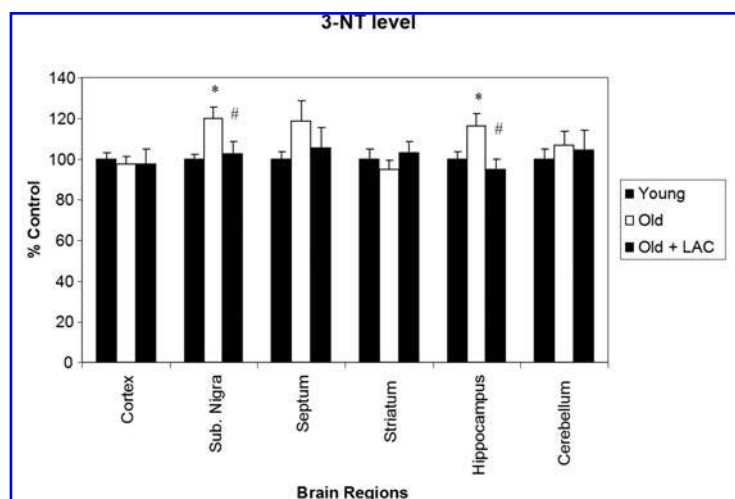


FIG. 3. Protein 3-NT levels. Results represent the average protein 3-NT levels in cortex, substantia nigra, septum pellucidum, striatum, hippocampus, and cerebellum of young rats, old rats, as well as the old rats treated with LAC. Error bars indicate the SEM for 5–7 animals in each group. Measured values are normalized to the appropriate age-matched control values. *, $p < 0.05$ when compared to young rats. #, $p < 0.05$ when compared to the old rats.

TABLE 1. ALTERED PROTEIN SPECIFIC CARBONYL LEVEL IN AGED RAT HIPPOCAMPUS^a

SSP # ^b	Protein	6m ^c	28m ^c	28m +LAC ^c	<i>p</i> value: Young vs old	<i>p</i> value: Old vs old + LAC
2004	Hemoglobin (HMG)	100 ± 48	8569 ± 4334	2959 ± 2492	<i>p</i> = 0.06	<i>p</i> = 0.15
2206	Cofilin 1 (COF 1)	100 ± 48	8053 ± 4250	76 ± 27	<i>p</i> < 0.05	<i>p</i> < 0.05
7505	Actin (ACT)	100 ± 93	1173 ± 380	320 ± 184	<i>p</i> < 0.05	<i>p</i> = 0.7

^a*n* = 4; ^bSSP # is assigned by the PDQuest imaging software; ^cResults are expressed as mean ± SEM.

TABLE 2. ALTERED PROTEIN LEVELS IN AGED RAT HIPPOCAMPUS^a

SSP # ^b	Protein	6m ^c	28m ^c	28m +LAC ^c	<i>p</i> value: Young vs old	<i>p</i> value: Old vs old + LAC
3802	Mitochondrial aconitase (ACO 2)	100 ± 14	162 ± 21	134 ± 14	<i>p</i> < 0.05	<i>p</i> = 0.4
4201	Inositol monophosphatase (IMP)	100 ± 19	149 ± 12	240 ± 19	<i>p</i> < 0.05	<i>p</i> < 0.05
5603	α-Enolase (EN01)	100 ± 12	175 ± 15	159 ± 12	<i>p</i> < 0.01	<i>p</i> = 0.6
6602	Creatine kinase, B chain (CK-B)	100 ± 15	32 ± 12	89 ± 27	<i>p</i> < 0.05	<i>p</i> = 0.8
7703	Tubulin alpha-1 chain (TUB 1)	100 ± 18	34 ± 19	45 ± 33	<i>p</i> < 0.05	<i>p</i> = 0.9

^a*n* = 4; ^bSSP # is assigned by the PDQuest imaging software; ^cResults are expressed as mean ± SEM.

TABLE 3. PROTEIN SPECIFIC CARBONYL LEVEL IN AGED RAT CORTEX^a

SSP # ^b	Protein	6m ^c	28m ^c	28m +LAC ^c	<i>p</i> value: Young vs old	<i>p</i> value: Old vs old + LAC
1712	Heat shock cognate protein 70 (HSC 70)	100 ± 98	547 ± 52	223 ± 132	<i>p</i> < 0.05	<i>p</i> = 0.08
2212	Glyoxylase 1 (GOL 1)	100 ± 73	457 ± 38	288 ± 113	<i>p</i> < 0.05	<i>p</i> = 0.23
2502	beta-Actin (ACT)	100 ± 72	454 ± 38	176 ± 127	<i>p</i> < 0.05	<i>p</i> = 0.10
4407	3-Mercaptopyruvate sulfur transferase (MPST)	100 ± 13	151 ± 13	185 ± 127	<i>p</i> < 0.05	<i>p</i> = 0.80
6203	Peroxiredoxin 1 (PDX)	100 ± 71	450 ± 37	401 ± 15	<i>p</i> < 0.05	<i>p</i> = 0.29
6401	Phosphoribosyl pyrophosphate synthetase 1 (PPRPS1)	100 ± 72	454 ± 38	402 ± 15	<i>p</i> < 0.05	<i>p</i> = 0.26
6606	Fumarase (FUM)	100 ± 73	457 ± 38	285 ± 116	<i>p</i> < 0.05	<i>p</i> = 0.20

^a*n* = 3; ^bSSP # is assigned by the PDQuest imaging software; ^cResults are expressed as mean ± SEM.

