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Increased expression of heat shock proteins in rat brain during aging: relationship with mitochondrial function and glutathione redox state

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Abstract

It is generally recognized that lipid peroxides play an important role in the pathogenesis of several diseases and that sulfhydryl groups are critically involved in cellular defense against endogenous or exogenous oxidants. Recent evidence indicates that lipid peroxides directly participate in induction of cytoprotective proteins, such as heat shock proteins (Hsps), which play a central role in the cellular mechanisms of stress tolerance. Oxidative damage plays a crucial role in the brain aging process and induction of Hsps is critically utilized by brain cells in the repair process following various pathogenic insults. In the present study, we investigated, in rats 6, 12, and 28 months old, the role of heat shock expression on aging-induced changes in mitochondrial and antioxidant redox status. In the brain expression of Hsp72 and Hsc70 increased with age up to 28 months; at this age the maximum induction was observed in the hippocampus and substantia nigra followed by cerebellum, cortex, septum and striatum. Hsps induction was associated with significant changes in glutathione (GSH) redox state and HNE levels. Interestingly, a significant positive correlation between decrease in GSH and increase in Hsp72 was observed in all brain regions examined during aging. Analysis of mitochondrial complexes showed a progressive decrease of Complex I activity and mRNA expression in the hippocampus and a significant decrease of Complex I and IV activities in the substantia nigra and septum. Our results sustain a role for GSH redox state in Hsp expression. Increase of Hsp expression promotes the functional recovery of oxidatively damaged proteins and protects cells from progressive age-related cell damage. Conceivably, heat shock signal pathway by increasing cellular stress resistance may represent a crucial mechanism of defence against free radical-induced damage occurring in aging brain and in neurodegenerative disorders.

Keywords: Free radical; Oxidant/antioxidant balance; Heat shock proteins; Glutathione; 4-Hydroxynonenal

1. Introduction

Although the term ‘aging’ is generally understood in broad terms, the aging process is extremely complex and multifaced. 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 (Halliwell, 2001; Golden et al., 2002). Experimental evidence indicates that increased rate of free radical generation and decreased efficiency of the 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. Reduced glutathione (GSH) is the most prevalent non-protein thiol in animal cells. Its de novo and salvage synthesis serves 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. 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 (Butterfield et al., 2002a,b; Drake et al., 2002, 2003; Poon et al., 2004). Since ROS 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

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implicated in a variety of pathological conditions (Calabrese et al., 2003, Butterfield et al., 2002c). Cause and effect links between GSH metabolism and diseases such as cancer, neurodegenerative diseases and aging have been shown (Droge, 2002). 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 documentated to occur in brain during aging (Droge, 2002; Stadtman, 2001). 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 (Carney et al., 1991). Protein carbonyls can be generated by direct oxidative damage to proteins, by the binding to proteins of cytotoxic aldehyde such as 4-hydroxy-trans-nonenal (HNE), and by glycosidation of proteins (Hyun et al., 2003). 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.

It is well known that brain cells are continually challenged by conditions which may cause acute or chronic stress. To adapt to these environmental changes and survive different types of injuries, a network of different responses have evolved which detect and control diverse forms of cellular stress. One of these responses, known as the heat shock response, has attracted a great deal of attention as a fundamental mechanism necessary for cell survival under a variety of unfavorable conditions (Calabrese et al., 2000a). 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 (Calabrese et al., 2002a, 2000c). 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 (Calabrese et al., 2000a). 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 (Mayer, 2003; Motterlini et al., 2000; Calabrese et al., 2002b). This has opened new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear to be possible candidates for novel cytoprotective strategies. 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. 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. Therefore, alterations of the expression and function of stress proteins are supposed to be linked to the protein denaturation with aging. Recently, it was suggested that the basal Hsp70 increased by accumulation of modified proteins in aged rat kidney (Unno et al., 2000). Accumulation of denatured proteins in long-lived cells like neurons might be related to the decreased function of the brain with aging. In addition, mitochondrial respiratory chain is considered a powerful source of reactive oxygen species (ROS), and increasing body of evidence suggests that the dysfunction of cell energy metabolism is an important factor in the pathogenesis of most important neurodegenerative disorders (Calabrese et al., 2001). The implication of mitochondria both as producers and as targets of ROS has been, in fact, the basis for the mitochondrial theory of aging. Recent studies have confirmed that Complex I is a major source of superoxide production in several types of mitochondria and localized the oxygen-reducing site between the ferricyanide and the quinone reduction sites (Lenaz et al., 2002). In view of the recent findings suggesting that mitochondria are selective target of Hsp protection against oxidative insults (Tsuchiya et al., 2003), we investigated, in different brain regions of rats 6, 12, and 28 months old, the role of heat shock expression on aging-induced changes in mitochondrial and antioxidant status. We found that heat shock protein expresion in some brain regions, such as cortex, substantia nigra (S. nigra), striatum, septum, and hippocampus, were 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 increase in GSSG and HNE levels. The possible implications of redox-dependent mechanisms and heat shock response in neurodegenerative disorders are discussed.

