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Redox Modulation of Heat Shock Protein Expression by Acetylcarnitine in Aging Brain: Relationship to Antioxidant Status and Mitochondrial Function

V. CALABRESE,[1] C. COLOMBRITA,[1] R. SULTANA,[1] G. SCAPAGNINI,[1] M. CALVANI,[2] D.A. BUTTERFIELD,[3] and A.M. GIUFFRIDA STELLA[1]

ABSTRACT

There is significant evidence to show that aging is characterized by a stochastic accumulation of molecular damage and by a progressive failure of maintenance and repair processes. Protective mechanisms exist in the brain which are controlled by vitagenes and include members of the heat shock system, heme oxygenase-I, and Hsp70 as critical determinants of brain stress tolerance. Given the broad cytoprotective properties of the heat shock response, molecules inducing this defense mechanism appear to be possible candidates for novel cytoprotective strategies. Acetyl-L-carnitine is proposed as a therapeutic agent for several neurodegenerative disorders, and the present study reports that treatment for 4 months of senescent rats with acetyl-L-carnitine induces heme oxygenase-1 as well as Hsp70 and SOD-2. This effect was associated with upregulation of GSH levels, prevention of age-related changes in mitochondrial respiratory chain complex expression, and decrease in protein carbonyls and HNE formation. We hypothesize that maintenance or recovery of the activity of vitagenes may delay the aging process and decrease the risk of age-related diseases. Particularly, modulation of endogenous cellular defense mechanisms *via* acetyl-L-carnitine may represent an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration. *Antioxid. Redox Signal.* 8, 404–416.

INTRODUCTION

AGING IS characterized by a general decline in physiological functions that affects many tissues and increases the risk of death. Mitochondria are important participants in the regulation of the reduction-oxidative status of the cell. As generators of reactive oxygen species (ROS), mitochondria have antioxidant defense systems to counteract oxidative stress. Lacking many endogenous antioxidant mechanisms, mitochondria depend on glutathione (GSH) for antioxidant defense (18, 30, 35, 50). Because mitochondria lack the enzymes needed to synthesize GSH, this tripeptide must be transported into the mitochondria. As such, increasing GSH levels in the mitochondria (32–34) may prove to be an important therapeutic approach to preventing cell death in oxidative stress-linked, age-dependent neurodegenerative disorders (13, 38). The role of mitochondria in the process of the age-dependent deterioration of tissues has become the focus of many studies with the gradually accepted idea that mitochondrial decay is a major contributor to aging (7, 24 46, 58). As the source of these toxic oxidants, mitochondria are also their potential victims (39, 67). Mitochondrial decay is also a contributor to acceleration of aging in the senescence-accelerated mouse and in stress (3, 4 17, 62). During the last few years, cellular oxidant/antioxidant balance has become the subject of intense study, particularly by those interested in

[1]Section of Biochemistry and Molecular Biology, Department of Chemistry, Faculty of Medicine, University of Catania, Catania, Italy.
[2]Department of Physiology, University of Rome Tor Vergata, Italy.
[3]Department of Chemistry, University of Kentucky, Lexington, Kentucky.

brain aging and in neurodegenerative mechanisms (15, 21–24). A number of experimental evidence indicate that increased rate of free radical generation and decreased efficiency of the reparative/ degradative mechanisms (63), such as proteolysis, both are factors which primarily contribute to age-related elevation in the level of oxidative stress and brain damage (40–43). With respect to this, it has been suggested that decreases in levels of enzymes which ordinarily protect neuronal cells against oxidative stress with age may be responsible for increased levels of free-radical damage in the brain, or that these enzymes themselves are susceptible to inactivation by free radical molecules which increase with age in the brain (9, 12). Alteration of proteins in the function and higher structure has been observed in aged organisms. As a consequence during aging a number of enzymes accumulate as catalytically inactive or less active forms (3, 15).

Cells respond to sublethal heat stress by preferential synthesis and accumulation of several members of functionally and compartmentally distinct families of heat shock (or stress) proteins (such as Hsp70, Hsp90, Hsp60, and Hsp27) (14, 15). Some of these have been implicated in the development of thermotolerance and resistance to other environmental stresses (47, 51). Evidence indicates that Hsp72 may contribute to cellular protection against a variety of stresses by preventing protein aggregation, assisting in the refolding of damaged proteins, and chaperoning nascent polypeptides along ribosomes (19, 26). Expression of the genes encoding Hsps has been found in various cell populations of central nervous system (CNS), including neurons, glia, and endothelial cells (20, 81). Hence, the heat shock response contributes to establishing a cytoprotective state in a variety of metabolic disturbances and injuries, including hypoxia, stroke, epilepsy, cell and tissue trauma, neurodegenerative disease, and aging (62, 79). This has opened new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear to be possible candidates for novel cytoprotective strategies (27, 68, 69, 77). Efficient functioning of maintenance and repair processes, which is critical for brain cellular survival, is accomplished by a complex network of the so-called longevity assurance processes, under control of several genes termed vitagenes. These include members of the heat shock protein system, such as Hsp70, Hsp60, and heme oxygenase-1 (HO-1) (1, 62, 64).

HO-1, also known as Hsp32, is emerging for its central role in brain stress tolerance, as it has been recently demonstrated that HO-1 induction, by generating the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin, could represent a protective system potentially active against a large array of brain oxidative injuries (26, 45, 49). Acetyl-L-carnitine (LAC) is proposed as a therapeutic agent for several neurodegenerative disorders (10, 25, 27). We have recently shown in astrocytes exposed to nitrosative stress that LAC, a well known metabolic and/or antioxidant modulator, protects against cytokine-mediated mitochondrial chain respiratory complex impairment and the associated increase in protein and lipid peroxidation. The increase in astroglial antioxidative potential observed after LAC treatment involves a secondary line of antioxidant defenses, represented by stress responsive genes, such as HO-1, and the mitochondrial Hsp60 and SOD (27). In view of the recent findings suggesting that mitochondria are a selective target of Hsp protection against oxidative insults (78) we investigated, in different brain regions of aging rats, the effect of LAC supplementation on the expression of Hsps, mitochondrial respiratory chain complexes and antioxidant status. We found that heat shock protein expression was higher in aged than young animals and this increase was associated with mitochondrial dysfunction and with disruption of thiol homeostasis, as indicated by a decrease in the GSH and complex I expression, and with increased glutathione disulfide (GSSG) and 4-hydroxy-transnonenal (HNE) levels. Treatment of rats with LAC resulted in upregulation of stress responsive genes, such as Hsp70, heme oxygenase, and SOD-2, as well as mitochondrial respiratory complex expression. This effect was associated with increased GSH levels and decreased HNE and carbonyls groups. The involvement of LAC on the redox-dependent mechanisms underlying activation of the heat shock response and the possible implications in neurodegenerative disorders are discussed.

MATERIALS AND METHODS

Chemicals

5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), 1,1,3,3-tetraethoxypropane, purified bovine blood SOD, NADH, reduced glutathione (GSH), oxidized glutathione (GSSG), β-NADPH (type 1, tetrasodium salt), glutathione reductase (GR, Type II, from Bakers Yeast), were from Sigma Chemicals Co. (St. Louis, MO, USA). Acetyl-L-carnitine (99.99% pure) was a generous gift from Sigma Tau Co. (Pomezia, Italy). All other chemicals were from Merck (Darmstadt, Germany) and of the highest grade available.