TABLE 4. ALTERED PROTEIN LEVEL IN AGED RAT CORTEX^a

SSP # ^b	Protein	6m ^c	28m ^c	<i>p</i> value: 28m +LAC ^c	<i>p</i> value: Young vs old	Old vs old + LAC
4303	F-actin capping protein beta subunit (CapZ)	100 ± 21	42 ± 9	75 ± 7	<i>p</i> = 0.06	<i>p</i> < 0.05
5211	Rab GTP dissociation inhibitor (GDI-2)	100 ± 34	72 ± 5	29 ± 8	<i>p</i> = 0.47	<i>p</i> < 0.05
7002	Ubiquitin (UBQ)	100 ± 12	141 ± 19	41 ± 25	<i>p</i> = 0.15	<i>p</i> < 0.05

^a*n* = 3; ^bSSP # is assigned by the PDQuest imaging software; ^cResults are expressed as mean ± SEM.

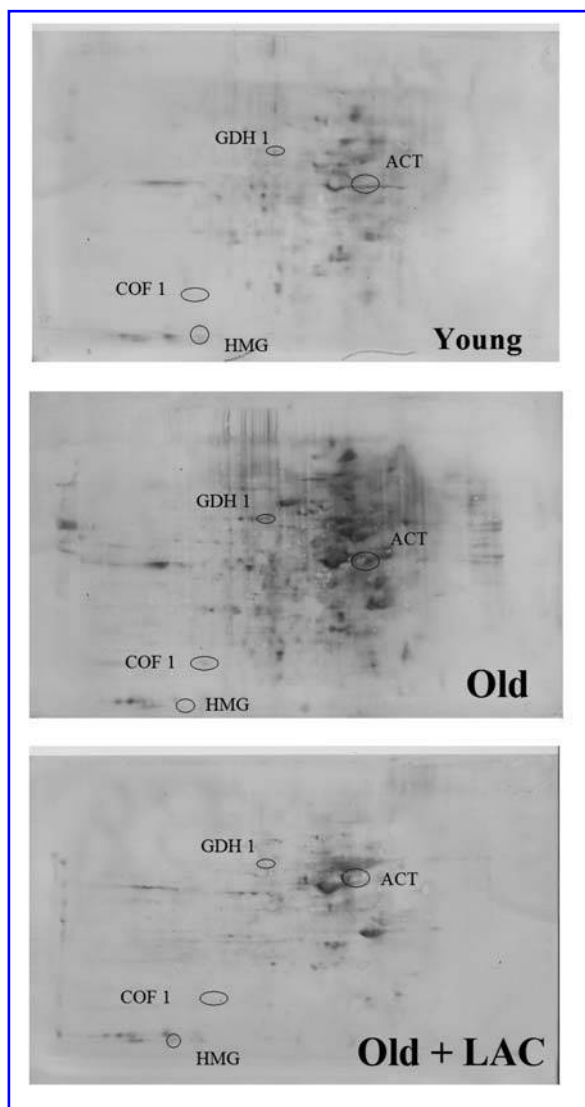


FIG. 4. Representative 2D Western blot of hippocampus. Representative Western blots showing oxidatively modified proteins from the rat hippocampus (HP). The blots show protein carbonyl western blots of HP from young rats (*top*), old rats (*middle*), as well as the old rats treated with LAC (*bottom*). *Circled proteins* show significant changes in oxidative modification in the HP of aged rats and/or aged rats treated with LAC. See text.