2. Materials and methods

2.1. 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 aged 6, 12, and 28 months, ($n = 8$ per 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 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 s time-variability for each sample across animals. Substantia nigra was dissected from the deepest part of the interpeduncolar fossa. In order to exclude the possibility that the small size of nigral or septal samples could affect results we analysed pooled samples from S. nigra or septum, in order to have a protein content comparable to that measured in cortex or striatum, and lipid peroxidation products

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 corn starch.
[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%.

as well as thiols or enzymes measured. In all these cases we did not find significant differences between pooled samples and those coming from a single experimental animal.

2.2. Reduced and oxidized glutathione assay

Reduced glutathione (GSH) and glutathione disulfide (GSSG) were measured by the NADPH-dependent GSSG reductase method as previously reported (Calabrese et al., 2002b). Specimens were homogenized at ice temperature for 10 s in 100 mM potassium phosphate, pH 7.5, which contained 12 mM disodium EDTA. For total glutathione, aliquots (0.1 ml) of homogenates were immediately added to 0.1 ml of a cold solution containing 10 mM DTNB and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The sample 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 of GSSG reductase in 100 mM potassium phosphate and 5 mM 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 mM NEM and 5 mM EDTA in 100 mM 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) 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 of GSSG reductase. The assay then proceeded as in the measurement of total GSH. Plasma GSH and GSSG were measured as described before, 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 nmol, and 0.010 and 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 milligram protein.

2.3. Western blot analysis

The tissue homogenate was centrifuged at 10,000 × *g* for 10 min and the supernatant was used for Hsc70, Hsp72, and HNE level determinations 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), transferred overnight to nitrocellulose membranes and the non specific binding of antibodies was blocked with 3% non-fat dry milk in PBS. Membranes were then probed with a monoclonal anti-Hsc70 antibody (Santa Cruz) that recognizes the constitutive form of Hsp70, or with a monoclonal anti-Hsp72 antibody (RPN 1197, Amersham) 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 Hsp72, constitutive Hsc70, and HNE were quantified by scanning Western blot imaged films with a laser densitometer (LKB-Ultroscan, XL model). Multiple exposure of each blot were used to ensure linearity of the film response. The investigator performing densitometry analysis was blinded to the identity of samples so as to minimize experimenter bias.

2.4. Real time quantitative RT-PCR

Total RNA from rat brain dissected areas was extracted using Trizol (Sigma, St. Louis, MI, USA) and treated with RNase-free DNase to remove any residual genomic DNA. Single-stranded cDNAs were synthesized incubating total RNA (1 μg) with SuperScript II RNase H-reverse transcriptase (200 U), oligo-$(dT)_{12–18}$ primer (100 nM), dNTPs (1 mM), 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 Hsp72 and mitochondrial Complexes I and IV are listed in Table 1. 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 and generated a 183 bp PCR product. Aliquots of cDNA (0.1 and 0.2 μg) and known amounts of external standard (purified PCR product, 10^2–10^8 copies) were amplified in parallel reactions using the FP and RP indicated in Table 2. Each PCR reaction (final volume 20 μl) contained 0.5 μM of primers, 2.5 mM Mg^{2+} and 1× Light-Cycler

Table 2
Oligonucleotide Primers for Hsp72 and mitochondrial complexes mRNAs

Name	Sequence
Hsp72-F	TTTTCTGGCTCTCAGGGTGT
Hsp72-R	CCTTGCATCCCTACAAACTGA
Complex 1 sub B13 (cyt)-F	ACTACTGGCCTGGTGGGATT
Complex 1 sub B13 (cyt)-R	CCACCCTGAAGCAAGTTTTC
Complex IV subIV (cyt)-F	ACATGAAGAGCAACCCCATA
Complex IV subIV (cyt)-R	AAGGGAATGGAGGAGACAAG
Complex IV sub3 (mit)-F	CCAAACCCATGCATACCATA
Complex IV sub3 (mit)-R	GTGGCCTTGGTATGTTCCTT
PGK1-F	AGGTGCTCAACAACATGGAG
PGK1-R	TACCAGAGGCCACAGTAGCT

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 (1 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 (1 cycle: 95 °C for 0 s; 70 °C for 10 s; 95 °C for 0 s); (iv) cooling (1 cycle: 40 °C for 3 min). 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 as 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.

2.5. Enzyme assays

Brain homogenate was centrifuged at $1000 \times g$ for 10 min. The nuclear pellet was washed once and the supernatants were centrifuged at $20{,}000 \times g$ for 30 min to obtain the supernatant and the mitochondrial pellet, respectively. The pellet was washed twice and the mitochondria were resuspended in 40 mM Tris–HCl (pH 6.8), 2.5% SDS. For the measurement of mitochondrial enzymatic activities, the pellet was homogenized in 0.8 ml of 0.9% NaCl and sonicated for 10 s at 0–2 °C in an ultrasonic disintegrator (power 150 W). Contamination of the mitochondrial extract by cytosol, determined by measurement of lactate dehydrogenase activity was estimated to be less than 10% of the measured activity. Cytochrome c oxidase (Complex IV, EC 1.9.3.1) and NADH-CoQ_1 reductase (Complex I, EC 1.6.99.3) activities were determined as previously dcscribed (Calabrese et al., 2000b).