Animals

All animal protocols were approved by the University of Catania Laboratory Animal Care Advisory Committee. Male 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 divided into three groups, 12 months (adult), 28 months (senescent), and 28 months plus acetyl-L-carnitine (n = 8 per each age group). Acetyl-L-carnitine to this group of animals was supplemented orally for the last 4 months at the dose of 150 mg/kg/day. The rationale for LAC supplementation was chosen on the basis of previous results (22, 48). All groups were fed *ad libitum* a certified diet prepared according to the recommendations of the American Institute of Nutrition, and the percentage energy composition is given in Table 1. 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.

Reduced and oxidized glutathione assay

Reduced glutathione (GSH) and glutathione disulfide (GSSG) were measured by the NADPH-dependent GSSG re-

Table 1. Percentage Energy Composition of the Diet

	(g/100 g)
Dextrin–Maltose*	53
Oil mixture†	25
Casein	22
D-L ethionine	0.5
Salt mixture (AIN 76)	3.5
Vitamin mixture (AIN 76)	1.2

*From corn starch;
†Olive oil/corn oil 2:1; 16:0 = 12.8%; 16:1 = 0.4%; 18:0 = 5.2%; 18:1 = 27.8%; 18:2 = 50.4%.

ductase method as previously reported (18). Specimens were homogenized at ice temperature for 10 s in 100 m*M* potassium phosphate, pH 7.5, which contained 12 m*M* disodium EDTA. For total glutathione, aliquots (0.1 ml) of homogenates were immediately added to 0.1 ml of a cold solution containing 10 m*M* DTNB and 5 m*M* EDTA in 100 m*M* potassium phosphate, pH 7.5. The samples were mixed by tilting and centrifuged at 12,000 *g* for 2 min at 4°C. An aliquot (50 µl) of the supernatant was added to a cuvette containing 0.5 U GSSG reductase in 100 m*M* potassium phosphate and 5 m*M* EDTA, pH 7.5 (buffer 1). After 1 min of equilibration, the reaction was initiated with 220 nmol of NADPH in buffer 1 for a final reaction volume of 1 ml. The formation of a GSH-DTNB conjugate was then measured at 412 nm. The reference cuvette contained equal concentrations of DTNB, NADPH, and enzyme, but not sample. For GSSG assay, aliquot (0.5 ml) of homogenate was immediately added to 0.5 ml of a solution containing 10 m*M* NEM and 5 m*M* EDTA in 100 m*M* potassium phosphate, pH 7.5. The sample was mixed by tilting and centrifuged at 12,000 *g* for 2 min at 4°C. An aliquot (500 µl) of the supernatant was passed at one drop/s through a SEP-PAK C18 Column (Waters, Framingham, MA, USA) that had been washed with methanol followed by water. The column was then washed with 1 ml of buffer 1. Aliquot (865 µl) of the combined eluates were added to a cuvette with 250 nmol of DTNB and 0.5 U GSSG reductase. The assay then proceeded as in the measurement of total GSH. GSH and GSSG standards in the ranges between 0 to 10 nmol and 0.010 to 10 nmol, respectively, added to control samples, were used to obtain the relative standard curves, and the results were expressed in nmol of GSH or GSSG, respectively, per mg protein.

Western blot analysis

The tissue homogenate was centrifuged at 10,000 *g* for 10 min and the supernatant was used for HO-1, Hsp72, and HNE determination, after dosage of proteins as described below. Aliquot (30 µg) of protein extract was separated by SDS-polyacrylamide gel electrophoresis using a miniprotean apparatus (BioRad, Hercules, CA), transferred overnight to nitrocellulose membranes. The nonspecific binding of antibodies was blocked with 3% nonfat dry milk in PBS. Membranes were then probed with anti-HO-1 antibody (Stressgen, Victoria, Canada) (1:1,000 dilution in Tris-buffered saline, pH 7.4) or with a monoclonal anti-Hsp72 antibody (RPN 1197, Amersham, Piscataway, NJ, USA) that recognizes only the inducible form. When probed for HNE, membranes were incubated for 2 h at room temperature with anti-HNE (anti-4-hydroxy-2-nonenal) Michael adducts (B 4067, Calbiochem, San Diego, CA). Goat polyclonal antibody specific for β-actin was used as loading control (1:1000). For detection, the membranes were incubated with a horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (IgG), followed by ECL chemiluminescence (Amersham). The amount of inducible HO-1, Hsp72, and HNE was quantified by scanning Western blot imaged films with a laser densitometer (LKB-Ultroscan, XL model, Pharmacia, Alameda, CA). Multiple exposure of each blot was used to ensure linearity of the film response.

Real-time quantitative RT-PCR

Total RNA was extracted using Trizol (Sigma) and treated with RNase-free DNase to remove any residual genomic DNA. Single-stranded cDNAs were synthesized by incubating total RNA (1 µg) with SuperScript II RNase H reverse transcriptase (200 U), oligo-(dT)12–18 primer (100 n*M*), dNTPs (1 m*M*), and RNase inhibitor (40 U) at 42°C for 1 h in a final volume of 20 µl. Reaction was terminated by incubating at 70°C for 10 min. Forward (F) and reverse (R) primers used to amplify HO-1, Hsp72, SOD-2, and mitochondrial complex I and IV are shown in Table 2. To control the integrity of RNA and for differences attributable to errors in experimental manipulation from tube to tube, primers for rat phosphoglycerate kinase 1 (PGK 1) a housekeeping gene that is consistently expressed in brain tissues, were used in separate PCR reactions (PGK-F: AGGTGCTCAACAACATGGAG, PGK-R: TACCAGAGGCCACAGTAGCT, GenBank accession No. M31788), and the expected amplification products for this gene was 183 bp. Aliquots of cDNA (0.1 µg) and known amounts of external standard (purified PCR product, 10^2 to 10^8 copies) were amplified in parallel reactions using the forward and reverse primers. Each PCR reaction (final volume, 20 µl) contained 0.5 µ*M* of primers,

Table 2. Sequences of Oligonucleotide Primers for HO-1, Hsp72, SOD-2, Complex I, Complex IV and PKG1 mRNAs

Name	*Sequence*
HO1-F	TGCTCGCATGAACACTCTG
HO1-R	TCCTCTGTCAGCAGTGCCT
HSP72-F	TTTTCTGGCTCTCAGGGTGT
HSP72-R	CCTTGCATCCCTACAAACTGA
SOD2-F	GGCCAAGGGAGATGTTACAA
Complex1 sub B13 (cyt)-F	ACTACTGGCCTGGTGGGATT
Complex1 sub B13 (cyt)-R	CCACCCTGAAGCAAGTTTTC
Complex IV subIV (cyt)-F	ACATGAAGAGCAACCCCATA
Complex IV subIV (cyt)-R	AAGGGAATGGAGGAGACAAG
PGK1-F	AGGTGCTCAACAACATGGAG
PGK1-R	TACCAGAGGCCACAGTAGCT