DISCUSSION

This study demonstrated that oxidative modification of proteins (indicated by elevated protein carbonyl, 3-NT, and HNE-binding levels) is generally increased in all brain regions of old rats when compared to those of young control rats, except cerebellum. Moreover, most of these age-related oxidative stress indices can be reduced by *in vivo* administration of LAC. Other researchers reported that LAC improves memory and learning deficits, reduces oxidative stress, and enhances impaired plasticity of the central nervous system (CNS) in aged animals (33, 49, 51, 53, 56, 93). Among the brain regions, HP shows dramatic increases in protein carbonyl, HNE, and 3-NT levels in old rats, and such oxidative parameters were reduced by administration of LAC. Similar results are observed in the CX except for 3-NT levels. Since HP and CX are involved in learning and memory and showed significant alterations in oxidative parameters in our current study, we used a parallel proteomic analysis to identify the proteins that were increased in specific carbonyl levels as well as those with altered expression in these brain regions in old rats when compared to those in young rats. Moreover, we used the same technique to identify the proteins that have decreased specific carbonyl levels or altered expression levels in HP and CX of old rats treated with LAC when compared to those in old rats without LAC treatment. We found that the proteins that are oxidized or altered in expression as a function of age can be classified into three principal categories by their function: antioxidant, mitochondria function, and plasticity (Fig. 8A). The alteration in specific carbonyl level and expression level of certain proteins by the administration of LAC in the HP and CX of old rats are summarized in Figure 8B referring to these three categories.

Hippocampal proteins altered in oxidation level and expression level

Besides being involved in oxygen transport between the lung and tissues, HMG also participates in other cellular functions, such as: modulation of erythrocyte metabolism; an onset of erythrocyte senescence by HMG oxidation; enzymatic activities and interactions with drugs; and sources of physiologically active catabolites (31). Moreover, HMG can chelate nitric oxide to reduce oxidative stress (54). Upon oxidation, neuroglobin, a heme protein similar to HMG, acts as a guanine nu-

TABLE 5. SUMMARY OF HIPPOCAMPAL PROTEINS IN AGED BRAIN IDENTIFIED BY MASS SPECTROMETRY

<i>DB accession</i>	<i>Protein ID</i>	<i>MW</i>	<i>pI</i>	<i>% Coverage</i>	<i>No. of peptides matched</i>	<i>Mowse Score^a</i>	<i>Probability^b</i>
gi 37748075	Hemoglobin (HMG)	154.9	8.45	48	10	113	5.01×10^{-12}
gi 34865596	Cofilin (COF)	22.2	9.37	35	6	69	1.26×10^{-07}
gi 6671509	Actin beta (ACT)	42.1	5.29	26	9	86	2.51×10^{-09}
gi 38541404	Mitochondrial aconitase (ACO 2)	86.1	7.87	27	19	205	3.16×10^{-21}
gi 44888968	Inositol monophosphatase (IMP)	30.8	5.17	40	7	72	6.31×10^{-08}
gi 22096350	α -Enolase (ENO1)	47.5	5.84	41	12	93	5.01×10^{-10}
gi 125296	Creatine kinase, B chain (CK-B)	43.0	5.33	40	12	136	2.51×10^{-14}
A24903	Tubulin alpha-1 chain (TUB)	50.8	4.94	41	12	85	3.16×10^{-09}

^aMowse score greater than 57 indicating the probability of the false identification of the protein is less than 0.05, $p < 0.05$;
^bProbability that the reported identification of the proteins is a random event.

cleotide dissociation inhibitor (GDI) by inhibiting the rate of exchange of GDP for GTP (87), thus leading to protection against apoptosis (79). Therefore, the increased specific carbonyl level of HMG observed in brain-aging possibly leads to functional alteration of HMG and, thus, potentially to apopto-

sis in aged brains. However, such increased carbonyl level of HMG was decreased by treatment by LAC, consistent with the concept that LAC can reduce oxidative stress by diminution of the carbonyl level of HMG in brain aging, thus likely restoring HMG function and protecting neurons from apoptosis.

TABLE 6. SUMMARY OF CORTICAL PROTEINS IN AGED BRAIN IDENTIFIED BY MASS SPECTROMETRY

<i>DB accession</i>	<i>Protein ID</i>	<i>MW</i>	<i>pI</i>	<i>% Coverage</i>	<i>No. of peptides matched</i>	<i>Mowse Score^a</i>	<i>Probability^b</i>
gi 13242237	Heat shock cognate protein 70 (HSC 70)	71	5.37	20	12	64	3.98×10^{-07}
gi 46485429	Glyoxylase 1 (GOL 1)	21	5.12	37	9	83	5.01×10^{-09}
gi 71620	β -Actin (ACT)	42.1	5.29	24	7	92	6.31×10^{-10}
gi 4826659	F-actin capping protein beta subunit (Cap Z)	31	5.69	22	6	68	1.58×10^{-07}
gi 20304123	3-Mercaptopyrivate sulfurtransferase (MPST)	33.2	5.88	24	8	90	1.00×10^{-09}
gi 1707891	Rab GDP dissociation inhibitor beta (GDI)	51.2	5.66	15	6	69	1.26×10^{-07}
gi 16923958	Peroxiredoxin 1 (PDX)	22.3	8.27	23	6	92	6.31×10^{-10}
gi 10946854	Phosphoribosyl pyrophosphate synthetase 1	35.3	6.51	26	7	68	1.58×10^{-07}
gi 227665	Fumarase (FUM)	54.7	9.13	26	10	87	2.00×10^{-09}
gi 38373984	Ubiquitin (UBQ)	11.4	5.43	65	9	118	1.58×10^{-12}

^aMowse score greater than 57 indicating the probability of the false identification of the protein is less than 0.05, $p < 0.05$;
^bProbability that the reported identification of the proteins is a random event.