2.6. Determination of protein

Proteins were estimated by the method of (Smith et al., 1985), using bicinchoninic acid reagent.

2.7. Statistical examination

Results were expressed as means ± S.E.M. 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 one-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.

3. Results

3.1. 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 at 28 months, and all but cerebellum and septum showed the decline in GSH levels at 12 months (Fig. 1A and B). Conversely, the level of oxidized GSH, i.e., GSSG, increased with the same pattern (Fig. 1A and B). To test the hypothesis that this loss of redox status in the brain as a consequence of aging would induce an Hsp response, we measured the expression of Hsp72 in different brain regions in rats of 6, 12, and 28 months of age. Fig. 2A and B show that expression of Hsp72 was significantly elevated at 12 months of age, a finding that was replicated in 28-month rat brain.

3.2. Hsps distribution in brain aging

Consistent with the increased expression of Hsp72 with brain aging, the message for this response protein was also elevated, particularly at 28 months of age in the substantia nigra, hippocampus, and cerebellum regions (Fig. 3). To examine if these regions also had elevated Hsc70, the constitutive form of Hsp70, we measured expression of this protein in brain aging. Hsc70 also was increased with aging (Fig. 4).

3.3. 4-Hydroxynonenal analysis

One measure of oxidative stress in brain aging is protein oxidation (Butterfield et al., 1997a; Butterfield and Stadtman, 1997b). However, lipid peroxidation, indexed by HNE, can also occur in brain under oxidative stress (Lauderback et al., 2001). HNE, formed from arachidonic acid or other unsaturated fatty acids following free radical attack (Butterfield et al., 1997a), binds by Michael addition to proteins, particularly at cysteine, histidine, or lysine residues (Butterfield et al., 1997a). Examination of HNE

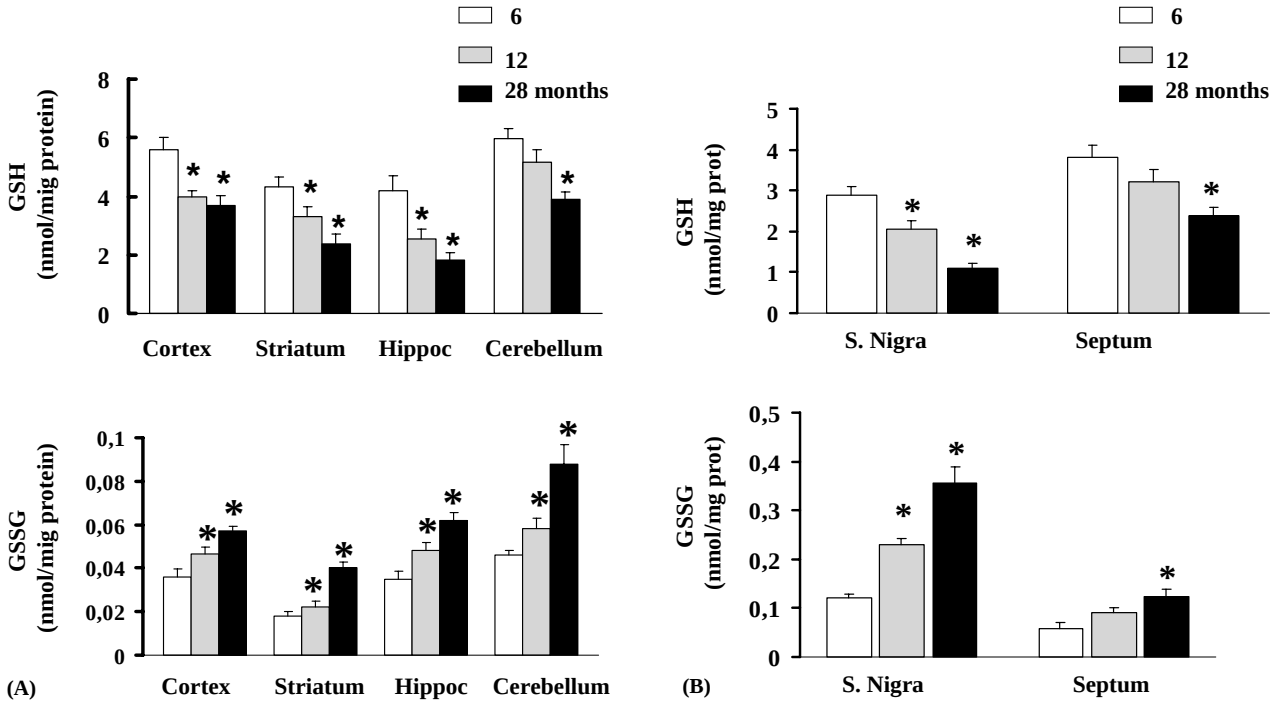

Fig. 1. Regional distribution of reduced glutathione (GSH) and oxidized glutathione (GSSG) in different brain regions as function of aging. GSH and GSSG in: (A) cortex, striatum, hippocampus (Hippoc), cerebellum, and (B) substantia nigra (S. nigra) and septum were measured as described in Section 2. Results are expressed in nmol/mg protein. Data are means ± S.E.M. of eight animals. *$P < 0.05$ vs. control.

levels in different brain regions in 6-, 12-, and 28-month old rat brains showed elevation of protein-bound HNE in all brain regions, but particularly striking in the hippocampus and substantia nigra regions (Fig. 5A and B, 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. 5A).