2.5 m*M* Mg2+, and 1× Light Cycler DNA Master SYBR Green (Roche Diagnostics, Indianapolis, IN, USA). PCR amplifications were performed with a Light Cycler (Roche Molecular Biochemicals) using the following four cycle programs: (i) denaturation of cDNA (one cycle: 95°C for 10 min); (ii) amplification (40 cycles: 95°C for 0 s, 58°C for 5 s, 72°C for 10 s); (iii) melting curve analysis (one cycle: 95°C for 0 s, 70°C for 10 s, 95°C for 0 s); (iv) cooling (one cycle: 40°C for 3 min). The temperature transition rate was 20°C/s except for the third segment of the melting curve analysis, where it was 0.2°C/s. Fluorimeter gain value was 6. Real-time detection of fluorimetric intensity of SYBR Green I, indicating the amount of PCR product formed, was measured at the end of each elongation phase. Quantification was performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards. For this analysis, fluorescence values measured in the log-linear phase of amplification were considered using the second derivative maximum method of the Light Cycler Data Analysis software (Roche Molecular Biochemicals). Specificity of PCR products obtained was characterized by melting curve analysis followed by gel electrophoresis, visualized by ethidium bromide staining, and DNA sequencing.

In situ hybridization

Brains from decapitated 6, 28, and 28 month-old Wistar rats treated with LAC (n = 6) were rapidly removed and frozen at −70°C. Coronal sections of 12 µm thickness were cut on a cryostat at -20°C, thaw mounted onto silanated glass slides (Digene, Gaithersburg, MD, USA), and stored at −70°C until hybridization. The sections were fixed in 4% paraformaldehyde in 1× PBS buffer (pH 7.3), acetylated, dehydrated in serial alcohol solutions, delipidated in chloroform, partially redehydrated, and air dried. *In situ* hybridization was performed as described previously (31). A 315 bp fragment of cDNA corresponding to bases 561–867 of the previously characterized rat HO-1 mRNA (GenBank accession No: NM_012580) was synthesized by PCR from rat brain RNA using specific primers (FP: 5′-CGCATGAACACTCTGGAGAT-3′; RP: 5′-CATGGCATAAATTCCCACTG-3′). A 319 bp fragment of cDNA corresponding to bases 287–605 of the previously characterized rat SOD2 mRNA (GenBank accession No: NM_017051) was synthesized by PCR from rat brain RNA using specific primers (FP: 5′- GGCCAAGGGAGATGTTACAA-3′; RP: 5′-CCACAGGCCTTATTCCACTG-3′).

These fragments, designed to specifically detect transcripts encoding, respectively, HO-1 and SOD2, were subcloned into the PCR 4Blunt-TOPO vector (Invitrogen, Carlsbad, CA, USA). The orientation of the inserts was determined by DNA sequencing. After linearization with the restriction enzymes *Pme*I and *Not*I and *Spe*I and *Not*I, respectively, the HO-1 and SOD2 cDNA fragment-inserted vectors were used as templates for riboprobe synthesis. The sense and antisense riboprobes were synthesized with T3 or T7 RNA polymerase (Ambion, Austin, TX, USA), respectively, in the presence of 1 µg cDNA (HO-1/SOD2) template, 0.5 m*M* ATP, 0.5 m*M* CTP, 0.5 m*M* GTP, and 2 m*M* [α-^{35}S] UTP (>1000 Ci/mmol, NEN). The transcribed products were purified on Sephadex G-25 spin columns (Roche) and probe labeling was assessed by scintillation counting. Fifty microliters of the HO-1 and SOD2 riboprobes (10^6 cpm/µl) were applied, respectively, to each slide holding three sections and hybridized in a mixture containing 20 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 300 m*M* NaCl, 50% formamide, 10% dextran sulfate, 13% Denhardt's solution, 4 mg/ml salmon sperm DNA, 10 mg/ml yeast total RNA, 10 mg/ml yeast tRNA, 100 m*M* DTT, 0.1% sodium dodecyl sulfate (SDS), 0.1% NTS. Hybridization was carried out at 55°C for 24 h. Sections incubated with the sense probe during hybridization were used as negative controls. After high-stringency post hybridization washes and RNase treatment, brain sections were dehydrated in graded ethanol. Slides were then exposed to film (hyperfilm Bmax; Amersham) for 24 h for the generation of autoradiograms. Hybridization with the sense riboprobe led to no detectable signal (data not shown).

Protein carbonyl measurement

Carbonyl groups were measured as already reported (32). Briefly, 200 m*M* 2,4-dinitrophenylhydrazine (DNPH) stock solution was diluted ten times with water. 5 µl of sample was incubated at room temperature with 5 µl 12% SDS and 10 µl of the diluted DNPH for 20 min with vortexing. The samples were neutralized with 7.5 µl of the neutralization solution (2 *M* Tris in 30% glycerol). 250 ng of the sample solution was loaded into the wells of the slot blot apparatus. Proteins were transferred directly to the nitrocellulose paper under vacuum pressure and standard immunochemical techniques were performed. A rabbit-anti DNPH polyclonal primary antibody and a goat anti-rabbit IgG alkaline phosphatase secondary antibody were used. Samples were developed using SigmaFast Tablets (BCIP/NBT substrate), and blots were scanned into Adobe PhotoShop and quantitated with Scion Image (PC version of Macintosh compatible NIH Image).

Alternatively, an aliquot (30 µg) of protein extract was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were then probed with anti-DNPH polyclonal primary antibody before detection with goat anti-rabbit IgG horseradish peroxidase conjugate (dil 1:2000) and subsequent ECL chemiluminescence (Amersham).

Protein determination

Proteins were estimated by the method of Smith (72) using bicinchoninic acid reagent.

Statistical examination

Results were expressed as means ± SEM of at least eight separate experiments. Statistical analyses were performed using the software package SYSTAT (Systat Inc., Evanston IL, USA). The significance of the differences, evaluated by two-way ANOVA, followed by Duncan's new multiple-range test, was considered significant at $p < 0.05$. Correlation analysis was considered statistically significant if the coefficient of determination R was ≥ 0.8.

RESULTS

Glutathione redox state analysis

When different brain regions were examined for GSH levels as a function of aging, all brain regions showed a diminution in GSH in senescent compared to adult animals (Fig. 1A). Conversely, the level of oxidized GSH (GSSG) increased with the same pattern (Fig. 1B) in all brain areas investigated. Treatment with LAC of senescent rats restored GSH levels in all brain regions examined (Fig. 1A) and maintained GSSG content similar to that found in control adult rats (Fig. 1B). To test the hypothesis that this loss of redox status in the brain as a consequence of aging would induce a Hsp response, we measured the expression of heme oxygenase-1 (HO-1) and Hsp72 in different rat brain regions of adult, senescent, and senescent given acetyl-L-carnitine for the last 4 months.

