Journal of Neuroscience Research 79:509–521 (2005)

Acetylcarnitine Induces Heme Oxygenase in Rat Astrocytes and Protects Against Oxidative Stress: Involvement of the Transcription Factor Nrf2

Vittorio Calabrese,[1]* Agrippino Ravagna,[1] Claudia Colombrita,[1] Giovanni Scapagnini,[1] Eleonora Guagliano,[1] Menotti Calvani,[2] D. Allan Butterfield,[3] and Anna Maria Giuffrida Stella[1]

[1]Department of Chemistry, Biochemistry and Molecular Biology Section, Faculty of Medicine, University of Catania, Catania, Italy
[2]Department of Physiology, University of Rome Tor Vergata, Rome, Italy
[3]Department of Chemistry, Center for Membrane Science, and Sander-Brown Center on Aging, University of Kentucky, Lexington, Kentucky

Efficient functioning of maintenance and repair processes seem to be crucial for both survival and physical quality of life. This is accomplished by a complex network of the so-called longevity assurance processes, under control of several genes termed *vitagenes*. These include members of the heat shock protein system, and there is now evidence that the heat shock response contributes to establishing a cytoprotective state in a wide variety of human conditions, including inflammation, neurodegenerative disorders, and aging. Among the various heat shock proteins, heme oxygenase-1 has received considerable attention; it has been recently demonstrated that heme oxygenase-1 induction, by generating the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin, could represent a protective system potentially active against brain oxidative injury. Acetyl-L-carnitine is proposed as a therapeutic agent for several neurodegenerative disorders. Accordingly, we report here that treatment of astrocytes with acetyl-L-carnitine induces heme oxygenase-1 in a dose- and time-dependent manner and that this effect was associated with up-regulation of heat shock protein 60 as well as high expression of the redox-sensitive transcription factor Nrf2 in the nuclear fraction of treated cells. In addition, we show that addition of acetyl-L-carnitine to astrocytes, prior to proinflammatory lipopolysaccharide- and interferon-γ-induced nitrosative stress, prevents changes in mitochondrial respiratory chain complex activity, protein nitrosation and antioxidant status induced by inflammatory cytokine insult. Given the broad cytoprotective properties of the heat shock response, molecules inducing this defense mechanism appear to be possible candidates for novel cytoprotective strategies. Particularly, manipulation of endogenous cellular defense mechanisms via acetyl-L-carnitine may represent an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration. We hypothesize that maintenance or recovery of the activity of vitagenes may delay the aging process and decrease the risk of age-related diseases.

Key words: heme oxygenase; Nrf2; mitochondrial dysfunction, oxidative stress; acetyl-L-carnitine; antioxidant defenses

The brain has a large potential oxidative capacity but a limited ability to combat oxidative stress (Calabrese et al., 2003a; Poon et al., 2004a, b). Under normal conditions, there is a steady-state balance between prooxidants and antioxidants necessary to ensure optimal efficiency of antioxidant defenses; however, when the rate of free radical generation exceeds the capacity of antioxidant defenses, oxidative stress ensues (Calabrese et al., 2001; Halliwell, 2002). To adapt to environmental changes and survive different types of injuries, brain cells have evolved networks of different responses that detect and control diverse forms of stress. One of these responses is the heat shock response. Heat shock proteins (Hsps) are proteins serving as molecular chaperones involved in the protection of cells from various forms of stress (Calabrese et al., 1998; Mosser and Morimoto, 2004). Among the various Hsps, Hsp32, also known as heme oxygenase-1 (HO-1), has received

*Correspondence to: Prof. Vittorio Calabrese, Department of Chemistry, Biochemistry Molecular Biology Section, Faculty of Medicine, University of Catania, Viale Andrea Doria, 6, 95100 Catania, Italy. E-mail: calabres@mbox.unict.it

Received 27 August 2004; Revised 25 October 2004; Accepted 27 October 2004

Published online 7 January 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20386

considerable attention; it has been recently demonstrated that HO-1 induction, by generating the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin, could represent a protective system potentially active against brain oxidative injury (Kravets et al., 2004; Schipper, 2004a).