3.4. Mitochondrial complex activity and expression analysis

As noted in the Section 1, mitochondria are a source of oxidative stress, but they also can be a target of free radial damage. The activities of mitochondrial Complex I and Complex IV of the electron transport chain (ETC) were

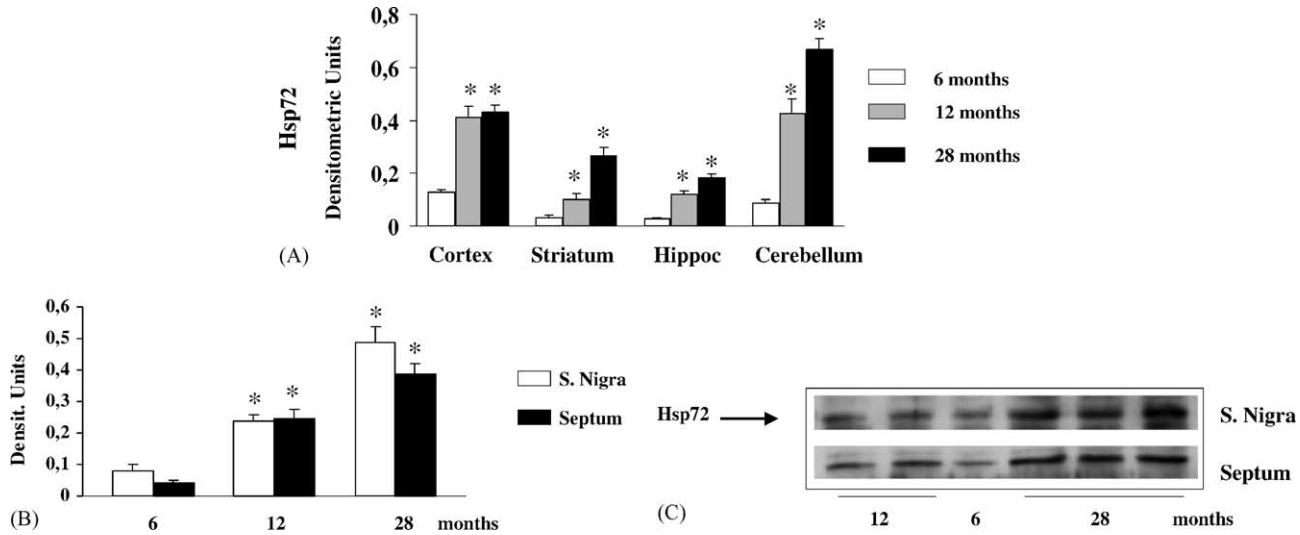

Fig. 2. Regional distribution in the levels of Hsp72 immunoreactive cytoplasmic material from different cerebral areas: (A) cortex; striatum, hippocampus (Hippoc), and cerebellum; (B) S. nigra and septum. The values are expressed as densitometric units obtained by scanning the Western blot luminographs with laser densitometer. Data are means ± S.E.M. of nine animals. (C) Representative Western blot is reported showing samples from S. nigra and septum probed with a monoclonal anti-Hsp72 antibody (RPN 1197, Amersham) that recognizes only inducible Hsp72. *$P < 0.05$ vs. control.

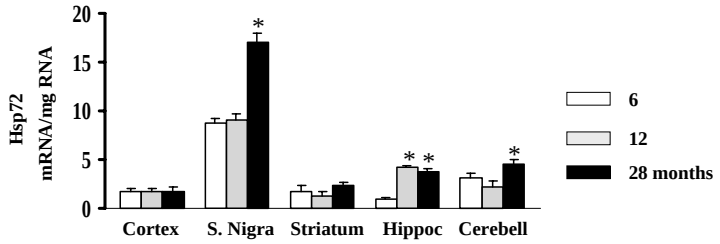

Fig. 3. Levels of Hsp72 mRNA in different cerebral areas as function of brain aging. Total RNA from rat brain dissected areas was extracted as described in Section 2 and quantification was performed by RT-PCR. Specificity of PCR products was characterized by melting curve analysis followed by gel electrophoresis, ethidium bromide staining, and DNA sequencing. $*P < 0.05$ vs. control.

examined in different brain regions as a function of brain aging. At 12 months of age, Complex I was not affected; however, at 28 months of age, brain mitochondria showed a decrease in Complex I activity in the hippocampus, substantia nigra, and septum. (Fig. 6A and B). Hippocampal Complex IV activity is elevated at 12 months and less so at 28 months, relative to Complex IV activity in hippocampus from 6-month old rat brain (Fig. 6A). In no other region studied was 12-month rat brain altered in its Complex IV activity. However, at 28 months substantia nigral and septum brain regions were decreased in Complex IV activity compared to that of 6-month old rat brain (Fig. 6B). We studied also the expression of brain Complex I and Complex IV mRNA during aging (Fig. 7A and B). Analysis of the nuclear B13 (39 kDa) Complex I subunit mRNA expression (Fig. 7A) showed no significant changes as function of aging in all brain regions examined, except for the hippocampus where we found increased mRNA levels at 12 months, followed by a significant decrease at 28 months. Likewise, Complex IV subunit IV (nuclear subunit, see Table 2) expression was up-regulated either at 12 or 28 months, as compared to 6 months old animals, in the hippocampus, whereas no significant changes were observed in all other brain regions examined (Fig. 7B). We also investigated the expression of the mitocondrial subunit III of Complex IV and no significant changes were observed in all brain regions examined during aging (Fig. 7B).