Hsps distribution in brain aging

Figure 2 shows expression of inducible Hsp70 during aging in the absence and presence of treatment with LAC. Expression of Hsp72 was significantly elevated at 28 months of age, compared to adult animals in the striatum, hippocampus, and cerebellum. Notably, treatment with LAC enhanced Hsp70 protein expression in the hippocampus, cerebellum, striatum, and cortex. We also measured HO-1 protein expression during aging and found a parallel increase in the synthesis of this heat shock protein, as observed with Hsp70, in all brain regions of senescent rats but the striatum (Fig. 3). Acetyl-L-carnitine to these groups of animals resulted in a further increase in HO-1 synthesis, particularly in the cere-

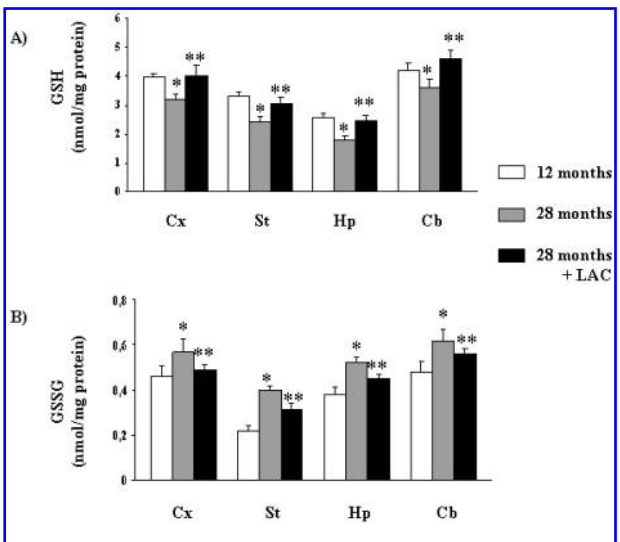

FIG. 1. Regional distribution of reduced glutathione (GSH) in different brain regions as function of aging. Effect of acetyl-L-carnitine. GSH (**A**) and GSSG (**B**) in cortex (Cx), striatum (St), hippocampus (Hp), and cerebellum (Cb) were measured as described in Materials and Methods. Results are expressed in nmol/mg protein. Data are means ± SEM of eight animals. (*) $p < 0.05$ vs. control adult 12-month-old rats; (**) $p < 0.05$ vs. senescent 28-month-old rats.

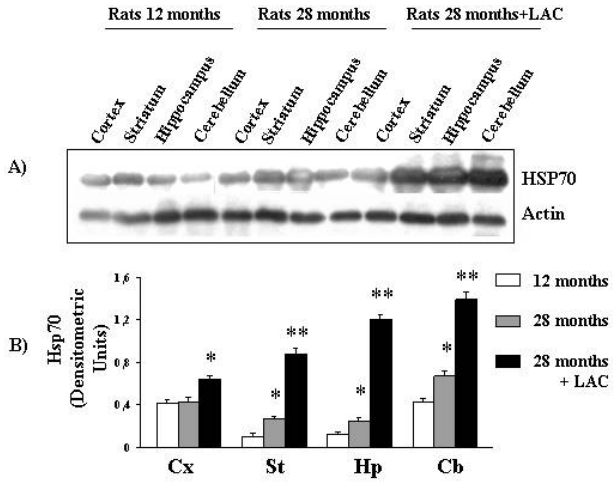

FIG. 2. Regional cerebral distribution in the levels of Hsp72 immunoreactive material. Cortex, striatum, hippocampus, and cerebellum were analyzed in adult, senescent, and senescent plus acetyl-L-carnitine-treated rats. The values are expressed as densitometric units obtained by scanning the Western blot luminographs with laser densitometer. (**A**) Representative Western blots show samples from cortex, striatum, hippocampus and cerebellum probed with a monoclonal anti-Hsp70 antibody (RPN 1197, Amersham) that recognizes only inducible Hsp70. Data are means ± SEM of eight animals (**B**). Cortex (Cx), striatum (St), hippocampus (Hp) and cerebellum (Cb). (*) $p < 0.05$ vs. adult; (**) $p < 0.05$ vs. senescent rats.

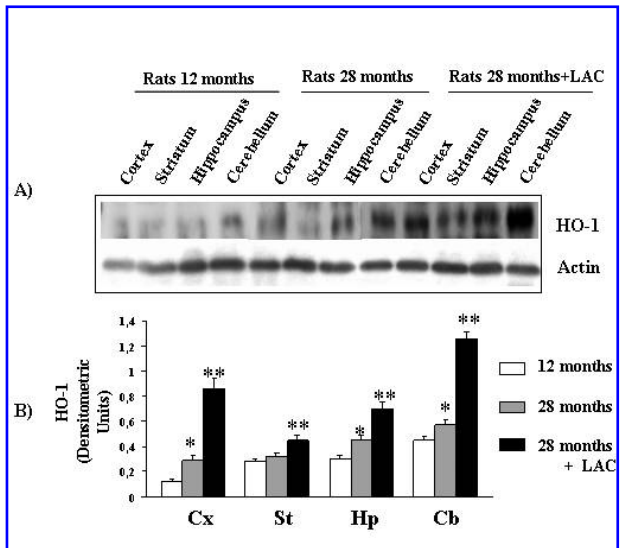

FIG. 3. HO-1 protein expression in adult (12 months) and senescent (28 months) rats. HO-1 expression in the absence and presence of acetyl-L-carnitine treatment in different cerebral areas: cortex, striatum, hippocampus, and cerebellum. The values are expressed as densitometric units obtained by scanning the Western blot luminographs with laser densitometer. (**A**) Representative Western blot is reported showing samples from all the brain areas. (**B**) Cortex (Cx), striatum (St), hippocampus (Hp), and cerebellum (Cb). Data are means ± SEM of eight animals. *$p < 0.05$ vs. adult control; (**) $p < 0.05$ vs. senescent rats.

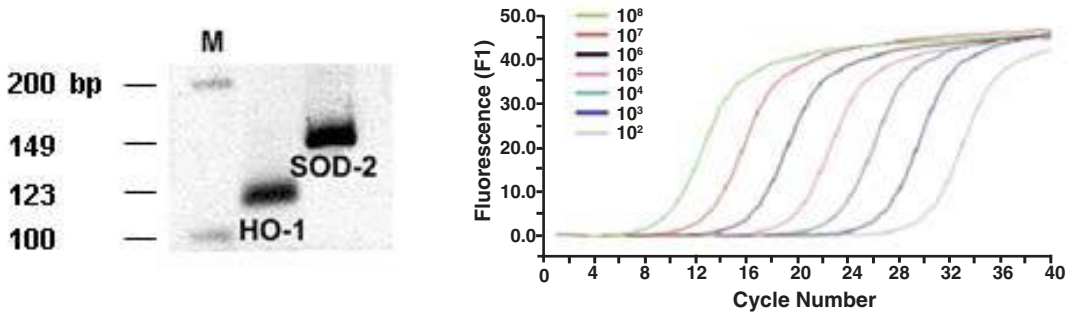

FIG. 4. Real-time quantification by RT-PCR. RT-PCR of HO-1 and SOD-2 mRNA levels in different brain regions of adult, senescent and senescent plus acetyl-L-carnitine-treated rats. Total RNA from different samples and known amounts of external standards (purified PCR product, 10^2 to 10^8 copies) were amplified in parallel reactions. Fluorimetric intensity of SYBR Green I, indicating the amount of PCR product formed, was measured at the end of each elongation phase. Quantification was performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards. Brain expression of HO-1 transcript relative to the expression of SOD-2 (mean ± SEM) in the hippocampus from senescent rats receiving acetyl-L-carnitine at the oral daily dose of 150 mg/kg is also shown.

bellum, followed by cortex, hippocampus, and striatum. Furthermore, expression of Hsps (Hsp70 and HO-1) in the hippocampus and cerebellum showed to correlate significantly with GSH/GSSG ratio, either in the absence ($R = 0.87$) or in the presence of treatment with LAC ($R = 0.98$).

