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FORUM REVIEW ARTICLE

Carnosinase Levels in Aging Brain: Redox State Induction and Cellular Stress Response

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Abstract

Carnosinase is a dipeptidase found almost exclusively in brain and serum. Its natural substrate carnosine, present at high concentration in the brain, has been proposed as an antioxidant *in vivo*. We investigated the role of carnosinase in brain aging to establish a possible correlation with age-related changes in cellular stress response and redox status. In addition, a stable HeLa cell line expressing recombinant human serum carnosinase CN1 was established. The enzyme was purified from transfected cells, and specific antibodies were produced against it. Brain expression of CN1, Hsp72, heme oxygenase-1, and thioredoxin reductase increased with age, with a maximal induction in hippocampus and substantia nigra, followed by cerebellum, cortex, septum, and striatum. Hsps induction was associated with significant changes in total SH groups, GSH redox state, carbonyls, and HNE levels. A positive correlation between decrease in GSH and increase in Hsp72 expression was observed in all brain regions examined during aging. Increased carnosinase activity in the brain can lead to decreased carnosine levels and GSH/GSSG ratio. These results, consistent with the current notion that oxidative stress and cellular damage are characteristic hallmarks of the aging process, sustain the critical role of cellular stress-response mechanisms as possible targets for novel antiaging strategies. *Antioxid. Redox Signal.* 11, 2759–2775.

Introduction

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 (9, 11, 27). Experimental evidence indicates that increased rate of free radical generation and decreased efficiency of the reparative/degradative mechanisms, such as antioxidant defense and proteolysis, are both factors that primarily contribute to age-related elevation in the level of oxidative stress and brain damage (12, 16). Among antioxidants, carnosine (β-alanyl-L-histidine, AH) has been described as a forgotten and enigmatic dipeptide (4, 36). Carnosine was first isolated from meat by Gulewitsch and Amiradzibi (28), and it is widely distributed in tissues (6, 19), including the central nervous system (59). In recent years, the occurrence of AH and its analogues homocarnosine (γ-aminobutyryl-L-histidine) and anserine (β-alanyl-1-methyl-L-histidine) in the CNS and their age-related alterations (33, 38) suggested a therapeutic potential in neurodegenerative diseases (34, 35). Carnosine has been shown to be neuroprotective because of its capacity to counteract both oxidative (42) and nitrosative stress (23, 57) related to several pathologic conditions (22,54), including ischemia (21, 60, 61).

The enigma of carnosine is particularly exemplified by its apparent antiaging actions; it suppresses cultured human fibroblast senescence and delays aging in senescence-accelerated mice (7) and *Drosophila* (67), but the mechanisms remain uncertain. In addition to the well-documented antioxidant, antiglycating, aldehyde-scavenging, and toxic metal ion-chelating properties of carnosine (37), its ability to influence the metabolism of altered polypeptides, whose accumulation characterizes the senescent phenotype, also should be considered (36). Exogenously administered AH can cross the blood–brain barrier (40), but its efficacy as drug is a challenge because of the presence of carnosinase enzymes acting as endogenous dipeptidases (53).

Two isoforms of these dipeptidases, belonging to the M20 metalloprotease family, have been purified and characterized (39, 45). The first enzyme, tissue carnosinase CN2 (EC 3.4.13.18) is a cytosolic form that displays broad substrate specificity and is ubiquitously expressed (45). The second enzyme is known as serum carnosinase, CN1 (EC 3.4.13.20),

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and exhibits narrow specificity (39). Recently, the two human isoforms were cloned and characterized (62); the crystal structures of mouse CN2 complexed with its inhibitor bestatin, together with Zn^{2+} at a resolution of 1.7 A and that with Mn^{2+} at 2.3 A, have been reported (65). However, sequence-based alignments of human CN1 and human CN2 with mouse CN2 display sequence identities of 53% and 91%, respectively.

It also was reported that CN1 differs from its cytosolic isoform, both for its unique ability to hydrolyze homocarnosine and for the absence in nonprimate mammals (39). The human CN1 is expressed predominantly in the liver and brain (62) and selectively secreted by brain cells into CSF. The increase of CN1 levels with age up to about 15 years in serum (44, 66) attracted our interest, prompting this work to acquire insight into the function of carnosinase and its possible relation with the redox status and cellular stress response in aging rat brain. For this purpose, a stable HeLa cell line expressing recombinant human serum carnosinase was established. The effect of nitrosative and oxidative stress was then tested on CN1-transfected and control cells. The enzyme was purified from the culture medium of stably transfected cells, and polyclonal specific anti-CN1 antibodies were produced. To understand the physiologic role of human serum carnosinase in aging and neurodegenerative processes, we evaluated for the first time the enzyme distribution, together with changes in redox state and cellular stress response, different rat brain areas of aged and senescent rats.

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 baker's yeast), SIN-1 (3-morpholinosydnonimine hydrochloride) were from Sigma Chemicals Co. (St. Louis, MO). All other chemicals were from Merck (Darmstadt, Germany) and of the highest grade available.

Cell cultures and treatment

HeLa cells were cultured in 25-cm^2 flasks with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and incubated with 5% CO_2 and at 37°C. The medium was replaced every 3 days until cells were confluent. Cells were harvested by using PBS 1X buffer containing 1 m*M* EDTA, washed twice in PBS 1x by centrifugation at 1,500 rpm, and at 4°C. Cell pellets and cell-culture medium were collected as necessary and used for cell transfection, enzymatic assay, Western blotting, and viability assay. Incubation of cells with 2 m*M* SIN-1, or with 0.7 m*M* H_2O_2 for 24 and 48 h, in the presence or absence of 3 m*M* carnosine, added 4 h before oxidant insult, was performed to test the viability of HeLa and HeLa-CN1 cells in response to oxidative or nitrosative stresses.

Recovery of cell lysate and cell-culture medium

To analyze CN1 expression and release with the Western blotting procedure and carnosinase activity with enzymatic assay, HeLa and HeLa-CN1 cells, lysates, and cell-culture medium were prepared. Cell protein extracts were obtained by sonication of cells resuspended in lysis buffer (50 m*M* Tris/HCl, pH 8.0, 1.5 mg/ml benzamidine, and 0.2 m*M* phenylmethanesulfonyl fluoride). After 30 min of centrifugation at 13,000 rpm and 4°C, supernatants were collected in new tubes. Culture medium was cleared of cell debris by centrifugation at 1,500 rpm, 4°C. Cell lysates and cell-culture medium were analyzed for CN1 release and enzymatic activity.

PCR amplification and cloning of human carnosinase CN1 cDNA

The coding sequence of CN1 was amplified from a human brain cDNA library by using as forward, the primer 5′-AAAGTCGACATGGATCCCAAACTCGG-3′, and as reverse, the primer 5′-AAAAAGCTTCTCGAGTTAATGGAG CTGGGC-3′. The forward primer introduced a *Sal*I restriction site, whereas the reverse primer introduced a *Hin*dIII and an *Xho*I restriction site. The PCR reaction was in a volume of 50 μl and was performed by using the program: 1 cycle at 94°C, 2 min; 35 cycles at 94°C, 30 sec, 65°C, 45 sec, 72°C 1 min 45 sec, one final extension cycle at 72°C, 5 min. After digestion with the appropriate restriction enzymes, the PCR product was cloned in pcDNA 3.0 expression vector and finally sequenced.

Expression of CN1 in eukaryotic cells

For transient transfection of HeLa cells, HeLa cells were plated a day before transfection into a six-well culture plate (0.5×10^6 cells/well). Cells were transfected with 4 μg of the pcDNA 3.0 expression vector carrying a CN1 coding sequence and 10 μl of lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells and cell-culture medium were harvested after 24, 48, and 72 h of transfection. Expression of recombinant CN1 protein was verified with ELISA, Western blotting, and enzymatic assay on HeLa cell lysate and cell-culture medium. A stable transfected HeLa cell line expressing CN1 protein was obtained by limiting dilution technique in the presence of selective antibiotic G418 (400 μg/ml; Invitrogen) after 15 days of incubation. Positive clone selection was performed with ELISA, Western blotting, and enzymatic assay on HeLa cell lysate and cell-culture medium. The stable clone is called HeLa-CN1 in the following text.

Cell-viability assay

Cell viability was estimated with MTT (3-[4,5-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide). In brief, untransfected HeLa and HeLa-CN1 cells were plated into 96-multiwell plates to have 5×10^3 cells/well and maintained in culture overnight. Cells were incubated with SIN-1 (2 m*M*) (Sigma) or H_2O_2 (0.7 m*M*) for 24 and 48 h in the presence or absence of 3 m*M* carnosine. Four hours before the end of the treatment with SIN-1, 10 μl of MTT (5 mg/ml) (Sigma) was added for each well of 96-multiwell plates plated with untransfected HeLa and HeLa-CN1 cells. At the end of the treatment, the medium was removed, and 100 μl per well of DMSO (Sigma) was added. After 15 min of incubation at room temperature, optical density was read at 570 nm, and the percentage of viable cells was calculated. Furthermore,

the statistical significance was evaluated a t test, and p values <0.05 were considered statistically significant.

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 12 months old (aged) and 28 months old (senescent) ($n =$ eight per each age group), were fed *ad libitum* a certified diet prepared according to the recommendations of the AIN, and the percentage energy composition is given in Table 1. After the animals were killed, brains were quickly removed and dissected into the cerebral cortex, hippocampus, septal area, and striatum, according to a standardized procedure, in a cold anatomic chamber and by following a protocol that allows a maximum of 50-s time variability for each sample across animals. Substantia nigra (SN) was dissected from the deepest part of the interpeduncular fossa. To exclude the possibility that the small size of nigral or septal samples could affect results, we analyzed pooled samples from SN or septum, to have a protein content comparable to that measured in cortex or striatum, and lipid peroxidation products as well as thiols, enzymes, and trace metals measured. In all these cases, we did not find significant differences between pooled samples and those coming from a single experimental animal.

Free and protein-bound sulfhydryl groups assay

Protein and nonprotein sulfhydryl (SH) compounds in different brain regions and in liver were estimated by the DTNB-based method of Sedlak and Lindsay (56). The content of SH groups was expressed in nanomoles per milligram of protein.