4. Discussion

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 ROS, mitochondria have antioxidant defense systems to counteract oxidative stress. Lacking many endogenous antioxidant mechanisms, mitochondria depend on glutathione (GSH) as an endogenous combatant against H_2O_2 (Coll et al., 2003; Fernandez-Checa et al., 1998). 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 may prove to be an important therapeutic approach to prevent cell death in oxidative stress-linked, age-dependent neurodegenerative disorders (Gegg et al., 2003). 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 (Calabrese et al., 2001). As the source of these toxic oxidants, mitochondria are also their potential victims (Sastre et al., 2003). Mitochondrial decay is also a contributor to acceleration of aging in the senescence-accelerated mouse and in stress (Ames, 2003; Atamma et al., 2002). During the last few years, cellular oxidant/antioxidant balance has become the subject of intense

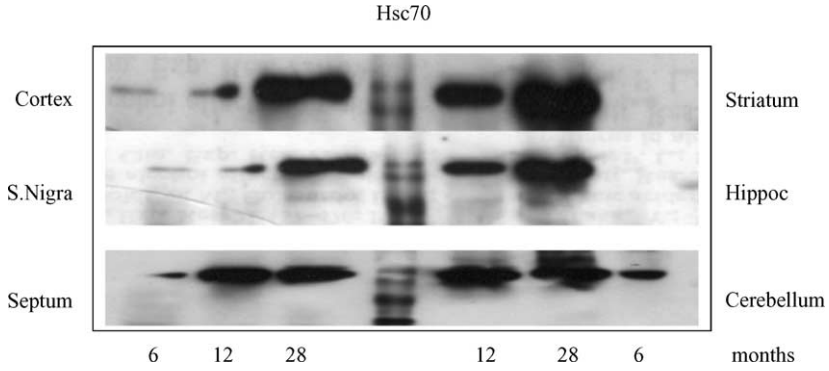

Fig. 4. Levels of Hsc70 immunoreactive cytoplasmic material extracted from different cerebral areas as function of brain aging. Representative Western blots of cortex, S. nigra, septum, striatum, hippocampus (Hippoc), and cerebellum samples probed with a monoclonal anti-Hsc70 that recognizes the constitutive form of Hsp70.