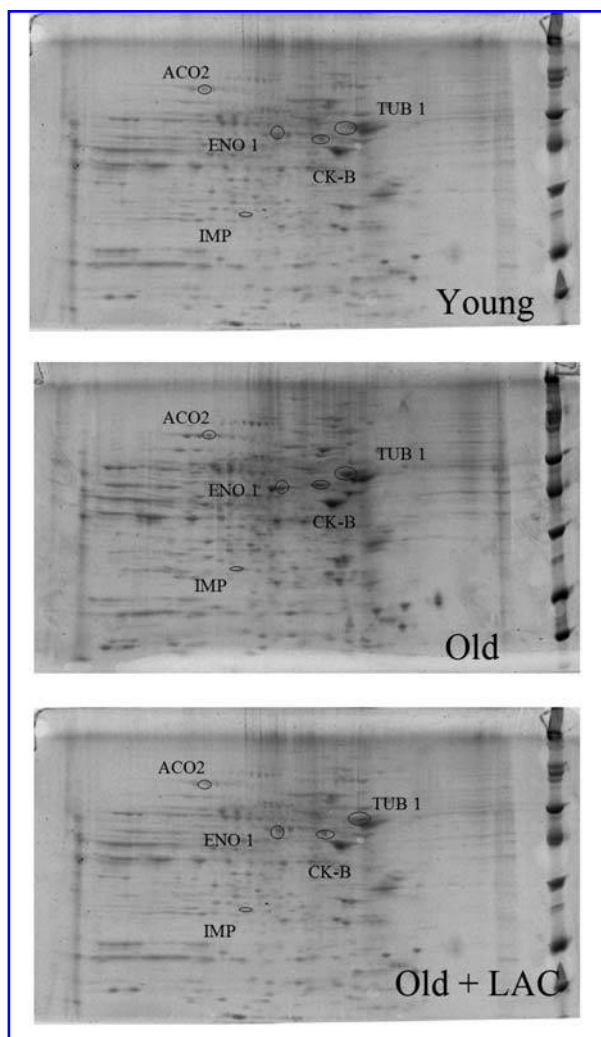


FIG. 5. Representative 2D gel of hippocampus. Representative 2D gel electrophoresis pattern of proteins from hippocampus of young rats (*top*), old rats (*middle*), as well as the old rats treated with LAC (*bottom*). See text.

COF1 is a putative actin binding protein involved in the regulation of actin polymerization and depolymerization. COF1 is accumulated in the nucleus of senescent fibroblast cells (47), suggesting that COF1 is altered as a function of age. Since actin polymerization and depolymerization are significant processes that are involved in neuronal plasticity and memory consolidation (48), increased oxidative modification of brain COF1 in old rats observed in our study may explain the increased susceptibility of age-related decline of cognition in aged animals. Our study shows that LAC treatment can decrease the oxidative modification of COF1; therefore, one can speculate that LAC enhances the neuroprotective effect of COF1 and promotes COF1-mediated ability to maintain neuronal plasticity.

ACT is a part of the cytoskeletal network responsible for cell structure and motility as well as synaptic plasticity in dendritic spines (29, 55). Oxidative modification of ACT in the muscles of aged rats (32) and decreased levels of actin in aged cultured neurons indicate that the oxidation of ACT may accelerate its degradation as a function of age (6). Consistent