Reduced and oxidized glutathione assay

Reduced glutathione (GSH) and glutathione disulfide (GSSG) were measured with the NADPH-dependent GSSG reductase method, as previously reported (17). Plasma GSH and GSSG were measured as described, except that DTNB or NEM solutions were directly added to equal volumes of whole blood and, after tilting, centrifuged at 2000 g for 6 min at 4°C. GSH and GSSG standards in the ranges between 0 and 10 nM and 0.010 and 10 nM, respectively, added to control samples, were used to obtain the relative standard curves, and the results were expressed in nanomoles of GSH or GSSG, respectively, per milligrams protein.

Table 1. Diet Composition (g/100 g)

Dextrin–maltose[a]	53
Oil mixture[b]	25
Casein	22
D-L Methionine	0.5
Salt mixture (AIN 76)	3.5
Vitamin mixture (AIN 76)	1.2

[a]From cornstarch.
[b]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%.

Western blot analysis

The tissue homogenate was centrifuged at 10,000 g for 10 min, and the supernatant was used for HO-1, Hsp72, Hsp90, TRX1, TRX, carbonyls, and HNE levels determination, after dosage of proteins as described later. Aliquots (30 μg) of protein extract were separated with SDS-PAGE, transferred to nitrocellulose membranes, and then probed with antibodies. Appropriate secondary antibodies were used, and the immunoreactivity was visualized by using ECL (Amersham Biosciences, Piscataway, NJ). The following primary antibodies were used: anti-HO-1, anti-TRX, anti-TRX1, and anti-Hsp90 antibodies (Stressgen, Victoria, BC, Canada) (1:1,000 dilution in Tris-buffered saline, pH 7.4) or with a monoclonal anti-Hsp72 antibody (RPN 1197; Amersham) that recognizes only the inducible form.

Anti-carnosinase CN1 polyclonal antibodies were produced in house. Two peptides (peptides $_{144}$L13K$_{156}$ and $_{279}$P13E$_{292}$) were selected from the most antigenic regions of CN1 amino acid sequence, as analyzed with two different software applications (http://bio.dfci.harvard.edu/Tools/antigenic.pl and http://www.imtech.res.in/raghava/bcepred/). The synthetic peptides, conjugated with ovalbumin (OVA) at the C terminal through a cysteine residue, were used to raise polyclonal antibody in rabbit. Purified antibody was stored at −20°C at a final concentration of 1 mg/ml. Rabbit serum of preimmunized and immunized animals was tested with ELISA and Western blotting procedure to verify antibody titer and specificity.

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). A rabbit-anti DNPH polyclonal primary antibody (Chemicon, Rosemont, IL) specific for DNP-protein adduct (1:100) was used to detect 2,4-dinitrophenylhydrazine (DNPH)-derivative carbonyl groups. Goat polyclonal antibody specific for β-actin was used as loading control (1:1,000). For detection, the membranes were incubated with a horseradish peroxidase–conjugated sheep anti-mouse immunoglobulin G (IgG), followed by ECL chemiluminescence (Amersham). The amounts of inducible HO-1, Hsp72, CN1, Hsp90, TRX1, TRX, carbonyls, and HNE were quantified with scanning Western blot–imaged films with a laser densitometer (LKB-Ultroscan, XL model). Multiple exposures of each blot were used to ensure the linearity of the film response.

Immunohistochemistry

Rat brains ($n = 6$) were quickly removed and frozen at −80°C. Cryostat sections, 8 μm thick, were fixed by immersion in 4% paraformaldehyde. The nonspecific binding sites were blocked with 5% γ-globuline in PBS for 20 min. Cryostat sections were then immunostained with rabbit polyclonal antibody against HO-1 (1:1,000) overnight at 4°C in humid atmosphere. Then the slides were washed, and a secondary anti-rabbit IgG conjugated with fluorescein (Vector Laboratories, Burlingame, CA) was added (dilution, 1:400) and incubated at room temperature for 1 h in the dark. Negative controls were carried out by omitting the primary antisera. The slides were then washed 3 times in PBS for 5 min and sealed with the "mounting media" Vectashield (Vector Laboratories). The immune reaction was revealed and photographed

with a Zeiss Axioskop fluorescence microscope with a 40× objective (Zeiss, Oberkochen, Germany).

ELISA assay

Cell lysate, cell-culture medium, homogenate rat brain areas, and purification fraction were analyzed with ELISA assay for CN1 expression and release. In brief, a solution containing antigen (10 μg) in carbonate-coating buffer (0.15 *M* Na_2CO_3, 0.35 *M* $NaHCO_3$), pH 9.6, was coated into a 96-well plate and incubated overnight at 4°C. The next day, the plate was washed 4 times with PBS 1X-Tween 0.05% and incubated with anti-CN1 polyclonal antibody diluted in PBS 1X-Tween 0.05% to 0.1% BSA for 2 h at room temperature. After four washes with PBS 1X-Tween 0.05% and incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody for 2 h at room temperature, the bound CN1 was revealed by oxidative action of HRP on the substrate *ortho*-phenylendiamine (OPD) in the presence of peroxide. The reaction was stopped by addition of a stop solution (0.2N H_2SO_4), and the absorbance was measured at 490 nm.

Enzyme purification

The purification of CN1 was performed by using an automated chromatographic workstation (BioRad BioLogic Duoflow). The cell-culture medium of the clone HeLa-CN1 (200 ml) was cleaned of cells and debris with centrifugation, and the supernatant was filtered with a 0.45-μm filter disk (Corning). The resulting solution was concentrated with a Centrifugal Ultrafree system equipped with CX-10 membrane (Millipore) and initially purified by DEAE Sephacel (GE Healthcare) (100 ml) equilibrated with buffer A (50 m*M* Tris/HCl, pH 8.0). The elution was carried out with a multistep NaCl gradient: (a) linear gradient from buffer A to 130 m*M* NaCl in the same buffer over three column volumes (CV); (b) isocratic step of buffer A with 130 m*M* NaCl in buffer A over 25 CV; (c) linear gradient from 130 to 250 m*M* NaCl in buffer A over 13 CV; (d) linear gradient from 250 m*M* to 1 *M* NaCl in buffer A over 5 CV. A 1 *M* NaCl isocratic step was finally applied to elute the bound material. The flow rate of the eluents was 1.2 ml/min, and 4-ml fractions were collected.

All the fractions containing carnosinase activity were pooled, concentrated by ultrafiltration, and dialyzed with cellulose membrane (Sigma; MWCO 10,000) against buffer A. The final sample was loaded onto a Uno Q1 column (Biorad). The running method was the same as the one used for the previous purification. The 3-ml fractions were tested for the carnosinase activity, and those containing CN1 were pooled, dialyzed, and concentrated with ultrafiltration.

Protein identification with mass spectrometry

The investigated band corresponding to CN1 was excised from the SDS gel, washed, in-gel reduced by DTT, *S*-alkylated with iodoacetamide, and subjected to in-gel trypsin digestion (58). The gel pieces were swollen in 50 m*M* NH_4HCO_3 and 12.5 ng/μl trypsin (modified porcine trypsin, sequencing grade; Promega, Madison, WI) in an ice bath. After 30 min, the excess of trypsin was removed, and 50 μl of 50 m*M* NH_4HCO_3 was added to the gel. Digestion proceeded at 37°C overnight. The solution containing tryptic peptides (10 μl) was used for micropurification (desalting/concentration) of peptides with a homemade 5-mm nanocolumn packed with C18 resin (POROS R2) (Applied Biosystems, Foster City, CA) in a constricted GELoader tip (Eppendorf Scientific, Westbury, NY), according to Gobom *et al.* (26). The columns were washed with 10 μl of 0.1 % trifluoroacetic acid (TFA), and the bound peptides subsequently eluted directly onto the MALDI target with 0.6 μl of matrix solution [10 mg/ml α–cyano-4-hydroxycinnamic acid in 70% (vol/vol) CH_3CN and 0.1% TFA (wt/vol)] and deposited directly onto the MALDI target.

MALDI mass spectra were acquired in positive ion reflectron-delayed extraction mode over an m/z range from 600 to 4,000, by using a Voyager DE-PRO time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA) equipped with UV nitrogen laser (337 nm). The m/z software (Proteometrics, New York, NY) was used to analyze MS spectra. MALDI-TOF spectra were internally calibrated by using porcine trypsin autolysis products (m/z 842.51 and 2211.10). The General Protein/Mass Analysis for Windows (GPMAW) software (http://welcome.to/gpmaw) was used for all sequence handling and storage.

Carnosinase assay

The solution containing carnosinase (0.1–5 μl) was incubated with carnosine (10 m*M*) in 50 μl of 50 m*M* Tris/HCl, pH 8.0, for 2 h at 37°C. The reaction was stopped by adding 10 μl of 0.5N TCA. After centrifugation (15,000 *g* for 5 min), the final mixture was used to assay the histidine content with spectrofluorimetry (44), in 96-well plates. Then 90 μl of 2 *M* NaOH and 90 μl of 0.05% m*M* *o*-pthaldialdehyde (OPA) were added to the deproteinized sample, incubated at 37°C for 15 min until the addition of 90 μl of 4 *M* H_3PO_4. After 15 more minutes at 37°C, the solution was left at room temperature for 30 min before fluorescence measurement (λ_{exc}, 340 nm, and λ_{em}, 450 nm), carried out on Cary Eclipse Fluorescence spectrophotometer equipped with a Microplate reader (Varian, Palo Alto, CA).

Statistical examination

Results were expressed as mean ± SEM of at least eight separate experiments. Statistical analyses were performed by using the software package SYSTAT (Systat Inc., Evanston, IL). The significance of the differences, evaluated with 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 or more.