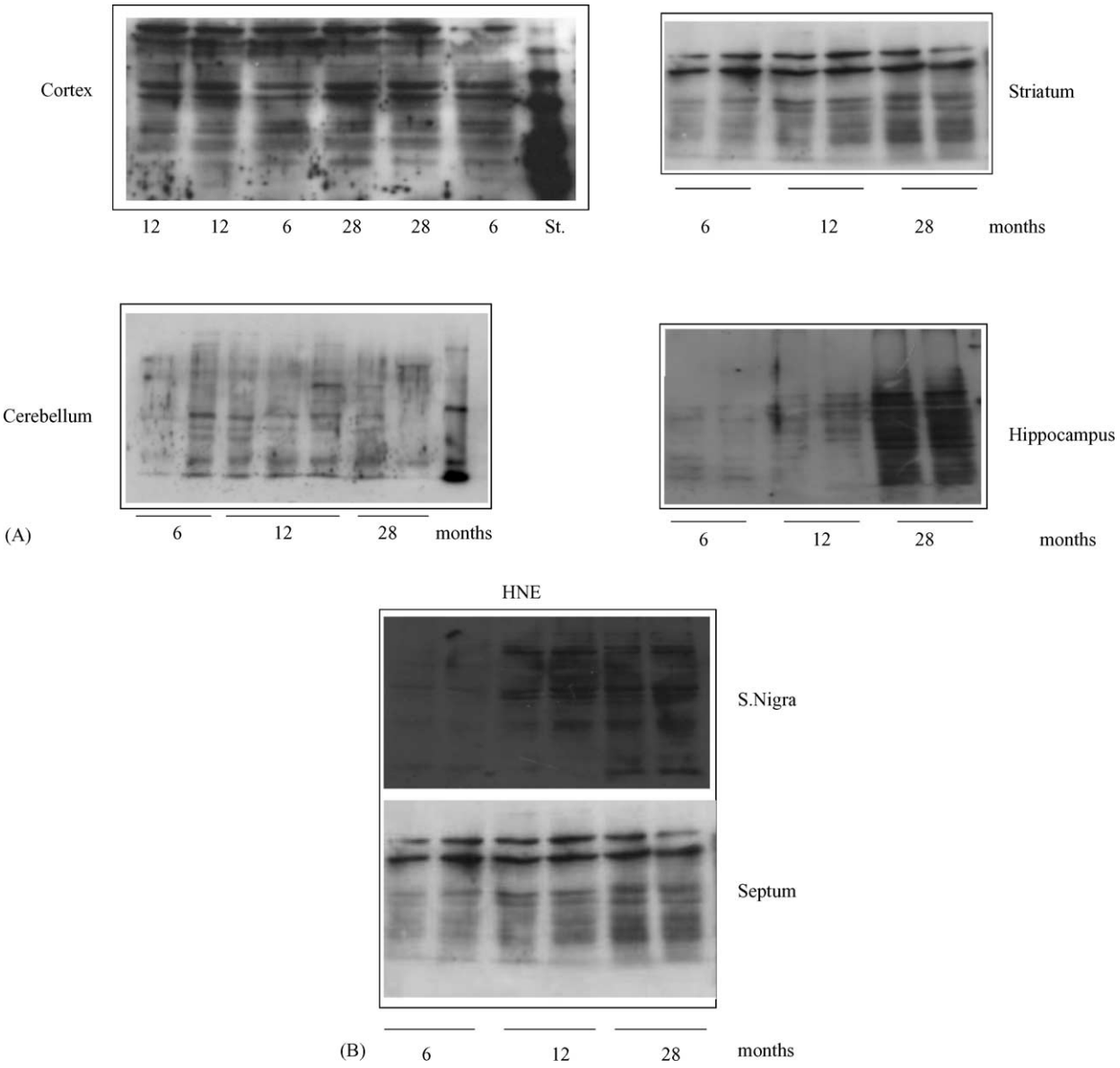

Fig. 5. Representative Western blots of: (A) cortex, striatum, hippocampus, cerebellum, and (B) S. nigra and septum samples probed with a monoclonal anti-4-hydroxy-2-nonenal that recognizes Michael adducts as a measure of protein oxidation by oxidative stress in brain aging.

study, particularly by those interested in brain aging and in neurodegenerative mechanisms (Atamma et al., 2002; Hyun et al., 2002; Lee et al., 2001). A number of experimental evidence indicate that increased rate of free radical generation and decreased efficiency of the riparative/degradative mechanisms, such as proteolysis, both are factors which primarily contribute to age-related elevation in the level of oxidative stress and brain damage (Hyun et al., 2003; Halliwell, 2002). 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 (Butterfield, 2004; Butterfield et al., 2003). 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 (Calabrese et al., 2000a).

Cells respond to sub-lethal 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). Some of these have been implicated in the development of thermotolerance and resistance to other environmental stresses (Mattson et al., 2002). 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. Expression of the genes