Heme oxygenase is a stress protein that has been implicated in defense mechanisms against agents that may induce oxidative injury, such as endotoxins, cytokines, and heme (Foresti et al., 2003; Chen and Regan, 2004), and its induction represents a common feature in a number of neurodegenerative diseases (Maines, 2002; Poon et al., 2004a). Interestingly, the spatial distribution of HO-1 expression in diseased brain is essentially identical to that of pathological expression of tau (Takeda et al., 2000a). In Alzheimer's disease (AD) cortex and hippocampus, HO-1 has been shown to be overexpressed and to colocalize to senile plaques and neurofibrillary tangles (Schipper et al., 2000, 2004b). Successful transduction of the human HO-1 gene into neuroblastoma cells resulted in a stable increase of HO activity associated with a dramatic decrease in the level of tau protein. This result demonstrates that expression of tau protein and HO-1 may be regulated by oxidative stresses in a coordinated manner and play a pivotal role in the cytoprotection of neuronal cells (Takeda et al., 2000b). Similarly, up-regulation of HO-1 in the substantia nigra of Parkinson's disease subjects has been demonstrated. In these patients, nigral neurons containing cytoplasmic Lewy bodies exhibited in their proximity maximal HO-1 immunoreactivity (Schipper et al., 2000; Yoo et al., 2003). There is now evidence to suggest that the HO-1 gene is redox regulated and contains in its promoter region the antioxidant-responsive element (ARE), similar to other antioxidant enzymes (Alam and Cook, 2003; Balogun et al., 2003). The HO-1 gene is, in fact, modulated by redox-sensitive transcription factors that recognize specific binding sites within the promoter and distal enhancer regions of the HO-1 gene (Alam, 2002). These include Fos/Jun [activator protein-1 (AP-1)], nuclear factor-κB (NFκB), and the more recently identified Nrf2 proteins (Alam and Cook, 2003; Balogun et al., 2003). In addition, HO-1 is rapidly up-regulated by oxidative and nitrosative stresses as well as by glutathione depletion (Naughton et al., 2002; Shih et al., 2003; Calabrese et al., 2004a). Given the broad cytoprotective properties of the heat shock response, there is now strong interest in discovering and developing pharmacological agents capable of inducing the heat shock response (Calabrese et al., 2003a, b, Calabrese et al., 2004a–c).

Acetyl-L-carnitine (LAC) has been proposed as a therapeutic agent for several neurodegenerative disorders (Calabrese et al., 2003c; Beal, 2003). Acetyl-L-carnitine is normally synthesized in several organs, particularly in brain and liver, by the enzyme acetyl-L-carnitine transferase. The role of carnitine and its derivatives as an obligate cofactor in mitochondrial fatty acid β-oxidation is well known (Wutzke and Lorenz, 2004). In addition, the carnitine system is involved in membrane stabilization and repair, in the intracellular communication of acyl groups, upon which depend peroxisomal fatty acid oxidation, the metabolism of branched chain amino acids, or removal of disruptive acyl CoAs such as acylcarnitines (Calabrese and Rizza 1999a, b; Calabrese et al., 2004d). LAC has been proposed as a therapeutic agent for several disease paradigms (Pisano et al., 2003; Galeotti et al., 2004). LAC in fact was reported to prevent, in nonhuman primates, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurological injury to the substantia nigra as well as to increase cellular respiration, mitochondrial membrane potential, and cardiolipin levels in hepatocytes of 24-month-old rats (Beal, 2003). These biochemical effects are paralleled by increases in ambulatory activity of aged rats (Liu et al., 2002).

We have recently shown that both curcumin and caffeic acid phenethyl ester (CAPE), two phenolic natural compounds well known for their antioxidative, antiinflammatory and anticancer properties, potently induce HO-1 expression and activity in rat astrocytes (Scapagnini et al., 2002). Here we report results that extend our previous findings, examining in astrocytes the effects of acetyl-L-carnitine on the expression on heme oxygenase and other Hsps, such as Hsp60 as well as on the antioxidative potential of astrocytes exposed to lipopolysaccharide (LPS)- and interferon-γ (INFγ)-induced nitrosative stress.