Real-time quantification of HO-1 and SOD-2, as well as Hsp72 mRNA levels in different brain regions of aging rats was accomplished by RT-PCR. Total RNA from different samples and known amounts of external standards (purified PCR products, 10^2 to 10^8 copies) were amplified in parallel reactions (Fig. 4). The sequences of oligonucleotide primers used to amplify rat RNA for HO-1, Hsp72, SOD-2, as well as mitochondrial complexes (see below) and PKG1 are illustrated in Table 2. Consistent with the increased expression of Hsp72 and HO-1 with brain aging, the message for these response proteins was also elevated in senescent animals treated with LAC, particularly in the hippocampus and cerebellum regions (Figs. 5A and 5B). The expression of SOD-2, the mitochondrial isoform of superoxide dismutase was also examined as function of brain aging (Fig. 5C). Compared to adult rats, in senescent animals SOD-2 did not show significant differences in the cortex and striatum, whereas in the hippocampus was significantly diminished ($p < 0.05$) and increased in the cerebellum. Treatment with LAC unmodified SOD-2 expression in the striatum and cerebellum, but increased significantly in the hippocampus and cortex. This

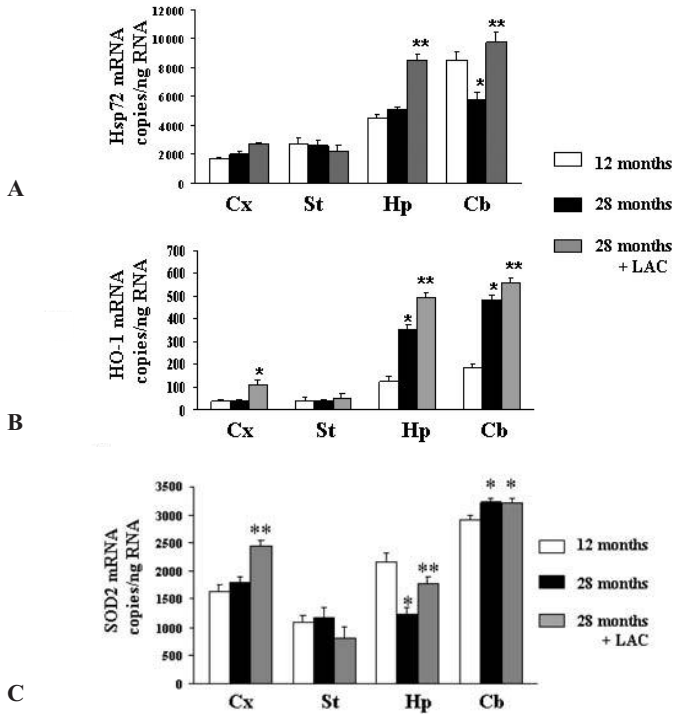

FIG. 5. Hsp72, HO-1, and SOD-2 levels in aging brain. Levels of (**A**) Hsp72, (**B**) HO-1, and (**C**) SOD-2 mRNAs in different cerebral areas as function of brain aging, and the effect of acetyl-L-carnitine treatment. Total RNA from rat brain dissected areas was extracted as described in Materials and Methods and quantification was performed by RT-PCR. Specificity of PCR products was characterized by melting curve analysis followed by gel electrophoresis, ethidium bromide staining, and DNA sequencing. Cortex (Cx), striatum (St), hippocampus (Hp) and cerebellum (Cb). (*) $p < 0.05$ vs. control; (**) $p < 0.05$ vs. 28-month-old senescent rats.

finding was also confirmed by *in situ* hybridization analysis (Fig. 6), which showed higher riboprobe signal intensities for both HO-1 and SOD-2 mRNAs induced by LAC in the cerebellum and hippocampus, compared with mRNA signal measured in senescent untreated animals.

Hydroxynonenal and protein carbonyl analysis

One measure of oxidative stress in brain aging is protein oxidation (8, 12). However, lipid peroxidation, indexed by HNE, can also occur in brain under oxidative stress (59–61). HNE, formed from arachidonic acid or other unsaturated fatty acids following free radical attack (33), binds by Michael addition to proteins, particularly at cysteine, histidine, or lysine residues (59, 63). Examination of HNE levels in different brain regions in adult and senescent rat brains showed elevation of protein-bound HNE in all brain regions, but particularly striking in the striatum and hippocampus regions, and to a lesser extent in the cortex (Fig. 7), while the cerebellum, consistently with higher level of GSH found in the same areas, showed the lowest HNE content as function of brain aging (Fig. 7). In these brain regions treatment with LAC resulted in a marked decrease in HNE-dependent protein adduct formation. Additionally, a significant increase in the amount of protein carbonyls ($p < 0.004$) (Figs. 8A and 8B), an index of protein oxidation (8,16) was found in all brain regions examined in senescent rats as compared to adult controls, but not in the cerebellum, confirming HNE data. Protein carbonyl formation induced by aging was prevented by pretreatment with LAC (Fig. 8B), particularly in the hippocampus (Fig. 8A).

Mitochondrial complex expression analysis

As noted in the Introduction, mitochondria are a source of oxidative stress, but they also can be a target of free radical damage. The expressions of mitochondrial Complex I and Complex IV of the electron transport chain (ETC) were examined in different brain regions as a function of brain aging. Senescent brain mitochondria showed a decrease in Complex I (nuclear B13, 39 kDa subunit) mRNA expression only in the hippocampus (Fig. 9A). Cerebellar Complex IV expression (nuclear subunit IV, see Table 2) is elevated in senescent rats, relative to adult animals (Fig. 9B). In no other region studied was senescent rat brain altered in its Complex IV expression, confirming our previous data on expression and activity of this mitochondrial respiratory complex during brain aging. However, treatment with LAC increased expression of complex I in the hippocampus and cerebellum, whereas complex IV was decreased in the hippocampus and increased in the cerebellum by the same treatment (Fig. 9B).