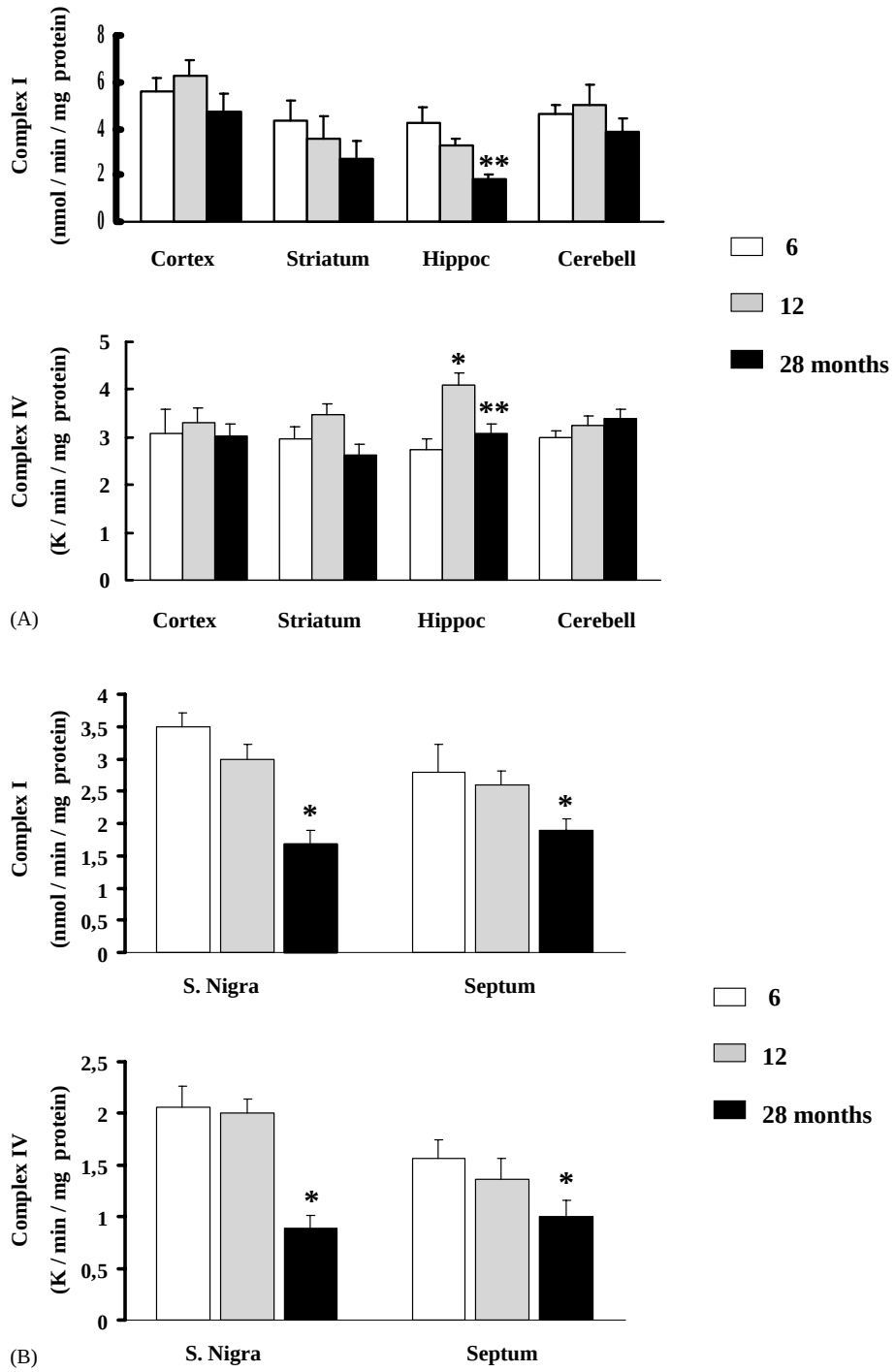

Fig. 6. Mitochondrial Complex I and Complex IV enzyme activities examined in different brain regions: (A) cortex, striatum, hippocampus, cerebellum; (B) S. nigra and septum; as a function of brain aging. Specific activities were measured as reported in Section 2 and expressed in nmol/min/mg protein (Complex I) or K/min/mg protein (Complex IV). Data are means ± S.E.M. of eight animals. $*P < 0.05$ vs. control; $**P < 0.05$ vs. 12 months.

encoding Hsps has been found in various cell populations of central nervous system (CNS), including neurons, glia, and endothelial cells (Calabrese et al., 2002b). In the nervous system Hsps are induced in a variety of pathological conditions, including cerebral ischemia, epilepsy and trauma, neurodegenerative, and metabolic disorders (Yenari, 2002). Stress proteins, which have a role to protect proteins from denaturation, may respond to the protein denaturation with aging. We found that the basal level of Hsp72, constitutively expressed Hsp70 (Hsc70), in 28-month-old rats was significantly higher in some parts of the brain than that in 6-month or 12-months old rats. These results suggest that the expression of Hsp70 increases with aging and may have a role to suppress protein denaturation. Furthermore,