Results

A stable clone expressing carnosinase CN1

Human serum carnosinase CN1 cDNA was amplified with PCR from a human brain cDNA library by using primers corresponding to the start and the stop codons of the coding sequences. PCR amplification product of 1.5 Kb was obtained and cloned in pcDNA 3.0. Sequence analysis of the clone confirmed the CN1 coding sequence. Transfection of HeLa cells with pcDNA carrying CN1 coding sequence was performed. CN1 expression and functionality in transiently transfected HeLa cells was evaluated with enzymatic assay. As reported in Fig. 1, carnosinase activity was found both in cell extract and in the cell-culture medium after 24 and 48 h of transfection. Moreover, CN1 enzymatic activity was demon-

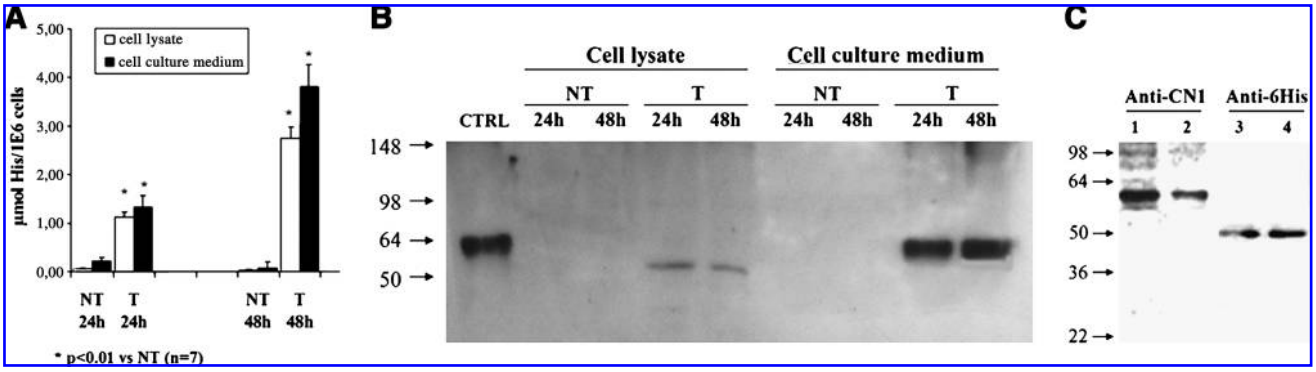

FIG. 1. Carnosinase activity in cellular lysate and in culture medium of untransfected (NT) and transiently transfected (T) cells after 24 and 48 h. (**A**) HeLa cells were transiently transfected with CN1-pCDNA3.0. At the indicated time, aliquots were collected, and carnosinase enzymatic assay was performed on the cellular lysates or the culture medium. (**B**) Western blot in lysate and culture medium of untransfected (NT) and HeLa-CN1 (T) cells after 24 and 48 h of transfection. Samples were immunostained with anti-CN1 (1:1,000). Pooled fractions of UnoQ purification were used as a control (CTRL). (**C**) Western blot of protein extract from transiently transfected HeLa-CN1 cells (line 1 and line 2) and *Escherichia coli* transformed with pQE31 carrying CN1 DNA. Samples were immunostained with anti-CN1 (line 1 and line 2) and anti-6xHis (line 3 and line 4) (1:1,000).

strated to be higher in the cell-culture medium than in cell-protein extracts (Fig. 1A). Furthermore, an increase of carnosinase activity was detected after 48 h from transfection. After 48 h, the decrease of carnosinase activity paralleled the increase of cellular death.

We selected and established a stable HeLa cell line expressing the recombinant human serum carnosinase CN1 (clone Hela-CN1). CN1 expression in cell-protein extracts and its secretion in the cell-culture medium was confirmed with Western blotting. A faint protein band of ~55 kDa was

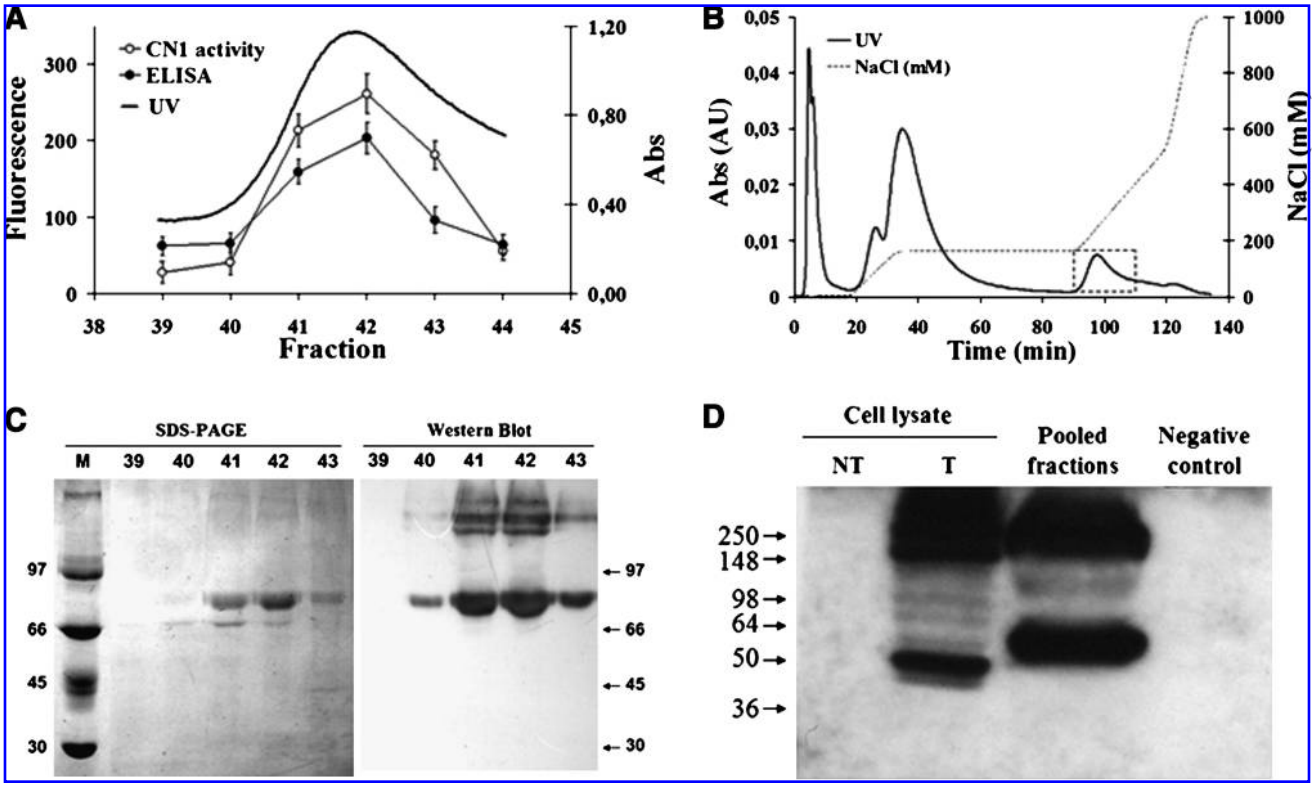

FIG. 2. (**A**) **Chromatographic profile of HeLa-CN1 culture medium purified with DEAE Sephacel (GE Healthcare).** The peak of CN1 is shown by square-shaped dashed line. (**B**) ELISA measurements (○), carnosinase enzyme activity (●), compared with UV (280 nm) trace (—) of fractions containing CN1 derived from DEAE Sephacel chromatography. (**C**) SDS-PAGE and Western blot analysis of fractions containing CN1 derived from DEAE Sephacel chromatography. Samples were immunostained with anti-CN1 (1:1,000). (**D**) Western blot of lysate and culture medium of untransfected (NT) and HeLa-CN1 (T) cells, compared with pooled factions of UnoQ chromatography and a negative control (**C**). Samples were immunostained with anti-CN1 (1:1,000).

detected in protein extracts derived from transfected HeLa cells, and a strong protein band of approximately 64 kDa was identified in the cell-culture medium collected from the same cell culture (Fig. 1B). We supposed that the different molecular masses between the secreted human serum carnosinase CN1 and the protein present in the cell were the result of posttranslational glycosylation events, as reported in the literature (62). To confirm this observation, we cloned the CN1 coding frame in a bacterial expression vector and compared the electrophoretic behavior of CN1 expressed in these various contexts (Fig. 1C). Again, the bacterial CN1 extract contained a protein with a faster electrophoretic migration than the eukaryotic product, because in bacteria, the posttranslational modification events strongly differ from those of eukaryotes. This experimental evidence suggests that the recombinant carnosinase CN1 obtained shows features of the human protein.

Purification and characterization of recombinant human serum carnosinase CN1

To pursue CN1 protein-purification steps, we used the enzymatic assay and an immunologic assay based on a polyclonal-specific antibody anti-CN1. This antibody was obtained in rabbit. High serum reactivity and specificity was demonstrated by using the anti-CN1 polyclonal antibody in Western blots against protein extracts from eukaryotic and bacterial cells expressing recombinant human serum carnosinase CN1 (Fig. 1B and C).

Human serum carnosinase CN1 was purified from the supernatant of a stably transfected cell culture. The procedure was similar to that previously reported (62), with exception that we used only two instead of three chromatographic steps. The chromatographic profile of the first purification step is reported in Fig. 2A: fractions were analyzed with ELISA and enzymatic assay (Fig. 2B). With both methods, the human serum carnosinase CN1 was identified in fractions from 39 to 44, reaching the highest concentration in fraction 42. These data were confirmed with SDS-PAGE and immunoblotting (Fig. 2C) performed on the same fractions. The complete procedure for the purification of the enzyme from the cell-culture medium of the clone HeLa-CN1 is summarized in Table 2. The separation procedures with DEAE Sephacel resulted in 38-fold purification and 59% of the enzyme activity. CN1 was finally 57-fold purified after the UnoQ purification step, with a yield of 11%. Fractions from UnoQ chromatography containing elevated concentrations of carnosinase activity were pooled and analyzed with Western blotting (Fig. 2D). The anti-CN1 antibody detected a protein band of approximately 64 kDa, as expected, corresponding to recombinant human serum carnosinase CN1 secreted by HeLa cells in cell-culture medium. The presence of an aggregate at higher molecular weight was probably the result of the very high concentration of the protein in the gel.