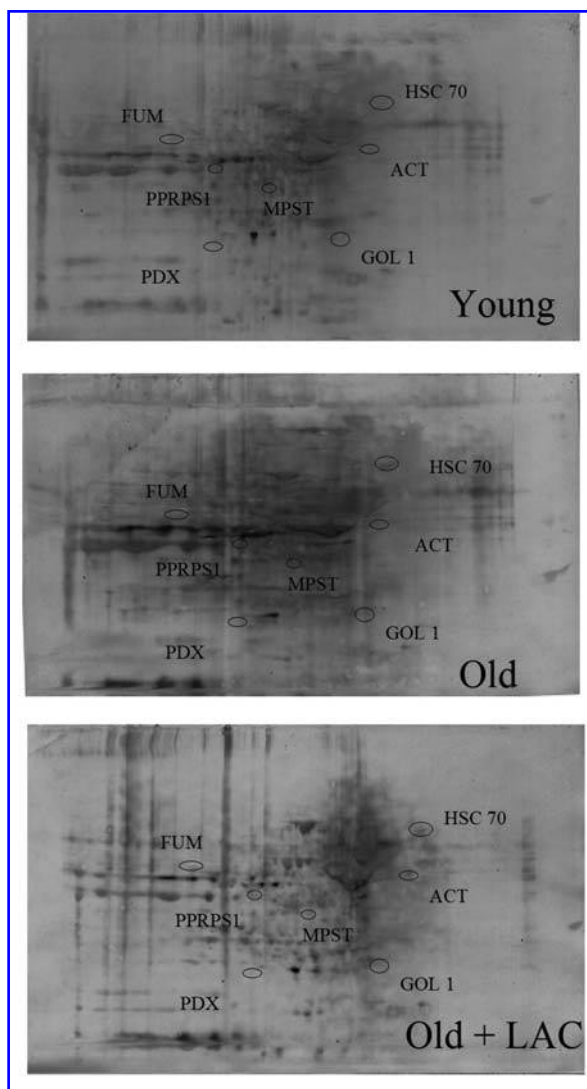


FIG. 6. Representative 2D Western blot of cortex. Representative 2D Western blots from cortex (CX) from young rats (*top*), old rats (*middle*), as well as the old rats treated with LAC (*bottom*). Circled proteins show significant oxidative modification in the aged rats and/or aged rats treated with LAC. The proteins in CX after 2D Western blot from the CX of the rats (*bottom*) described in this study are indicated. See text.

with this notion, we showed that ACT is oxidatively modified in the HP of old rats. The oxidative modification of ACT may lead to its structural alteration, thereby affecting actin filament architecture and leading to severe disarrangement of the cytoskeleton in aged brain. Moreover, structural alteration of ACT can impair neuronal plasticity, thus increasing the susceptibility of aged brains to age-related cognitive decline. However, *in vivo* LAC treatment decreases the oxidative modification of ACT, suggesting LAC protects aged HP by maintaining neuronal plasticity.

ACO2 is a mitochondrial matrix enzyme involved in the Krebs cycle. Selective oxidation during aging (25, 92) and oxidative inactivation of ACO2 by peroxynitrite leads to mitochondrial dysfunction (71). Also, stress-mediated induction of ACO2 (74) suggests that increased expression of ACO2 is a compensatory response to its oxidative inactivation. Consistent

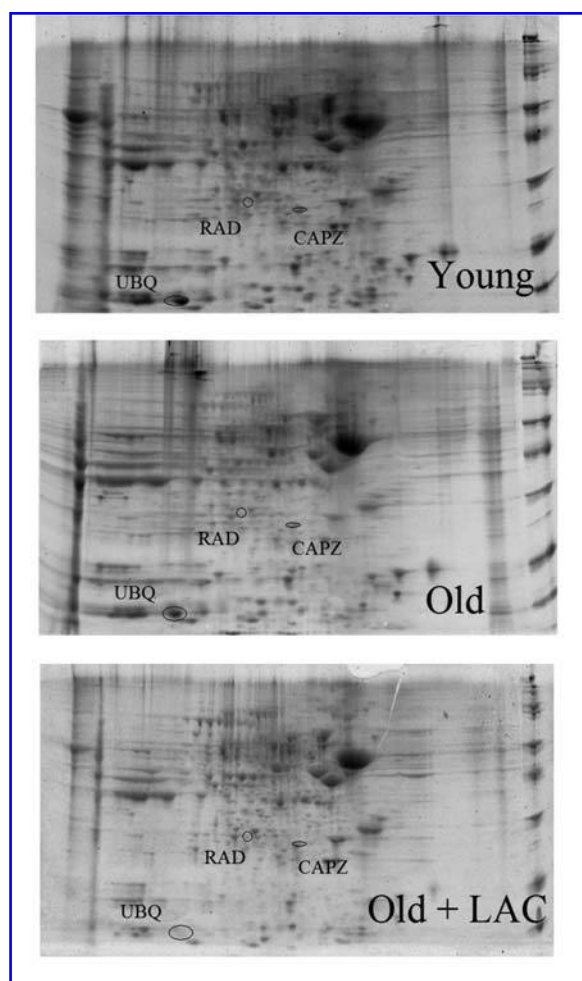


FIG. 7. Representative 2D gel of cortex. Representative 2D gel electrophoresis pattern of proteins from cortex from young rats (*top*), old rats (*middle*), as well as the old rats treated with LAC (*bottom*). The proteins in cortex after 2D electrophoresis described in this study are indicated. See text.

tent with this notion, our current study showed that protein level of ACO2 is increased in HP of old rats. However, LAC treatment reduces the increased expression level of ACO2 in aged brains, suggesting LAC modulates oxidative stress and may restore the normal mitochondrial functions.