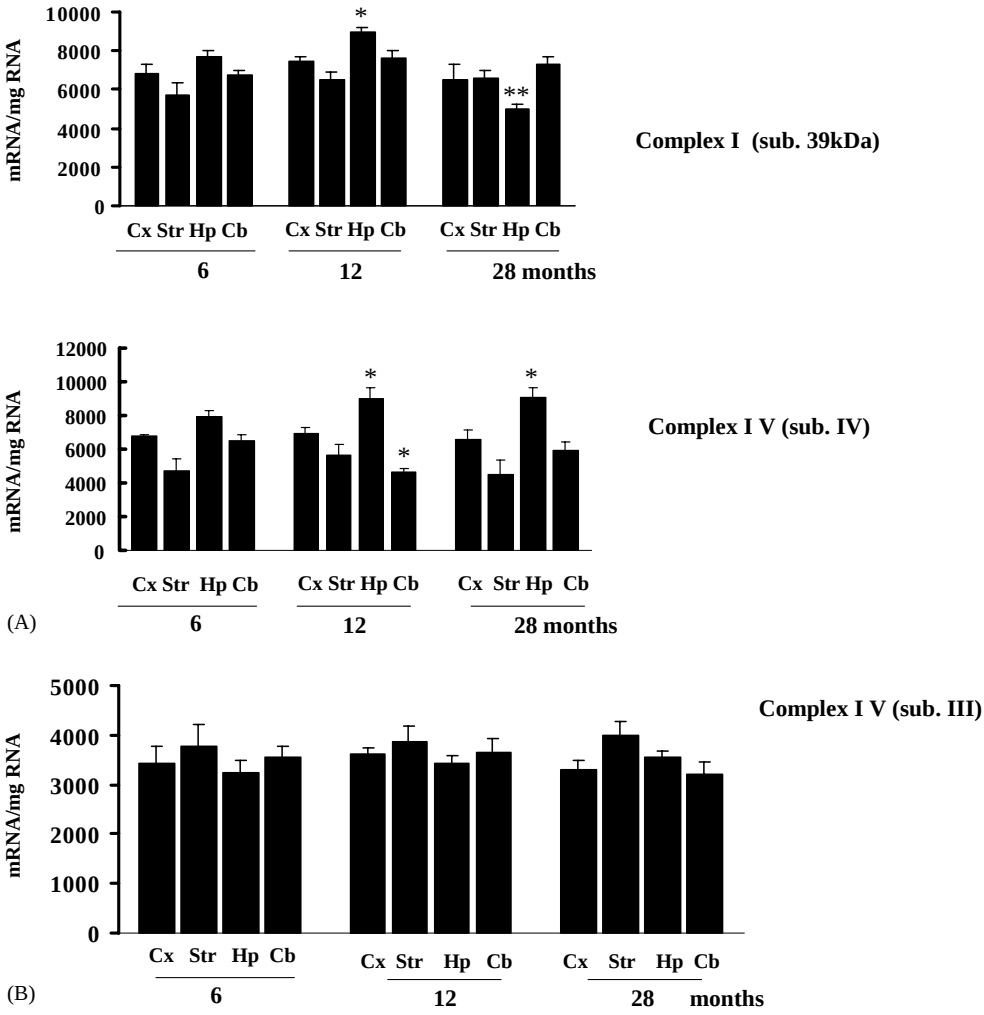

Fig. 7. Mitochondrial (A) Complex I (nuclear B13, 39 kDa subunit) and Complex IV (nuclear subunit IV); (B) Complex IV (mitochondrial subunit III) mRNA expressions examined in different brain regions as a function of brain aging. Total RNA from rat brain dissected areas was extracted as described in methods 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. *$P < 0.05$ vs. control; **$P < 0.05$ vs. 12 months.

increasing evidence indicates that the rate of neuronal aging and death greatly varies among cerebral structures, with some nuclei being particularly vulnerable and others very resistent to age-related influences. In view of the evidence that lipoperoxidation in the brain is an event showing regional variability, it has been suggested that the susceptibility to metabolic disfunctions and cellular damage of the different neuronal systems is, at least in part, dependent upon the basal rate of endogenous lipoperoxidation, which in the CNS presents regional specificity (Calabrese et al., 2002a; Calabrese and Fariello, 1988; Butterfield, 2002d).

In the present study we show that hydroxynonenal increase significantly in 28-months old animals compared to those aged 12 or 6 months. This was particularly significant in the hippocampus and substantia nigra, where incresed lipid-dependent protein oxidation was associated with a significant decrease in Complex I and Complex IV mitocondrial enzyme activities. Our data, consistently with our previous finding suggesting that induction of Hsps in the brain exhibit regional specificity, are relevant to mechanisms of cytoprotection and indicate that a different threshold might exist among various brain areas for the citoprotective heat shock response to endogenous oxidative stress (Calabrese et al., 2002a). Moreover, in the present study we provide experimental evidence that endogenous oxidative stress, by increasing as function of brain aging, affects with different severity the different areas of the brain. In 28 months old animals, higher hydroxynonenal levels were found in the S. nigra, hippocampus and septum than in the cortex, striatum and cerebellum. In the S. nigra, the high levels of HNE were positively correlated with Hsp expression and decrease of GSH. In addition, S. nigra exhibited a greater decrease of Complex I and Complex IV activities than the other regions examined. These data are consistent with the notion that S. nigra suffer more severe conditions of oxidative stress conditions than other regions of the brain.

As a dopamine-rich brain area, it undergoes to prooxidant actions of endogenous catechols, such as DOPA and dopamine, which are cytotoxic in vitro and in vivo (Spencer et al., 2002), presumably by formation of covalent bonds between their quinone forms and macromolecules of vital importance, primarily represented by thiol groups (Marshall et al., 1999). Nigral cells also contain neuromelanin, a pigmented substance related to lipofuscin and derived from dopamine. The synthesis of neuromelanins from dopamine is known to produce more oxidative damage than the synthesis from other catecholamines (Xin et al., 2000) and, in addition, neuromelanins polymerize from pheomelanin in a process that requires cysteine for synthesis, thus competing with γ-glutamyl cysteine synthetase which utilizes cysteine for GSH synthesis. Conceivably, all this could force the nigral GSH system towards a condition of increased demand and decreased synthetic capability, which can explain the particular vulnerability of this region to peroxidative injury which increases over the lifetime (Calabrese et al., 2002a).

Our findings may be relevant to theories connecting aging and neuronal degeneration with oxidative damage. S. nigra neurons are depleted during physiological aging and even more so in all neurodegenerative processes associated with Parkinsonian symptoms (McNaught et al., 2001). Furthermore, the septal region, a predominantly cholinergic structure, showed higher levels of peroxides, GSSG, inducible Hsp70 and iron than other brain regions examined and this is relevant to the finding that cholinergic neurons are primarily affected in primary degenerative dementia of Alzheimer's type (Smith et al., 1995). All these factors suggest that these areas are particularly vulnerable to endogenous as well as stress-induced enhancement of lipoperoxidation and that this intrinsically reduced capability to cope with perturbations of oxidant/antioxidant cellular balance may constitue the starting point for pathological consequences leading to neural degeneration and death.

Finally, the finding that protection by heat stress against myocardial dysfunction may be partially due to enhancement of mitochondrial energetics (Sammut et al., 2001; Li et al., 2002) pose intriguing implications on the importance of nutritional antioxidants as basal therapeutic intervention aimed at ensuring optimal efficiency of those mechanisms participating to induction of cytoprotective proteins (Hsps) or, more in general, involved in the cellular pathways of stress tolerance (Butterfield et al., 2002a; Scapagnini et al., 2002; Colombrita et al., 2003), to limit deleterious consequences of free radical-mediated reactions associated with aging and age related disorders.

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