The identity of CN1 was finally verified with mass spectrometry. The MALDI-TOF mass spectrum of trypsin-digested CN1 (Fig. 3) shows peaks of the most abundant tryptic peptides. They cover >23% of the entire amino acid sequence.

Characterization of HeLa-CN1 clone: carnosine influence in the nitrosative stress

The HeLa-CN1 clone was assayed with respect to its viability in the absence and presence of oxidative and nitrosative stress. Figure 4 shows that HeLa-CN1 cells have a lower cell survival than do nontransfected cells or HeLa cells transfected with an empty or a mock vector. This is an interesting finding, apparently indicating that the stable clone tends to live for a shorter time. Addition of carnosine does not counteract this effect, either in the cells stably expressing CN1 or in untransfected cells (Fig. 5). Conversely, viability of HeLa and HeLa-CN1 cells is markedly influenced by both oxidative and nitrosative stress. In the presence of 0.7 m*M* H_2O_2 or 2 m*M* SIN-1, a reduction of about 65% and 45%, respectively, of the number of viable cells was observed in both the untrasfected and transfected cells present (Fig. 5). Notably, viability in HeLa cells measured after H_2O_2 treatment was significantly higher than the viability of HeLa-CN1 cells after the same oxidant insult, indicating an increased susceptibility to oxidative stress of cells overexpressing carnosinase. Moreover, H_2O_2 and SIN-1 induced a decrease in cell survival, which occurred in a time- and dose-dependent manner (data not shown). Preincubation of the cells with carnosine is able to restore viability of control cells incubated with SIN-1, but not the viability of cells overexpressing carnosinase (HeLa-CN1 cells), a finding consistent with the possibility that the increased carnosinase activity, by degrading carnosine, abolishes its protective action (Fig. 5). However, addition of 2 m*M* carnosine before oxidative stress induced by H_2O_2 did not change the susceptibility of HeLa and HeLa-CN1 cells to the effects of H_2O_2. It is possible that, at this concentration of H_2O_2, carnosine is not able either to influence the cascade of events associated with oxidative stress, or to cope with oxidative stress itself.

Regional distribution of carnosinase activity and expression in aged and senescent rat brain

Expression and enzyme activity of CN1 were investigated in different brain regions of aged and senescent rats (Fig. 6A and B). In aged animals, CN1 expression was threefold higher in the SN compared with all other brain regions

Table 2. Purification of Serum Carnosinase from the Cell-culture Medium of the Clone HeLa-CN1

Fraction	*Total protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Purification (Fold)*	*Yield (%)*
Cell-culture medium	1,729	3,090	2	1	100
DEAE Sephacel	27	1,833	68	38	59
Uno Q1	3	350	102	57	11

One enzyme unit is defined as the enzymatic activity that catalyzes the hydrolysis of 1 μmol carnosine in 1 h under the standard conditions.

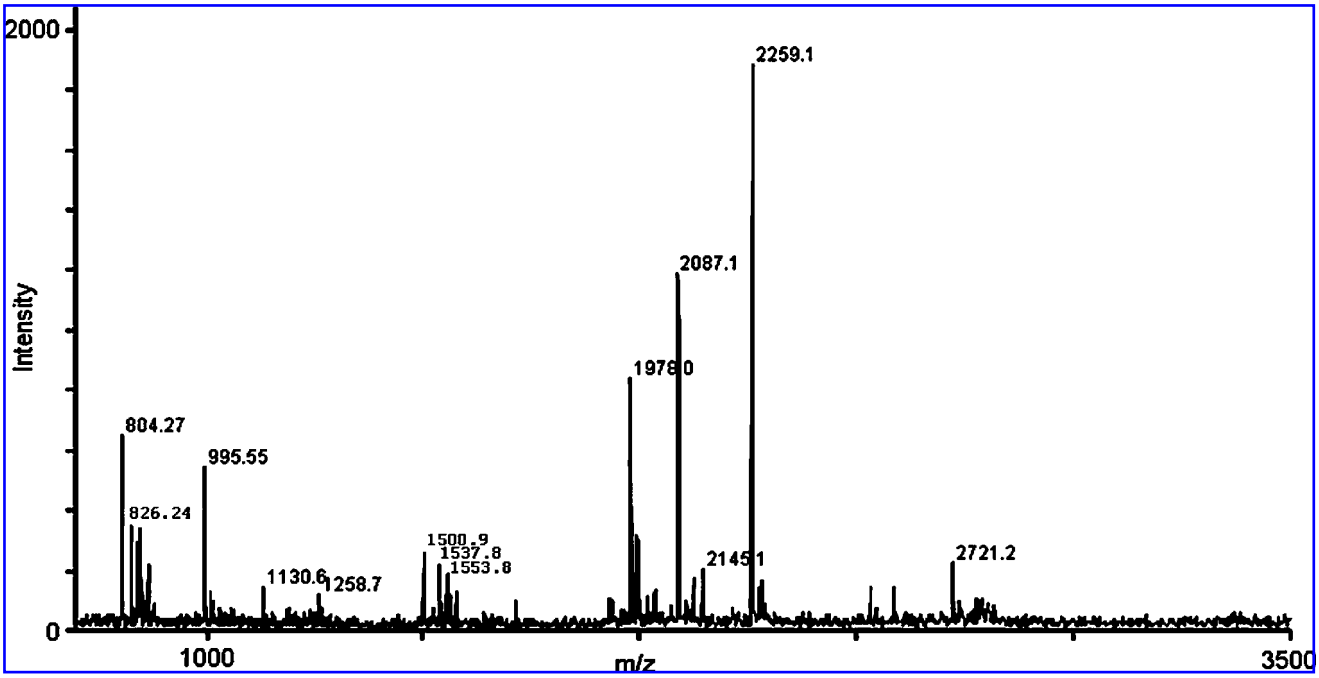

FIG. 3. MALDI-TOF mass spectrum of trypsin-digested CN1. MALDI-TOF mass spectrum of trypsin-digested CN1.The protein was previously excised from the SDS gel, washed, in-gel reduced by DTT, and *S*-alkylated with iodoacetamide. The General Protein/Mass Analysis for Windows (GPMAW) software (http://welcome.to/gpmaw) was used for all sequence handling and storage.

examined, which showed comparable activities (Fig. 6A). Senescent rats, as compared with aged rats, exhibited a significant ($p < 0.05$) increase in CN1 expression in all brain regions but the cortex. Consistent with CN1 protein expression, the enzyme activity was significantly higher in all brain regions of senescent rats compared with that in aged animals, with the maximal induction observed in the SN, followed by the cortex, septum, hippocampus, cerebellum, and striatum (Fig. 6B).

Total sulfhydryl groups and glutathione redox state analysis

When different brain regions were examined for thiols, total SH groups, and GSH levels, as a function of age, all brain regions but the cerebellum showed a decrease in total SH groups (Fig. 7) as well as GSH in senescent compared with aged animals (Fig. 8A). Conversely, the level of oxidized glutathione (GSSG) increased in senescent compared with aged rats (Fig. 8B). To test the hypothesis that these changes of redox status in the aging brain would trigger a heat-shock response, we measured the expression of inducible Hsp70 (Hsp72) and HO-1 in different brain regions in aged and senescent rats.

Hsp distribution in brain aging

Figures 9A and 10A showed that protein expression of Hsp72 and HO-1 was significantly elevated in senescent compared with aged rats in all brain regions examined. Representative Western blots of samples from the hippocampus are shown in Figs. 9B and 10B, respectively. A positive correlation ($r = 0.87$) between the decrease in GSH and the increase in Hsp72 expression was observed in all brain regions examined during aging. Increased carnosinase activity in the brain can lead to decreased carnosine levels, with consequent

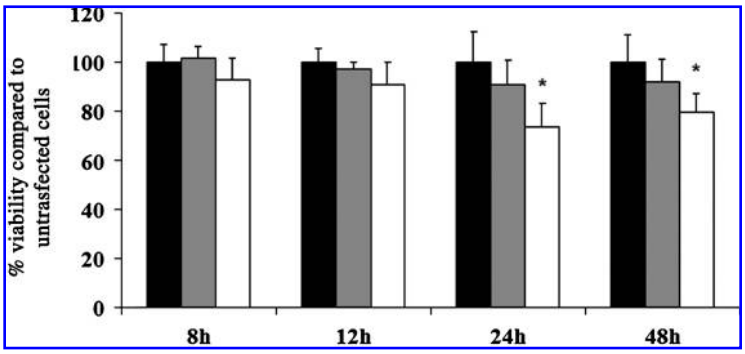

FIG. 4. Viability of the HeLa-CN1 clone. HeLa cells not transfected (HeLa NT), HeLa cells transfected with the empty plasmid pCDNA3.0 (MOCK) and with CN1-pCDNA3.0 (HeLa-CN1) were selected, and a clone stably expressing the enzyme was established. The cell viability was measured with MTT assay. Cell survival of carnosinase-expressing cells is significantly lower than control cells (*$p < 0.05$ *vs.* HeLa NT).

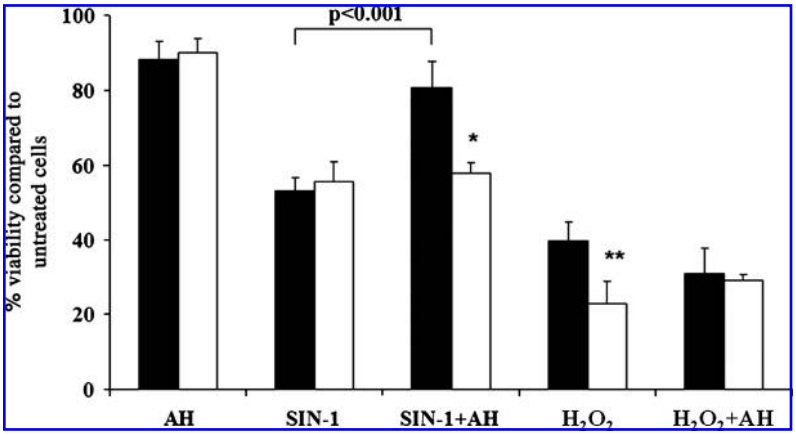

FIG. 5. Viability assay of HeLa and HeLa-CN1 cells after oxidative or nitrosative stress. HeLa and HeLa-CN1 cells were treated with H_2O_2 (0.7 m*M*) or SIN-1 (2 m*M*) in the absence or presence of 2 m*M* carnosine (AH), which was added 4 h before oxidant challenge. Cell viability was measured after 24 h with MTT assay. $^*p < 0.01$ and $^{**}p < 0.05$ *vs.* HeLa NT).

decrease of the GSH/GSSG ratio. This is consistent with the notion that the thiol redox switch acts as a signal for induction of cytoprotective genes modulating cellular stress tolerance.