DISCUSSION

Although the term "aging" is generally understood in broad terms, the aging process is extremely complex and multifaceted. Increasing evidence supports the notion that reduction of cellular expression and activity of antioxidant proteins and the resulting increase of oxidative stress are fundamental causes in the aging processes and neurodegenerative diseases (40). Experimental evidence indicates that increased rate of free radical generation and decreased efficiency of the

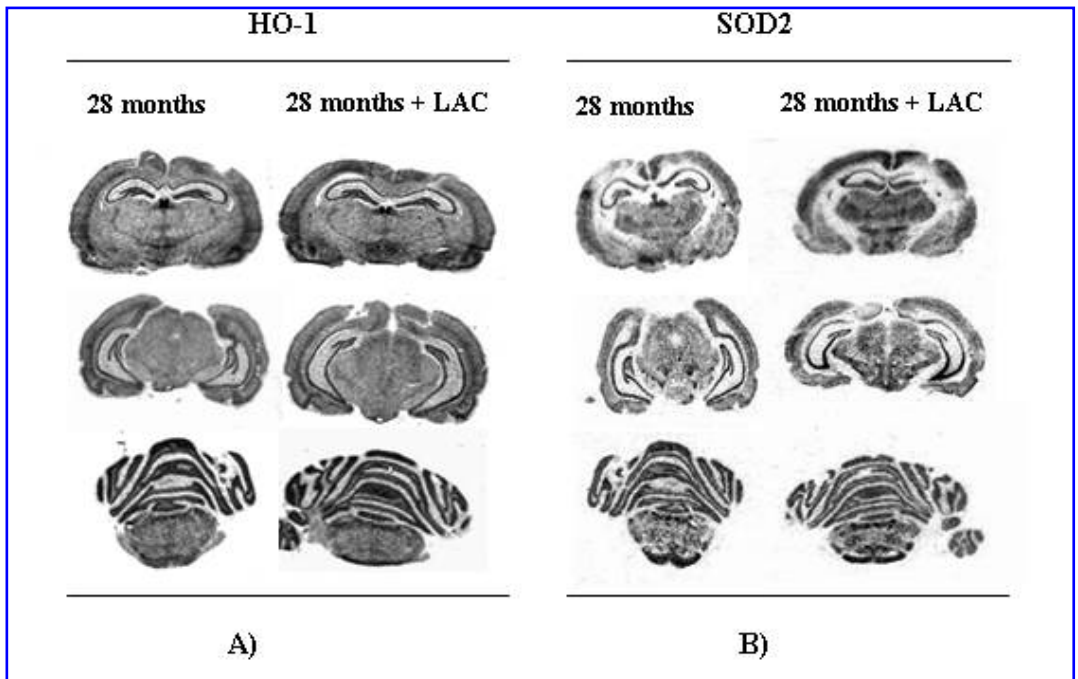

FIG. 6. *In situ* hybridization. Localization of HO-1 and SOD-2 mRNA in senescent and senescent plus acetyl-L-carnitine-treated rats. A 315 bp riboprobe for HO-1 and a 319 bp riboprobe for SOD-2 labeled with [α-^{35}S] UTP were synthesized and hybridized with coronal sections of the rat brain. After hybridization, the labeled HO-1 (**A**) and SOD-2 (**B**) mRNA signals were revealed by autoradiography.

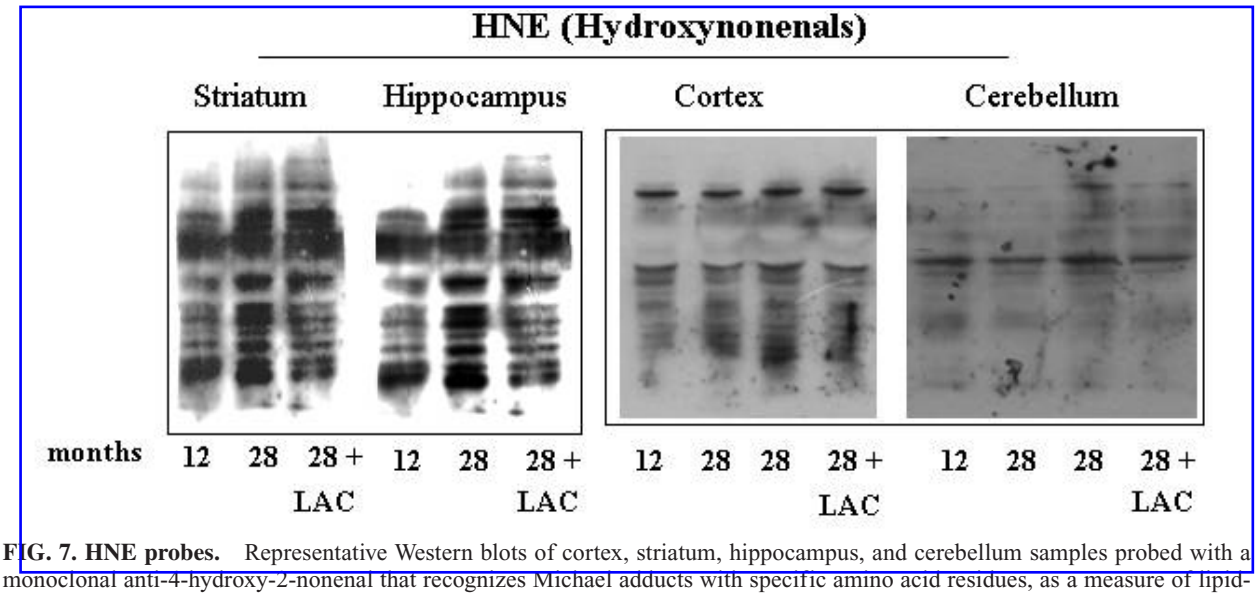

FIG. 7. HNE probes. Representative Western blots of cortex, striatum, hippocampus, and cerebellum samples probed with a monoclonal anti-4-hydroxy-2-nonenal that recognizes Michael adducts with specific amino acid residues, as a measure of lipid-derived oxidative stress, in the brain of adult and senescent rats, in the absence and presence of acetyl-L-carnitine treatment.

reparative/degradative mechanisms, such as antioxidant defense and proteolysis, both are factors which primarily contribute to age-related elevation in the level of oxidative stress and brain damage (53). Reduced glutathione (GSH) is the most prevalent nonprotein thiol in animal cells. Its *de novo* and salvage synthesis serve to maintain a reduced cellular environment and the tripeptide is a co-factor for many cytoplasmic enzymes and may also act as an important post-translational modification in a number of cellular proteins (70). The cysteine thiol acts, in fact, as a nucleophile in reactions with both exogenous and endogenous electrophilic species. As a consequence, reactive oxygen species (ROS) are frequently targeted by GSH in both spontaneous and catalytic reactions (11, 16, 18, 62). Since ROS have defined roles in cell signal-