IMP plays a significant role in the intracellular inositol level control by dephosphorylating inositol monophosphate to produce inositol (10), which can then be used to produce phosphatidyl inositol (PI) to activate the PI signal transduction pathway (11). IMP activity is decreased in aged mice brains when compared to adult mice brains (66). Decreased IMP activity could be brought about by the modification of the oxidation-sensitive thiol residues in IMP (43). Moreover, increased activity of IMP in the cerebral spinal fluid as a function of age suggests that active IMP is lost from the brains (11). Taken together, the increased protein level of IMP in aged HP in the current study is possibly a compensatory response to its loss of activity, consistent with a possible altered function in brain aging. LAC increased IMP expression even further in brain aging, consistent with a potential restoration of the PI signal transduction pathway in aged rat brain.

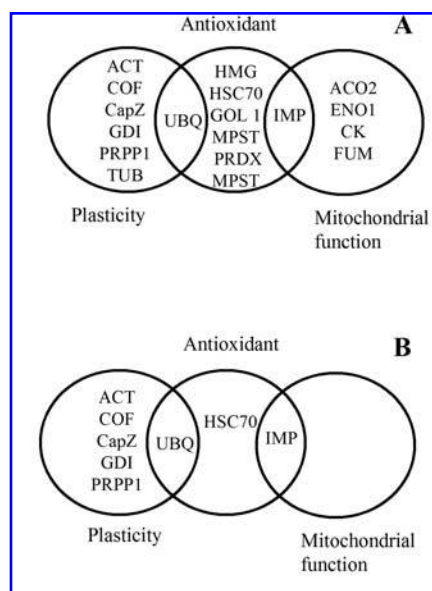


FIG. 8. Venn diagram of proteins described. (A) Venn diagram showing the altered proteins in age-impaired cellular processes: plasticity, antioxidant, and mitochondrial function. (B) Venn diagram showing the LAC-modulated proteins in this process. See text.

ENO1 is the α subunit of enolase, the other subunits being β - and γ -enolase. $\alpha\gamma$ and $\gamma\gamma$ isoforms are called neuron-specific enolases (NSE) (41). Decreased enolase activity results in reduced metabolism in brains (85). Also, the loss of the mitochondrial resident (α -ketoglutarate dehydrogenase complex, KGDHC)-enriched cells is proportional to the total loss of immunoreactivity to NSE, suggesting that enolase is not only involved in metabolism, but also in mitochondrial function (44). Additionally, the increased ENO1 specific carbonyl level in aged senescent-accelerated mice brains (69) suggests oxidative inactivation of ENO1 as a function of age. Here, we show that the protein level of ENO1 is significantly increased in HP of aged rats, suggesting the possibility that the increased level is a compensatory response to the loss of activity of enolase in brain aging.

CK, which is highly sensitive to oxidation, is found in cytoplasm and mitochondria of cells to catalyze the reversible transfer of high energy phosphoryl between the ATP and creatine phosphate (39, 77, 88, 91). Three protein subunits of CK are designated M (muscle), B (brain), and Mi (mitochondrial), which form three dimeric cytosolic (MM, BB, and MB) and distinct mitochondrial isoenzymes (Mi-CKs). It is also well established that oxidative inactivation of CK-B occurs in aging, AD, and other neurodegenerative diseases (3, 5, 7, 18, 36, 94). Moreover, the decreased expression of CK-B indicates that oxidative modification of this protein accelerates its degradation (26). Consistent with this notion, our study showed that levels of CK in aged rat brain is reduced significantly, and therefore likely affects its activity to produce ATP. However, such reduction in CK expression was reversed by LAC treatment, suggesting LAC treatment can improve mitochondrial function, and therefore, ATP production in the HP of aged rats.

TUB 1 is a structural protein that polymerizes to form microtubules of the cytoskeleton. TUB1 also interacts with

metabotropic glutamate receptor 7, which mediates a variety of responses to glutamate in brains (76). Therefore, alterations of TUB 1 not only change the cytoskeleton and intercellular trafficking, but also change the intercellular glutamate level control, thereby affecting interneuronal signaling and potentially promotes excitotoxicity. Consistent with the notion that the level of TUB 1 is significantly decreased as a function of age (62), our current study shows that the level of TUB 1 was decreased in aged rat HP, suggesting that the alteration of tubulin contributes to alteration of the cytoskeleton and glutamate dysregulation in aged brains.

Cortical proteins altered in oxidation level and expression level

CapZ is a subunit of an actin-binding protein that binds to the barbed end of actin filaments. CapZ regulates actin polymerization dynamics by attaching the actin filaments to the Z-line of myofibrils. CapZ also interacts with 5-hydroxytryptamine type 2C (5-HT_{2C}) receptors, which modulate a large variety of behavioral and physiological processes (13). Since actin polymerization dynamics contribute to memory consolidation (48), one can speculate that CapZ contributes to the receptor-mediated molecular learning and memory consolidation process. Here, we show that the CapZ expression is decreased as a function of age, suggesting an impaired actin polymerization process, with possibly impaired learning and memory in aged subjects. Moreover, we showed that the decreased level of Cap Z in CX of old rats can be elevated by treatment of LAC, suggesting that LAC can improve the actin polymerization process, thus improving learning and memory in aged brains.