Age-dependent modulation of HO-1 protein expression in rat brain

Further investigation of HO-1 protein expression was performed with immunohistochemistry analysis of the hippocampus and cortex, two brain regions selectively affected by age-dependent oxidative damage and cognitive decline. As representatively shown in Fig. 11, low HO-1 protein expression was found in the cortex and in the CA3 hippocampal area of aged rats, whereas senescent rats exhibited a significant increase in HO-1 expression in the hippocampus (CA3 area) but not in the cortex.

Hsp90, thioredoxin, and thioredoxin reductase expressions

We also evaluated levels of expression of Hsp90, thioredoxin (TRX), and thioredoxin reductase (TRX-Red) in the

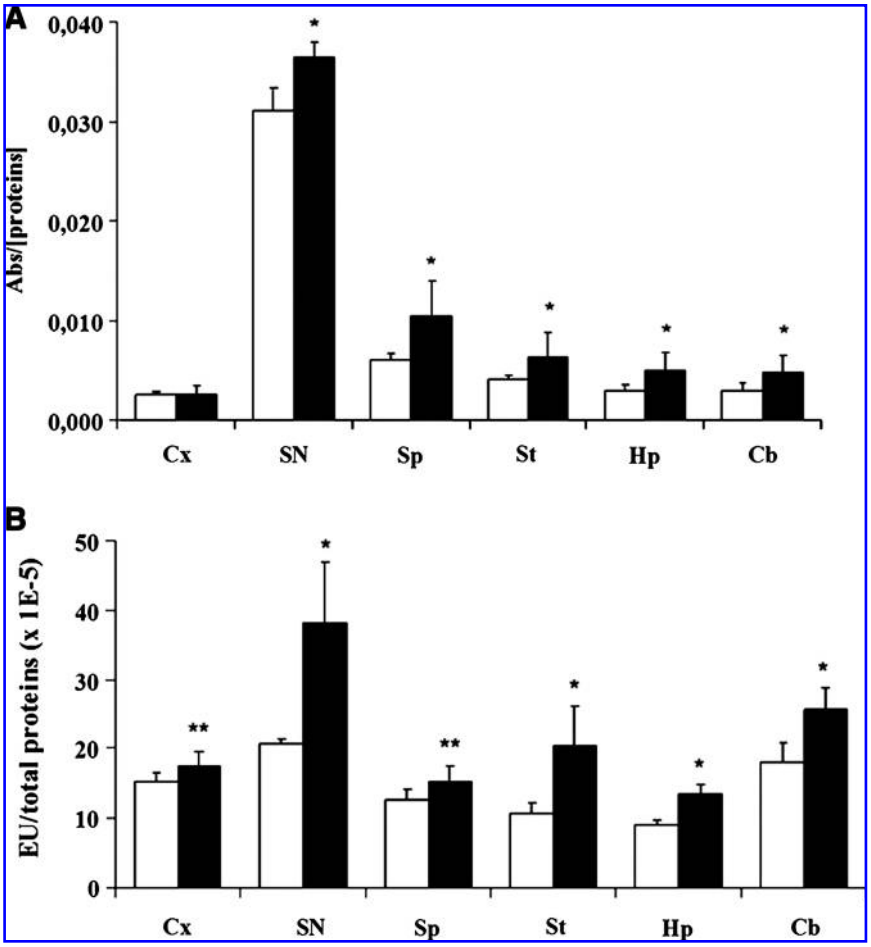

FIG. 6. Expression profiles (A) and enzyme activity (B) of CN1 in different brain regions. CN1 expression was evaluated with ELISA assay as described in Methods. Cortex (Cx), substantia nigra (SN), septum (Sp), striatum (St), hippocampus (Hp), and cerebellum (Cb). $^*p < 0.01$ and $^{**}p < 0.05$ *vs.* aged control). Open and solid bars represent aged and senescent, respectively.

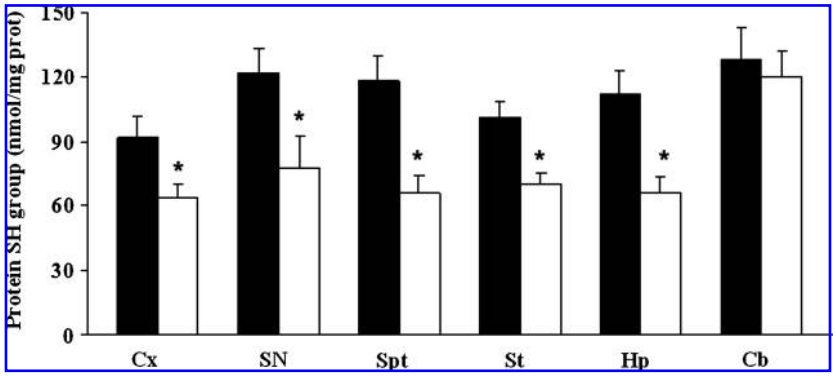

FIG. 7. Regional distribution of total sulfhydryl groups in different brain regions of senescent and aged rats. Total SH groups in cortex (Cx), substantia nigra (SN), septum (Spt), striatum (St), hippocampus (Hp), and cerebellum (Cb) were measured, as described in Methods. Results are expressed in nmol/mg protein. Data are expressed as mean ± SEM of eight animals. *$p < 0.05$ *vs.* aged (12-mo) rats. Solid and open bars represent aged and senescent, respectively.

brains of aged and senescent rats. Senescent rats showed, compared with aged rats, a significant decrease of Hsp90 in the cortex and hippocampus, an increase in the SN, but no significant changes were found in the septum, striatum, and cerebellum (Fig. 12A). These changes were associated with a decrease in the thioredoxin protein expression in all brain regions, particularly in the substantia nigra and in the hippocampus, except in the cerebellum (Fig. 13A). Consistently, TRX-Red expression was elevated in senescent rats compared with aged rats in all brain regions examined (Fig. 14A). Representative Western blot analysis of Hsp90, TRX, and TRX-Red proteins of samples from hippocampus are shown in Figs. 12B, 13B, and 14B, respectively.

Hydroxynonenal and protein carbonyl analysis

One measure of oxidative stress in brain aging is protein oxidation. However, lipid peroxidation, indexed by HNE, also can occur in the brain under oxidative stress. HNE, formed from arachidonic acid or other unsaturated fatty acids after free radical attack, binds by Michael addition to proteins, particularly at cysteine, histidine, or lysine residues. Examination of HNE levels in different brain regions in aged and senescent rat brains showed elevation of protein-bound HNE in all brain regions, except in the cerebellum, consistent with higher level of GSH found in the same region (Fig. 15A). In addition, a significant increase in the amount of protein carbonyls ($p < 0.005$) (Fig. 15B), an index of protein oxidation (8), was found in senescent compared with aged animals in all brain regions examined, but not in the cerebellum, confirming HNE data. Representative Western blot analysis of HNE and protein carbonyls of samples from the hippocampus are shown in Fig. 15C and D, respectively.

Discussion

Mammalian aging is characterized by a gradual and continuous loss, starting at full adulthood, of the quality of physiologic functions and responses. Losses appear to be more marked in the functions that depend on the integrated response

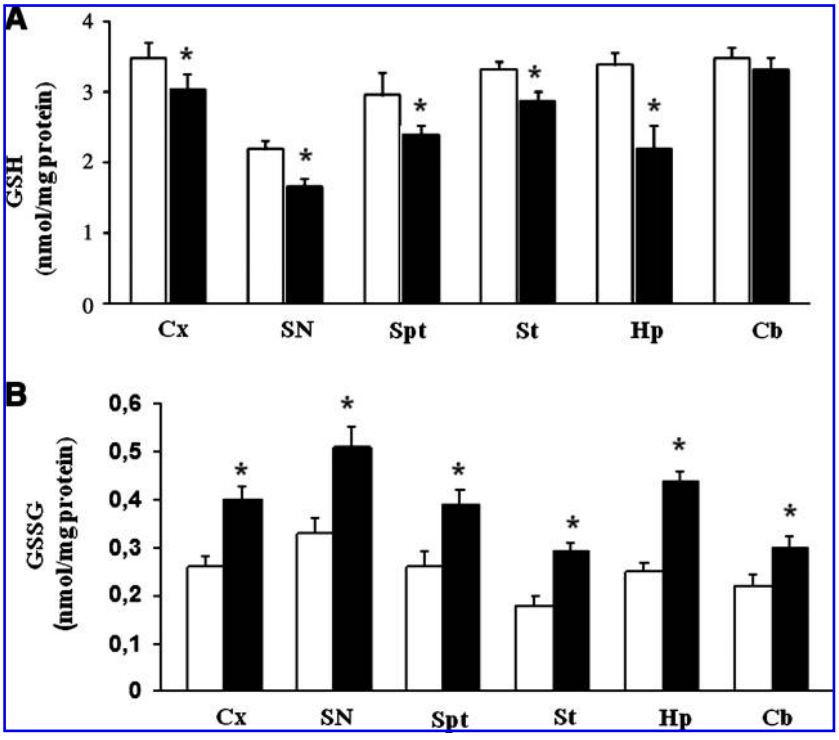

FIG. 8. Regional distribution of reduced (GSH) and oxidized (GSSG) glutathione in different brain regions of senescent and aged rats. GSH (**A**) and GSSG (**B**) in cortex (Cx), substantia nigra (SN), septum (Spt), striatum (St), hippocampus (Hp), and cerebellum (Cb) were measured as described in Methods. Results are expressed in nmol/mg protein. Data are expressed as mean ± SEM of eight animals. *$p < 0.05$ *vs.* aged (12-mo) rats. Open and solid bars represent aged and senescent, respectively.