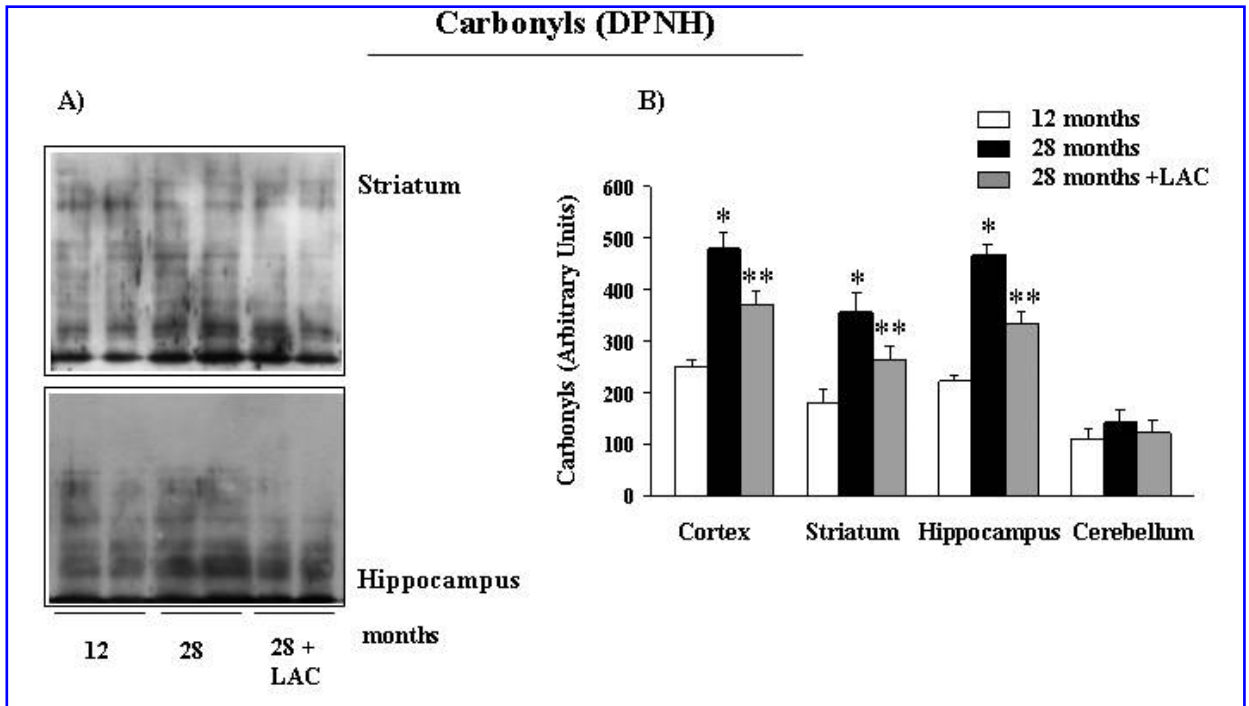

FIG. 8. Protein carbonyl groups. (**A**) Representative Western blots of samples from striatum and hippocampus probed with a polyclonal anti-DNPH that recognizes protein carbonyl groups. (**B**) Histograms are means ± SEM of eight animals. (*) $p < 0.05$ vs. control adult; (**) $p < 0.05$ vs. senescent rats.

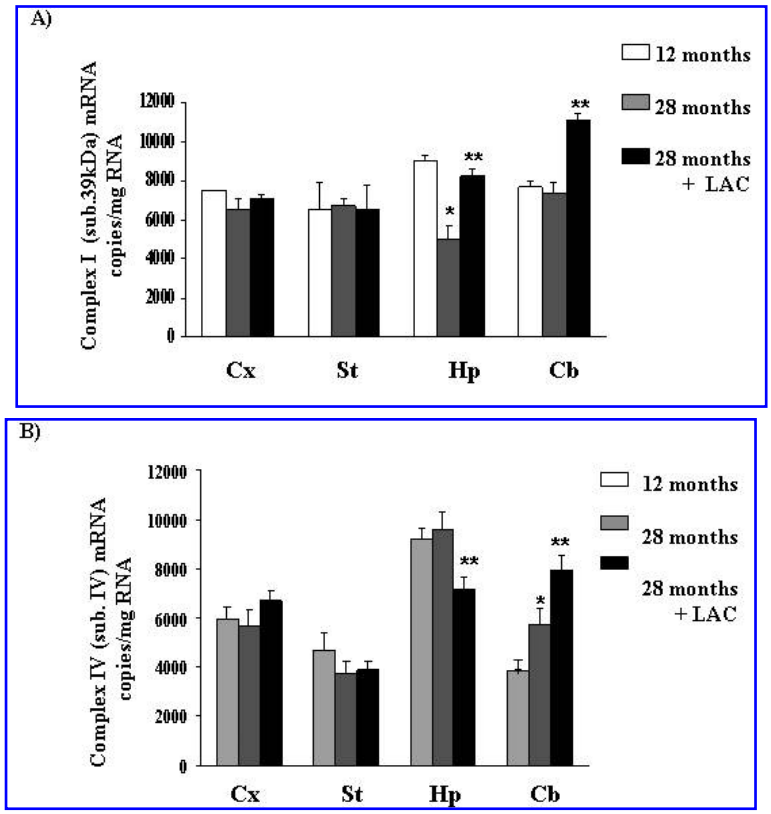

FIG. 9. Complex I and Complex II in aging brain. Mitochondrial (**A**) complex I (nuclear B13, 39 kDa subunit) and nuclear (**B**) complex IV (subunit IV) mRNA expressions examined in different brain regions as a function of brain aging. Total RNA from rat brain dissected areas was extracted as described in Materials and Methods and quantification was performed by RT-PCR. Specificity of PCR products was characterized by melting curve analysis followed by gel electrophoresis, ethidium bromide staining, and DNA sequencing. Cortex (Cx), striatum (St), hippocampus (Hp), and cerebellum (Cb). (*) $p < 0.05$ vs. adult control; (**) $p < 0.05$ vs. senescent animals.

ing events as well as in human disease pathologies, an imbalance in expression of GSH and associated antioxidant enzymes has been implicated in a variety of pathological conditions (22, 24). Moreover, an increase in protein oxidative damage, as indicated by the loss of protein sulfhydryl groups and by a decline in the activity of important metabolic enzymes, has been documented to occur in brain during aging (34, 75). The levels of oxidized proteins, exhibiting carbonyl groups, increase progressively with age in brain extracts of rats of different ages, and in old rats can represent 30%–50% of the total cellular protein (29). Protein carbonyls can be generated by direct oxidative damage to proteins, by the binding to proteins of cytotoxic aldehyde such as HNE, and by glycosidation of proteins (43). HNE, which is highly neurotoxic, avidly binds to proteins, and HNE–protein adducts are demonstrable in senile plaques and tangles in AD, tissues from ALS patients, and Lewy bodies in PD. The brain has a large potential oxidative capacity but a limited ability to combat oxidative stress (62–64). In normal conditions, there is a steady-state balance between pro-oxidants and antioxidants necessary to ensure optimal efficiency of antioxidant defenses; however, when the rate of free radical generation exceeds the capacity of antioxidant defenses, oxidative stress ensues (41, 42). To adapt to environmental changes and survive different types of injuries, brain cells have evolved networks of different responses that detect and control diverse forms of stress.

One of these responses is the heat shock response. Heat shock proteins are proteins serving as molecular chaperones involved in the protection of cells from various forms of stress (2, 18, 54, 66). In the central nervous system, heat shock protein (HSP) synthesis is induced not only after hyperthermia, but also following alterations in the intracellular redox environment, exposure to heavy metals, amino acid analogs or cytotoxic drugs (26, 62, 64). While prolonged exposure to conditions of extreme stress is harmful and can lead to cell death, induction of HSP synthesis can result in stress tolerance and cytoprotection against stress-induced molecular damage (81). Another Hsp member, Hsp32 also known as heme oxygenase-1 (HO-1), is receiving considerable attention, as it has been recently demonstrated that HO-1 induction, by generating the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin, could represent a protective system potentially active against brain oxidative injury (45, 49). Heme oxygenase is a stress protein that has been implicated in defense mechanisms against agents that may induce oxidative injury (71, 73, 74), such as endotoxins, cytokines, and heme (28, 36), and its induction represents a common feature in a number of neurodegenerative diseases (49, 64). There is now evidence to suggest that the HO-1 gene is redox regulated and contains in its promoter region the antioxidant responsive element (ARE), similar to other antioxidant enzymes (2, 6). HO-1 gene is, in fact, modulated by redox-sensitive transcription factors that recognize specific binding sites within the promoter and distal enhancer regions of the HO-1 gene (1). These include: Fos/Jun [activator protein-1 (AP-1)], nuclear factor-κB (NFκB) and the more recently identified Nrf2 proteins (2, 6). In addition, heme oxygenase-1 is rapidly upregulated by oxidative and nitrosative stresses, as well as by glutathione depletion (52, 55, 56). Given the broad cytoprotective properties of the heat shock response there is now strong interest in discovering and developing pharmacological agents capable of inducing the heat shock response (68, 69, 77).