Guanosine diphosphate (GDP) dissociation inhibitor 2 (GDI 2) regulates the GDP/GTP exchange reaction of Rab proteins involved in vesicle transport. This process is critical to the release of synaptic vesicles in synapses. Therefore, GDI 2 may play a regulatory role in plasticity of neurotransmission (37). Moreover, GDI 2 is oxidatively modified in aged muscle (40). Although no significant alteration was found in the aged brains in our current study, we found that LAC treatment can significantly reduce the level of GDI 2, suggesting that LAC regulates the plasticity of neurotransmission by altering the level of GDI 2.

UBQ is a polyubiquitin protein precursor that marks cellular proteins for degradation. UBQ is upregulated by stress and proteasome inhibition (63). Increased UBQ deposits are observed in dystrophic neurites of both normal aging and AD brains (57). Moreover, increased expression of UBQ in aged monkey brains suggest that UBQ upregulation is a response to age-related oxidative stress (78). Consistent with this notion, we found that mean protein expression of UBQ is increased by 41% in cortex of aged rats, although statistical significance was not reached. However, following LAC treatment, a statistically significant diminution of UBQ levels were found, suggesting both that the level of UBQ had been raised in brain aging and that LAC treatment modulates the oxidative stress in the CX of old rats.

Age-related oxidative stress induces heat shock proteins (HSP) in aged brains. HSP are molecular chaperones that mediate folding and assembly of other proteins (68). HSP-70 protects neurons against apoptosis by inhibiting the activation

of the caspase cascade (59). HSC 70, the constitutive isoforms of HSP 70, is recruited by the cell as a primary defense against stress conditions. HSC 70 is involved in the degradation of misfolded proteins by binding to a particular peptide region and labeling it for proteolysis (45). Therefore, it was suggested that HSC 70 may be involved in the structural maintenance of proteins by coupling with the proteasome (45). Alteration of HSC 70 and HSP 70 in aged brains is well established (reviewed in Ref. 17). Moreover, the diminished activity of HSC 70 in aging was suggested to be compensated by increased expression (24). The decreased activity is believed to be brought about by oxidative modification of HSC 70 (19). Consistent with this idea, the current study suggests that HSC 70 was oxidatively modified in the CX of old rats when compared to that in young rats ($p < 0.05$). This result is consistent with the notion that oxidative inactivation of HSC 70 in aged brains may cause impaired protein degradation and lead to accumulation of aggregated proteins, thereby increasing susceptibility to age-related cognitive decline. LAC treatment reduces the mean specific carbonyl level of HSC 70, albeit not at a statistically significant level, potentially protecting brains from age-related cognitive decline.

GOL 1 exhibits lactoylglutathione lyase activity that detoxifies toxic compounds. Some of these toxic compounds are produced by the action of reactive oxygen species (ROS) on biological molecules (9). Hypoxia-induced oxidative stress impairs the activity of GOL 1 (9), whereas antioxidant treatment can enhance the activity of GOL 1 as well as reducing oxidative stress parameters (60), indicating oxidative modification could inactivate GOL 1. Together, one can speculate that oxidative inactivation of GOL 1 is due to the age-related oxidative stress. Consistent with this notion, the current study demonstrated increased oxidative modification of GOL 1 in brain aging, suggesting the detoxification of toxic species in aged CX is impaired. This would lead to accumulation of toxic species and potentially neuronal death. Although not statistically significant, LAC treatment reduced the oxidative modification of GOL 1, putatively improving its activity and reducing the level of toxic species in aged brains.

MPST catalyzes the transfer of a sulfur ion from mercaptopyruvate to mercaptoethanol. This reaction is especially important in regulation of the glutathione level (90). The involvement of MPST in antioxidant metabolism is manifested by its increased expression response to oxidative stress (89). Our study shows that MPST is oxidatively modified and possibly inactivated in aged CX, suggesting decreased antioxidant metabolism in aged CX. Consistent with this notion, the glutathione level is also decreased in aged brains (52). Lower levels of glutathione, possibly due in part to oxidative inactivation of MPST, not only leads to loss of a reducing cellular component, but also could affecting expression and activity of protective enzymes (81).