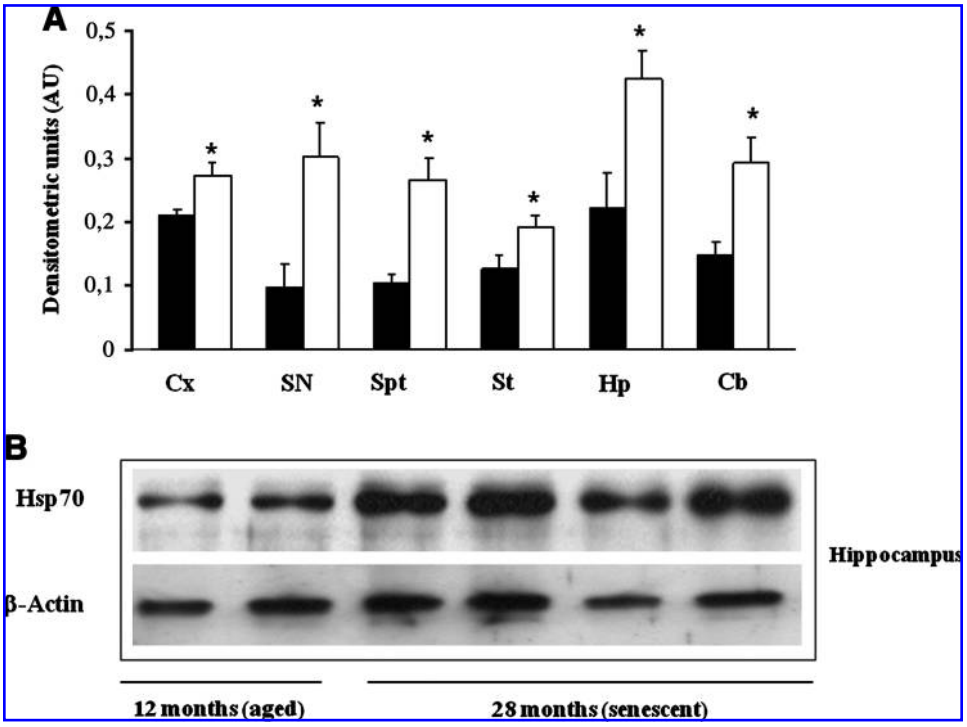

FIG. 9. Regional distribution in the levels of Hsp72 immunoreactive material in different brain regions of aged and senescent rats. Hsp72 from cortex, substantia nigra, septum, striatum, hippocampus, and cerebellum samples, in aged and senescent rats, were analyzed as described in Methods. (**A**) Values are expressed as densitometric units obtained by scanning the Western blot luminographs with a laser densitometer. (**B**) Representative Western blots of samples from hippocampus. Data are expressed as mean ± SEM of eight animals. Cortex (Cx), substantia nigra (SN), septum (Spt), striatum (St), hippocampus (Hp), and cerebellum (Cb). $*p < 0.05$ *vs.* aged rats. Solid and open bars represent aged and senescent, respectively.

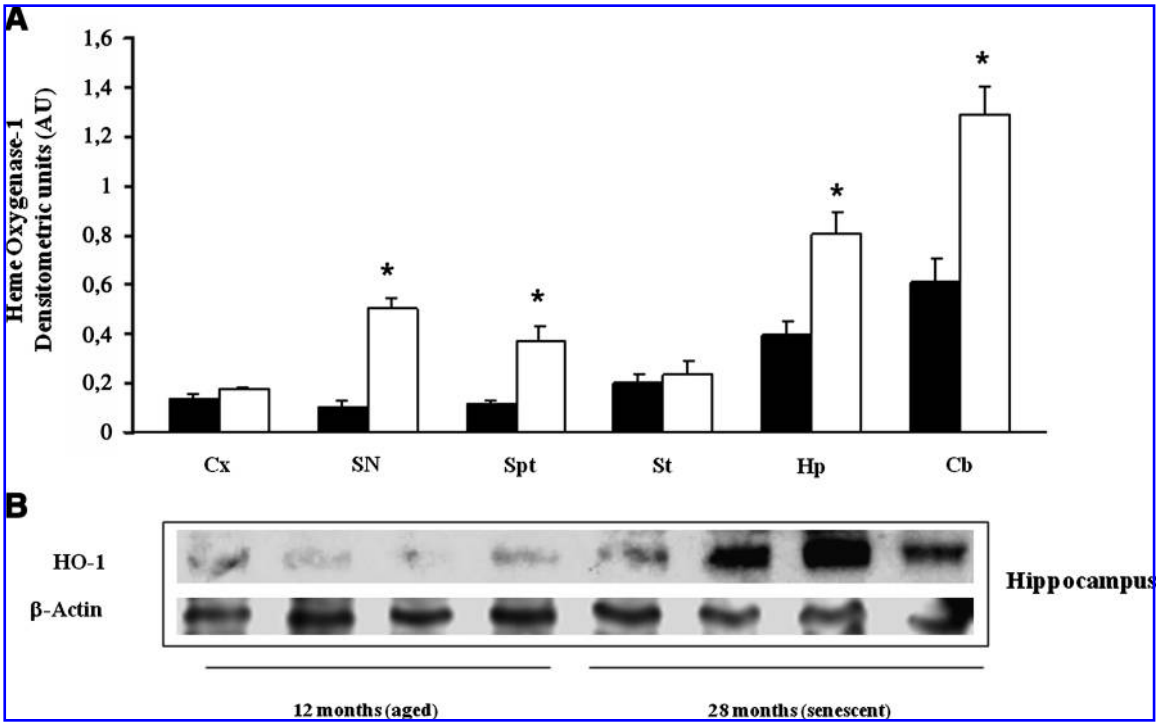

FIG. 10. Regional distribution in the levels of HO-1 immunoreactivity in different brain regions of aged and senescent rats. HO-1 from cortex (Cx), substantia nigra (SN), septum (Spt), striatum (St), hippocampus (Hp), and cerebellum (Cb samples, in aged and senescent rats, were analyzed as described in Methods. (**A**) Values are expressed as densitometric units obtained by scanning the Western-blot luminographs with a laser densitometer. (**B**) Representative Western blots of samples from the hippocampus. Data are expressed as mean ± SEM of eight animals. $*p < 0.05$ *vs.* aged rats. Solid and open bars represent aged and senescent, respectively.

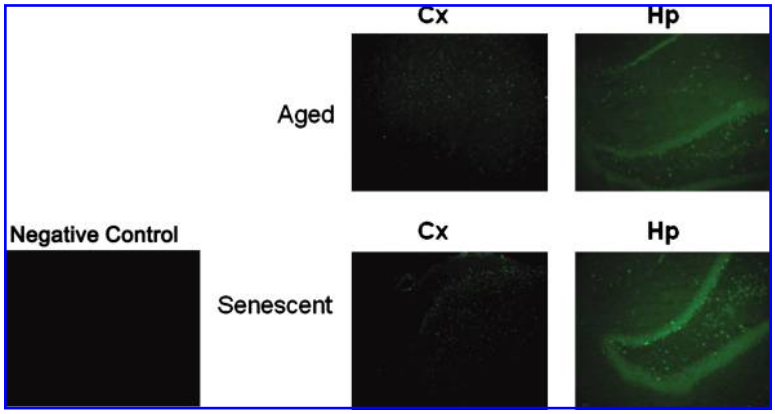

FIG. 11. Representative immunohistochemical analysis of HO-1 protein expression in cortex and in the CA3 hippocampal area of aged and senescent rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

of the central nervous system than in the functions of the renal or cardiovascular systems (9, 49). The free radical theory of aging, based on the pioneer works of Gerschman *et al.* (25) and Harman (30, 31), considers that aging is caused by the continuous loss of molecular fidelity, with inactivation of biologically essential macromolecules and subcellular structures, due to chemical modifications produced by reactions mediated by oxygen and nitrogen free radicals. A further refinement of this hypothesis is the mitochondrial theory of aging, as mitochondria are considered pacemakers of tissue aging, owing to their continuous production of superoxide radical and of nitric oxide (NO) and to the mitochondrial sensitivity to free radical–mediated oxidative damage (32, 43, 49).

In the present study, we expressed and purified a recombinant form of human carnosinase (CN1; EC 3.4.13.20), with a fast protocol that can allow the preparation of substantial amounts of this enzyme in a short time. Polyclonal monospecific antibodies to CN1 also were prepared in rabbit. A stable cell clone was established, expressing constant levels of the enzyme. The use of these tools is a necessary prerequisite to the study of the molecular features of the enzyme and to the elucidation of its role in cell physiology and in pathologic events, with special attention to the redox equilibrium in nerve cells. CN1 is indeed an enzyme found almost exclusively in brain tissue and serum (39). It has been suggested that the relatively high concentrations of this enzyme in human brain and CSF indicate that it may be synthesized in the brain and secreted into the CSF (39). Here we report an increased expression and activity of carnosinase (CN1) in the aging brain. Notably, the maximal induction was observed in