In aged animals, various denatured proteins such as enzymes with lowered activity, unfolded proteins, and proteins modified by oxidation and glycation have been detected. These abnormal proteins may lead to protein aggregation, cell damage, and decreased function of organs (65, 79). Stress proteins (molecular chaperones) are thought to have a role in protecting cells from damages through defense against denaturation, and restoration or resolution of denatured proteins (55, 57). Therefore, alterations of the expression and function of stress proteins are supposed to be linked to the protein denaturation with aging. Moreover, it has been suggested that the basal Hsp70 increased by accumulation of modified proteins in aged rat kidney (79). We have demonstrated that the basal level of Hsp72, constitutively expressed Hsp70 (Hsc70), in senescent 28-month-old rats was significantly higher in some parts of the brain than that in adult 12-month-old rats. These results suggest that the expression of Hsp70 increases with aging and may have a role to suppress protein denaturation. In addition, strong evidence exists that indicates an attenuated response of brain tissue to acute stresses in aged rats, which would explain the reduced stress resistance of aging brain to pathogenetic conditions associated to free radical attack and oxidative damage (24).

In the present study we show that oxidative stress increases during aging in brain, as revealed by decreased GSH content and increases in GSSG, as well as lipid and protein oxidation markers, such as hydroxynonenals and protein carbonyls. These changes were particularly significant ($p < 0.05$) in the brain regions of hippocampus and striatum and to a lesser extent in the cortex, while cerebellum exhibited high resistance to these oxidative changes. Increased oxidative stress was associated with upregulation of stress responsive genes such as Hsp70 and HO-1. In addition, in the brain region of hippocampus a significantly ($p < 0.05$) decreased expression of SOD-2 was observed. Notably, in these experimental conditions we found that treatment of rats with acetyl-L-carnitine resulted in upregulation of protective antioxidant genes, such as Hsp70, HO-1, and SOD-2, as well as prevention of age-dependent changes in mitochondrial chain respiratory complex expression. This effect was associated with increased GSH levels and decreased HNE and carbonyls groups. Previous studies have correlated the expression and activity of HO-1 to that of SOD-2, suggesting a reciprocal influence between these two genes (37). In our study, by performing *in situ* hybridization, we provide strong evidence that, after LAC treatment, both HO-1 and SOD-2 mRNA signals were highly concentrated in the hippocampus, particularly in the CA1, CA2, and CA3 areas and in the dentate gyrus, in the granule cell layer of the cerebellum, and in some specific regions of the brain cortex. A parallel increase in the expression of HO-1 and SOD-2 could be extremely relevant in the regulation of intracellular redox homeostasis, especially under conditions of attenuated cellular stress responsiveness, such as in aging brain.

Acetyl-L-carnitine is proposed as a therapeutic agent for several neurodegenerative disorders (7, 17, 20, 27). This compound was reported to prevent, in nonhuman primates, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurological injury to the substantia nigra, as well as to increase cellular respiration, mitochondrial membrane potential, and cardiolipin levels in hepatocytes of 24-month-old rats (7). These biochemical effects are paralleled by increases in ambulatory activity of aged rats (48). There is now evidence to suggest that the carnitine system is involved in membrane stabilization and repair processes, and in the metabolism of disruptive acyl CoAs such as acylcarnitines (23, 25). Carnitine and LAC attenuate neuronal damage produced by 3-nitroproprionic acid, rotenone, and MPTP (7). Moreover, LAC induced, after ischemia reperfusion in rats, a more rapid recovery of ATP, PCr, and lactate levels (5, 80).

The results from our study show for the first time that LAC treatment of aging rats induces HO-1 and Hsp70 heat shock proteins in the brain. Hsp70 is encoded in the nucleus and resides both in the cytosol and in the mitochondria (17). Hsp70, under normal conditions, form the chaperonin complex, which is implicated in protein folding and assembly within cytosol, but also cellular organelles, such as endoplasmic reticulum and mitochondria (44). Most mitochondrial proteins are synthesized in the cytosol and must be imported into the organelles in an unfolded state (44). During translocation, the proteins interact with Hsp70. ATP-dependent binding and release of Hsp70 provide the major driving force for complete transport of polypeptides into the matrix. Most imported polypeptides are released from soluble Hsp70; however, a subset of aggregation-sensitive polypeptides must be transferred from Hsp70 to Hsp60 for folding (57). Owing to the close functional interaction between these two chaperonin systems, it is likely that upregulation of Hsp70 may be a fundamental site targeted by LAC action, with consequent restoration of mitochondrial complex function. The data presented here are consistent with the notion that LAC plays a crucial role in the regulation of critical vitagenes (HO-1, Hsps, and SOD) and expands our previous finding indicating, in astrocytes exposed to oxidative stress, an increased astroglial antioxidative potential, which involves the redox sensitive transcription factor Nrf-2 and the consequent activation of stress responsive genes such as HO-1, the mitochondrial Hsp60, and SOD (27, 70). Although speculative, it is conceivable to suggest that during aging acetylcarnitine, by promoting acetylation of DNA-binding proteins, may promote the upregulation of these stress responsive genes through modulation of the redox-sensitive status and activity of specific transcription factors, such as HSF for Hsp70 and Hsps60 and, respectively, Nrf-2 for HO-1 and SOD-2.

Collectively, the data presented in this paper are consistent with the activation of protective genes such as heme oxygenase, Hsp70, and SOD-2 in response to exogenous acetyl-L-carnitine treatment during aging and sustain the emerging notion that the pharmacological activity attributed to several well-known or newly discovered drugs could rely on their intrinsic ability to activate specific components of the cellular stress response (20, 21, 26, 77). This highly inducible system, therefore, should be seriously considered as a target for novel therapeutic interventions focusing on the capability that compounds such as nutritional polyphenol antioxidants or acetyl-L-carnitine have to upregulate the vitagene system as a means to limit deleterious consequences of oxidative and nitrosative stress associated with aging and age-related disorders (76).

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ABBREVIATIONS

ARE, antioxidant responsive element; GSH, glutathione; Hsp60, heat shock protein 60; Hsp70, heat shock protein 70; HO-1, heme oxygenase-1; LAC, acetyl-L-carnitine; NFκB, nuclear factor-κB; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species.

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Address reprint request to:
Prof. Vittorio Calabrese
Section of Biochemistry & Molecular Biology
Department of Chemistry
Faculty of Medicine
University of Catania.
Viale Andrea Doria N° 6
95100 Catania, Italy

E-mail: Calabres@mbox.Unict.it

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