PDX is an oxidative stress-inducible antioxidant protein with peroxidase and heme-binding activities. PDX is also involved in heme metabolism (38). This enzyme is essential to cellular antioxidant defense (61) and serves as a redox-regulating molecule against oxidative stress in aging (95). Our proteomics analysis demonstrated increased oxidative modification of PDX in CX of old mice, suggesting that redox regulation and antioxidant defense in aged rat CX is possibly impaired by PDX oxida-

tive inactivation, thus protecting aged brains from oxidative damage and subsequent cognitive decline.

PRPPS1 produces phosphoribosyl pyrophosphate that is required for *de novo* purine and pyrimidine biosynthesis. Mutations in PRPPS1 cause neurological impairment (73), indicating the activity of PRPPS1 is important to the proper function of the CNS. Here, we show that oxidative modification of PRPPS1 in brain aging could possibly inactivate this enzyme and decrease the availability of phosphoribosyl pyrophosphate, which is both a substrate and activator of the *de novo* and salvage pathways of purine and pyrimidine synthesis (46). Therefore, oxidative modification of PRPPS1 may be related to impaired RNA and protein synthesis necessary for neuroplasticity observed in aged rats (28, 72).

FUM catalyzes the hydration of fumarate to L-malate in the Krebs's cycle. Severe neurological impairment caused by FUM deficiency suggests that FUM is essential for the integrity of the CNS (23). FUM contains an iron-sulfur cluster active site that is sensitive to oxidative modification (64). Therefore, the oxidative modification of FUM shown in our proteomics study may inactivate the enzyme and cause decreased ATP production, thus impairing the CNS functions in aged brains. The addition of exogenous thioredoxin to FUM in mitochondria displays a protective effect against oxidative stress (27). Similarly, using LAC treatment can reduce oxidative stress and the oxidative modification of FUM, though not statistically significant, thereby potentially improving cortical functions.

Summary of findings

The proteins identified in our study are involved in three impaired processes in aged brains: antioxidant, mitochondria function and plasticity (Fig. 8). Impaired antioxidant capacity increases oxidative stress and cellular dysfunction (67, 68). Mitochondrial dysfunction mediates increased oxidative stress and apoptosis cascades as well as impaired energy metabolism (22). Impaired plasticity leads to age-related learning and memory deficits (21). All of these processes contribute to the functional decline of the CNS in aged subjects (22, 30, 72). Treatment by LAC can improve the decline of these functions. Evidence shows that oxidative stress is reduced and antioxidant levels are increased in aged brains treated with LAC (65, 80). Furthermore, improvement of mitochondrial dysfunction by LAC is well established and recently reviewed (33, 34). Moreover, LAC improves neuronal plasticity, thus ameliorating learning and memory deficits in aged brains (21, 50, 93). Therefore, our results support the point of view that treatment of LAC reduces oxidative stress by improving antioxidant defense and mitochondrial function in the CNS, thereby improving the neuroplasticity and learning and memory deficits in aged subjects. Moreover, we pinpoint that the improved antioxidant, mitochondrial function and plasticity by LAC treatment in aged brain is, at least partially, due to the alteration of the oxidation or the expression of ACT, COF, CapZ, GDI, PRPP1, TUB, HMG, UBQ, HSC70, GOL 1, MPST, PRDX, MPST, ACO2, IMP, ENO 1, CK, and FUM. To our knowledge, this is the first study to identify specific protein targets in HP and CX in brain aging and those proteins that are positively affected by LAC in brain aging. Our results provide a valuable insight into the

mechanism of age-related protein oxidation and the effect of LAC on oxidative stress reduction and functional improvements in the HP and CX of aged brains. We posit that LAC should be considered as a potential therapeutic contributor for the treatment of cognitive decline in aging and age-related neurodegenerative disorders.

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ABBREVIATIONS

ACO 2, aconitase; ACT, β -actin; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; BCA, bicinchoninic acid; CAPZ, F-actin capping protein beta subunit; CB, cerebellum; CK-B, creatine kinase B chain; CNS, central nervous system; CX, cortex; COF-1, cofilin 1; DNP, 2,4-dinitrophenyl hydrazine; DNPH, 2,4-dinitrophenylhydrazine; ENO1, α -enolase; FUM, fumarase; GDI, guanine nucleotide dissociation inhibitor; GDI 2, FaiRab GDP dissociation inhibitor β ; GOL 1, glyoxylase 1; HMG, hemoglobin; HNE, 4-hydroxynonenal; HP, hippocampus; HSC 70, heat shock cognate protein 70; IMP, inositol monophosphatase; LAC, L-acetylcarnitine; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MPST, 3-mercaptopyruvate sulfurtransferase; 3-NT, 3-nitrotyrosine; PD, Parkinson's disease; PDX, peroxiredoxin 1; PPRPS1, phosphoribosyl pyrophosphate synthetase 1; SN, substantia nigra; SP, septum pellucidum; ST, striatum; TUB 1, tubulin alpha-1 chain; UBQ, ubiquitin.

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