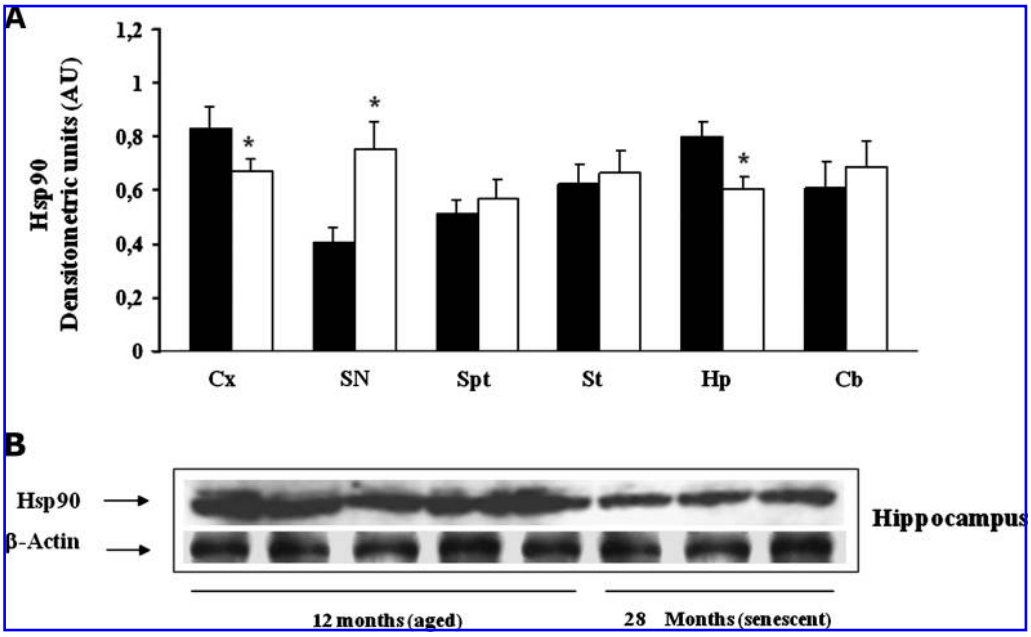

FIG. 12. Expression of Hsp90 in different brain regions of aged and senescent rats. (**A**) Regional Hsp90 protein distribution was estimated as described in Methods. (**B**) Representative Western blots of samples from hippocampus. Data are expressed as mean ± SEM of eight animals. Cortex (Cx), substantia nigra (SN), septum (Spt), striatum (St), hippocampus (Hp), and cerebellum (Cb). *$p < 0.05$ *vs.* aged rats. Solid and open bars represent aged and senescent, respectively.

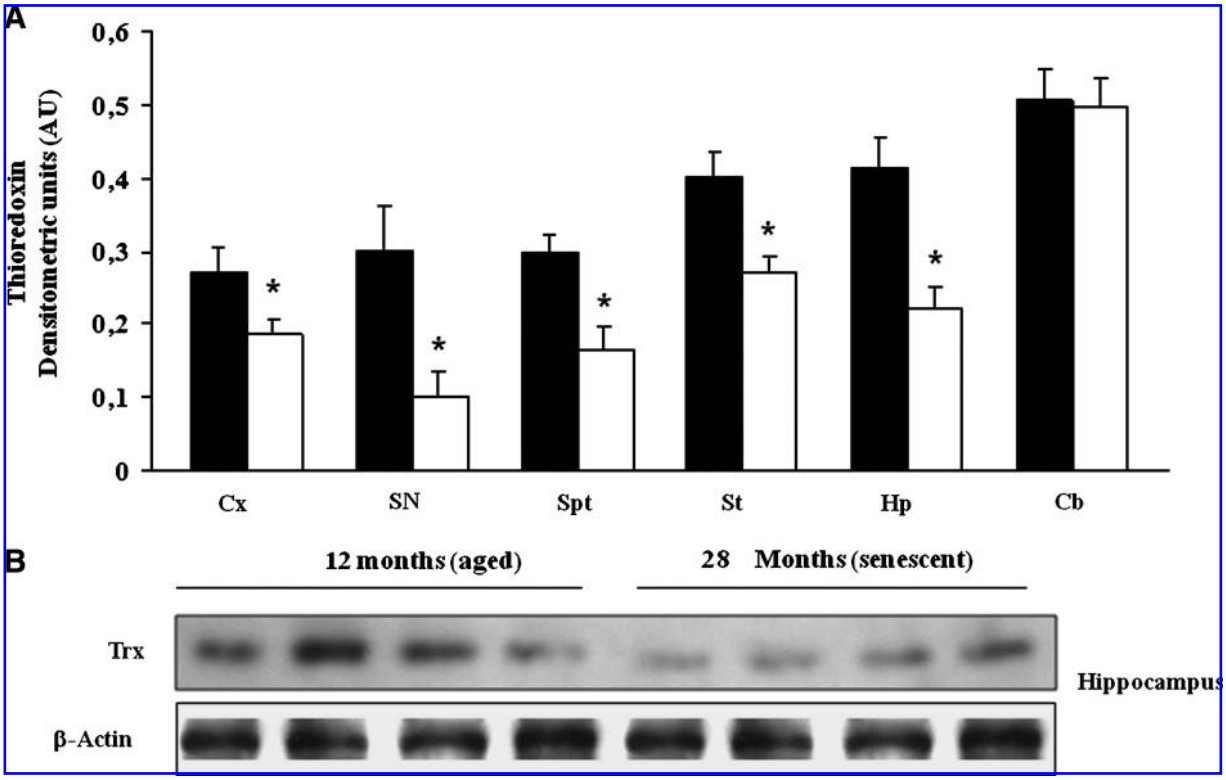

FIG. 13. Thioredoxin (TRX) expression in different brain regions of aged and senescent rats. (**A**) Regional distribution of TRX protein was estimated as described in Methods. (**B**) Representative Western blots of samples from hippocampus. Data are expressed as mean ± SEM of eight animals. Cortex (Cx), substantia nigra (SN), septum (Spt), striatum (St), hippocampus (Hp), and cerebellum (Cb). $^*p < 0.05$ *vs.* aged rats. Solid and open bars represent aged and senescent, respectively.

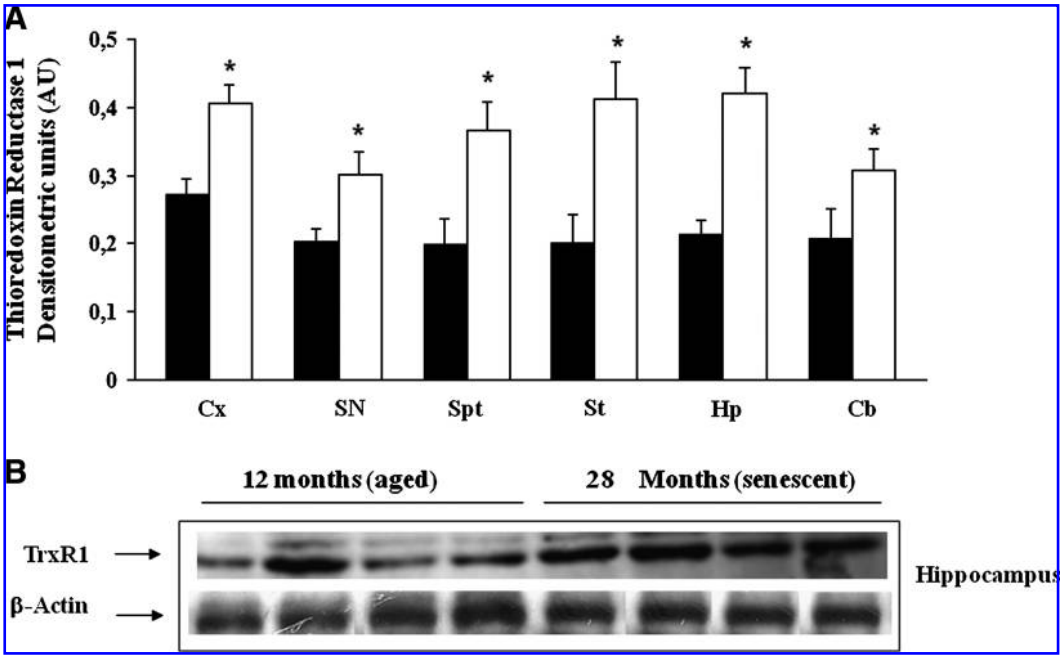

FIG. 14. Thioredoxin reductase expression in different brain regions of aged and senescent rats. (**A**) Thioredoxin reductase protein was estimated as described in Methods. (**B**) Representative Western blots of samples from hippocampus. Data are expressed as mean ± SEM of eight animals. $^*p < 0.05$ *vs.* aged rats. Cortex (Cx), substantia nigra (SN), septum (Spt), striatum (St), hippocampus (Hp), and cerebellum (Cb). $^*p < 0.05$ *vs.* aged rats. Solid and open bars represent aged and senescent, respectively.

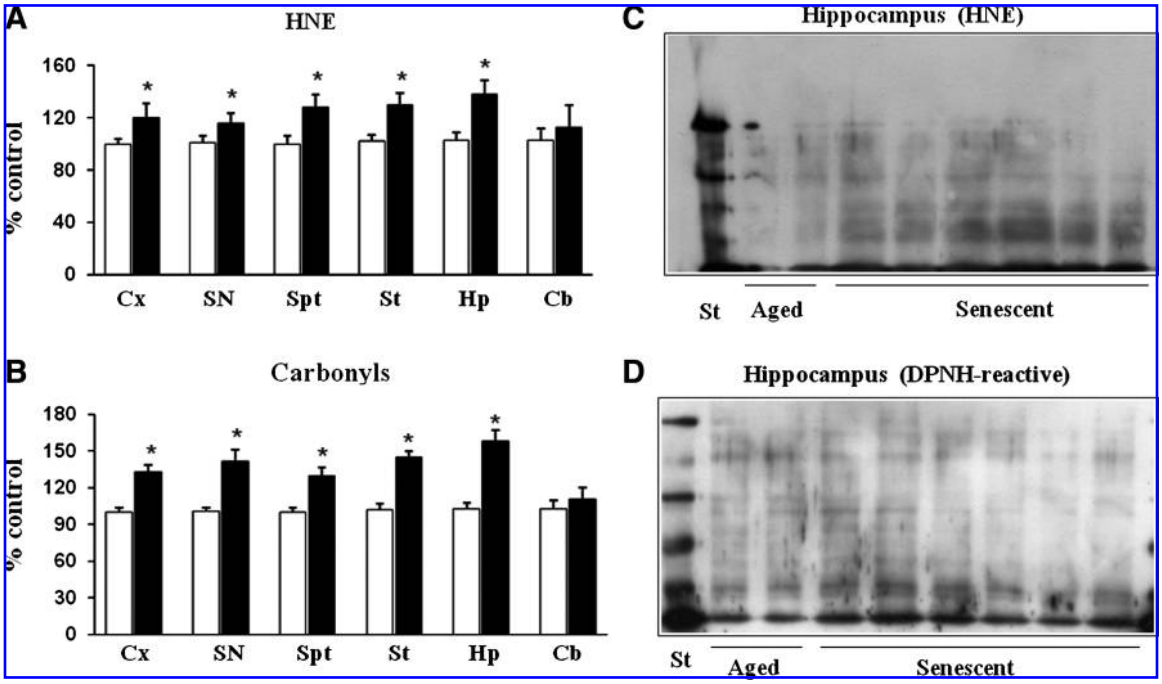

FIG. 15. Examination of HNE (A) and carbonyl (B) levels in different brain regions in aged and senescent rats. Cortex (Cx), substantia nigra (SN), septum (Spt), striatum (St), hippocampus (Hp), and cerebellum (Cb). Representative Western blot analysis of HNE (**C**) and protein carbonyls (**D**) of samples from hippocampus. Data are expressed as mean ± SEM of eight animals. *$p < 0.05$ *vs*. aged rats. Open and solid bars represent aged and senescent, respectively.

the SN and hippocampus, which are brain regions highly vulnerable to oxidant injury and aging effects. This may have important pathophysiologic implications, in light of the possibility that an increased expression and activity of CN1, by decreasing carnosine levels, can result in a significant decrease in antioxidative potential occurring during aging, as corroborated by a large body of literature.

Aging is characterized by a general decline in physiological functions that affects especially the brain, which is particularly susceptible to the effects of oxidant injury. In this context, 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 of the aging processes and neurodegenerative diseases (12, 16, 29). Reduced glutathione (GSH) is the most prevalent nonprotein thiol in animal cells. *De novo* and salvage synthesis of GSH maintains a reduced cellular environment in which the tripeptide is a cofactor for cytoplasmic enzymes and can act as an important posttranslational modifier in a number of cellular proteins (63). Owing to the cysteine thiol, it reacts as a nucleophile with exogenous and endogenous electrophilic species. As a consequence, reactive oxygen and nitrogen species (ROS, RNS) are frequently targeted by GSH in both spontaneous and catalytic reactions (47). Because ROS and RNS have defined roles in cell-signaling events as well as in human disease pathologies, an imbalance in expression of GSH and associated enzymes has been implicated in a variety of pathologic conditions (68). Thus, impaired function of the 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) (9, 18).

In the present study, we show that oxidative stress increases during aging in the brain, as revealed by decreased GSH content and increases in GSSG, as well as in 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 SN, and to a lesser extent in the cortex, septum, and striatum, whereas the cerebellum exhibited high resistance to these oxidative changes. Relevant to theory of aging, this is consistent with experimental findings showing that the hippocampus is atrophied during aging and in some neurologic diseases, all processes associated with memory and cognitive impairments (20, 50). Moreover, patients with early to moderate AD have a 25% decrease in hippocampal volume compared with that in healthy controls (41, 50) and show postmortem marked ultrastructural damage in hippocampal neurons, especially in mitochondria (51).

The most prominent biochemical symptom of aging and many age-related conditions is proteotoxic stress, resulting from the accumulation of altered proteins that arise from biosynthetic errors or deleterious postsynthetic polypeptide modifications or both (48). 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 heme oxygenase-1 (HO-1). Expression of the genes encoding Hsps has been found in various cell populations of central nervous system (CNS), including neurons, glia, and endothelial cells (13). In the nervous system, Hsps are induced in a variety of pathologic conditions, including cerebral ischemia, epilepsy, trauma, and neurodegenerative and metabolic disorders (14).

We found that the level of Hsp72 in senescent rats was significantly higher than that in aged rats, with the highest profile of expression in the hippocampus and SN, followed by the septum, cerebellum, cortex, and striatum. Similar patterns of expression as a function of aging were observed when testing for HO-1, indicating that these cytoprotective genes work together in the setting of cellular stress response. In addition, we show, also in aging brain, an increased expression of thioredoxin reductase, the latter possibly related to the decreased thioredoxin protein expression measured in all brain regions investigated. Although the expression of these two genes is known to be coordinately regulated, in our experimental conditions, a lack of coordination was found, consistently with that observed in AD brains (46). Interestingly, we found in the hippocampus a significant correlation between upregulation of Hsp72 and HO-1 and decrease in the content of protein sulfydryl groups, which followed parallel changes in GSH/GSSG redox states, confirming the generally accepted role of the redox state as a signal for the activation of protective responses, such as the synthesis of heat-shock proteins. Taken together, these results suggest that the expression of Hsps increases with age and occurs as a consequence of redox state perturbation, and this may have a role to limit the deleterious consequences associated with protein denaturation. In addition, we found, in senescent rats, compared with aged controls, a significant decrease of Hsp90 expression in the brain regions of the hippocampus and in the cortex, whereas in the SN, Hsp90s were upregulated.

This finding may be relevant to theories connecting aging to neurodegenerative disorders, as molecular chaperone Hsp90 has been demonstrated to be a target for *S*-nitrosylation, and *S*-nitrosylation of Hsp90 abolishes ATPase activity that is necessary for its chaperone function. Thus inactivation of Hsp90 may allow accumulation of tau and amyloid-β aggregates in the degenerating brain. In addition, increasing evidence supports the notion that inhibition of Hsp90 led to decreases in p-tau levels, suggesting that blockade of the Hsp90-mediated refolding pathway promotes p-tau turnover, through degradation. Remarkably, peripheral administration of a novel Hsp90 inhibitor promoted selective decreases in p-tau species in a mouse model of tauopathy, further suggesting a central role for the Hsp90 complex in the pathogenesis of tauopathies. In keeping with this notion, the decrease in Hsp90 expression observed in cognitive brain regions, such as the cortex and hippocampus, may be viewed as a compensatory mechanism to limit the deleterious consequences associated with pathologic hyperphosphorylation, such as that observed in AD brain and the SAMP8 mouse model of senescence (64). Therefore, pharmacologic modulation of cellular stress pathways is an emerging area in the treatment of human diseases, not only neurodegenerative disorders, but also cardiovascular disease and cancer. Relevant to this, we devoted our recent interest to the development of nutritional interventions able to target redox-sensitive cytoprotective genes, called vitagenes, involved in the homeostatic control of so-called longevity-assurance processes. In particular, the term vitagenes refers to a group of genes strictly involved in preserving cellular homeostasis during stressful conditions.

The vitagene family is actually composed of the heat-shock proteins (Hsp) Hsp32, Hsp70, the thioredoxin system, and the sirtuin system (1, 13). Dietary antioxidants, such as polyphenols, carnitines, and carnosine have recently been demonstrated *in vitro* to be neuroprotective through the activation of hormetic pathways, such as those including vitagenes (11, 12, 15, 16). Carnosine, in particular, it has been demonstrated to possess NO free-radical scavenging ability and NO-trapping capacity in cell-free experiments (52) and also is able to prevent, in astrocytes, the upregulation of iNOS and the induction of both HO-1 and Hsp70 after nitrosative stress (10). Here we found that the increase of carnosinases increases the susceptibility to toxic effects of oxidative stress, and this may be relevant to the free-radical theory of aging. In view of the finding that (a) neuronal NOS is increased in aging brain, (b) carnosine has been found to protect cells from Aβ–induced toxicity (24, 35), and that (c) plasma levels of carnosine are lower in AD patients than in age-matched controls (3), it is conceivable that the increase in carnosinase that shows regional specificity, is a factor contributing to age-related pathogenesis, which affects, with different severities, various regions of the aging brain. Relevant to this, histidine-containing dipeptides resistant to carnosinase hydrolysis have been recently developed (2, 5, 55), and their pharmacological application to neurodegenerative pathologies suggested. However, further studies are required to clarify important aspects of the respective roles of CN1 and its substrate carnosine in the CNS, particularly the functional relation between neuronal and glial cells with respect to carnosine synthesis and use in health and diseases states, such as brain aging and neurodegenerative disorders.

Acknowledgments

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Abbreviations Used

AD = Alzheimer's disease
AH = β-alanyl-L-histidine
AIN = American Institute of Nutrition
ALS = amyotrophic lateral sclerosis
ANOVA = analysis of variance
ANS = anserine (β-alanyl-1-methyl-L-histidine)
Cb = cerebellum
CN1 = serum carnosinase
CN2 = cytosolic carnosinase
CNS = central nervous system
CSF = cerebrospinal fluid
Cx = cortex
DEAE = diethylaminoethyl
DMEM = Dulbecco's modified Eagle's medium
DMSO = dimethyl sulfoxide
DNPH = 2,4-dinitrophenylhydrazine
DTNB = 5,5′-dithio-bis-(2-nitrobenzoic acid)
ECL = enhanced chemiluminescence
ELISA = enzyme-linked immunosorbent assay
GR = glutathione reductase
GSH reduced glutathione
GSSG = oxidized glutathione
HC = homocarnosine (γ-aminobutyryl-L-histidine)
HNE = hydroxynonenal
HO-1 = heme oxygenase-1
Hp = hippocampus
HRP = horseradish peroxidise
Hsp = heat shock protein
IgG = immunoglobulin G
MALDI-TOF = matrix-assisted laser desorption/ionization/time of flight
MTT = 3-[4,5-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide
NADH = β-nicotinamide adenine dinucleotide
β-NADPH = β-nicotinamide adenine dinucleotide phosphate
NEM = *N*-ethylmaleimide
NO = nitric oxide
OPA = *o*-pthaldialdehyde
OPD = *ortho*-phenylendiamine
OVA = ovalbumin
PBS = phosphate-buffered saline
PCR = polymerase chain reaction
PD = Parkinson's disease
RNS = reactive nitrogen species
ROS = reactive oxygen species
SDS = sodium dodecylsulfate
SDS-PAGE = sodium dodecylsulfate–polyacrylamide gel electrophoresis
SIN-1 = 3-morpholinosydnonimine hydrochloride
SN = substantia nigra
Sp = septum
St = striatum
TCA = trichloroacetic acid
TFA = trifluoroacetic acid
TRX = thioredoxin
TRX-Red = thioredoxin reductase

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