MATERIALS AND METHODS

Chemicals

5,5′-Dithiobis-(2-nitrobenzoic acid; DTNB), 1,1,3,3-tetraethoxypropane, purified bovine blood superoxide dismutase (SOD), NADH, reduced glutathione (GSH), oxidized glutathione (GSSG), β-NADPH (type 1, tetrasodium salt), glutathione reductase (GR; type II from baker's yeast), N(G)-monomethyl-l-arginine [L-NMMA, a nonisoform-specific nitric oxide synthase (NOS) inhibitor], and glucose oxidase (GOX, which generates hydrogen peroxide in the culture medium) were from Sigma (St. Louis, MO). Zinc protoporphyrin IX (ZnPP IX), a specific inhibitor of HO activity, was from Porphyrin Product (Logan, UT). LAC (99.99% pure) was a generous gift from Sigma Tau Co. (Pomezia, Italy). All other chemicals were from Merck (Darmstadt, Germany) and were of the highest grade available.

Cell Cultures and Treatment

Rat type 1 astrocytes (DI TNC1) were purchased from the American Type Culture Collection (Manassas, VA) and cultured according to the manufacturer's instructions. Cells were grown in 75-cm^2 flasks and maintained at 37°C in a humidified atmosphere of air and 5% CO_2. Confluent cells were exposed to various concentrations of LAC and/or LPS and INFγ, in the absence or in the presence of L-NMMA, or to the effects of heat shock (HS). After each treatment, cells were harvested for the determination of HO activity, HO-1 mRNA levels, and HO-1 or Hsp60 protein expression. NOS in astrocyte cultures was induced by treatment for 12 hr with increasing concentrations of LPS (0.01 μg/ml, 0.1 μg/ml, or 1 μg/ml;

Sigma) and INFγ (1 U/ml, 10 U/ml, or 100 U/ml; Genzyme). The specificity of the effect was tested by the addition to the culture medium of the NOS inhibitor L-NMMA (Sigma), at a final concentration 1 mM, or of LAC, at a final concentration 50 μM. In addition, astrocyte cultures were treated with 1 μg/ml LPS or 100 U/ml INFγ separately or in combination of both for different times. For HS treatment, culture flasks were sealed with parafilm and immersed in a precision shaker bath maintained at 42°C ± 0.01°C for 30 min. Subsequently, the parafilm was removed and cells were returned to the incubator for 2 hr before harvesting. After treatments, the cells were washed with phosphate-buffered saline (PBS), scraped, and pelleted. The cellular pellet was resuspended in 0.32 M sucrose, 1 mM EDTA, 10 mM Tris (pH 7.4), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and homogenized.

Western Blot Analysis

Samples of astrocytes were analyzed for HO-1, Hsp60, inducible NOS (iNOS), and Nrf2 protein expression, as well as nitrotyrosine protein, by using a Western immunoblot technique as described previously (Calabrese et al., 2000b, 2002a). In brief, an equal amount of proteins (30 μg) for each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred overnight to nitrocellulose membranes, and the nonspecific binding of antibodies was blocked with 3% nonfat dried milk in PBS. Immunodetection of iNOS and protein nitrotyrosine were performed with a polyclonal rabbit anti-iNOS antibody (sc-649; Santa Cruz Biotechnology, Santa Cruz, CA) and a polyclonal rabbit anti-nitrotyrosine antibody (06-284; Upstate Biotechnology, Lake Placid, NY), respectively. When probed for HO-1 or Hsp60, membranes were incubated for 2 hr at room temperature with anti-HO-1 or anti-Hsp60 antibodies (Stressgen, Victoria, British Columbia, Canada; 1:1,000 dilution in Tris-buffered saline, pH 7.4), respectively, whereas Nrf2 transcription factor was immunodetected in the nuclear pellet by using anti-Nrf2 antibodies (sc-13032) purchased from Santa Cruz Biotechnology. Goat polyclonal antibody specific for β-actin was used as a loading control (1:1,000). Blots were then visualized with either an amplified alkaline phosphatase kit from Sigma (Extra 3A) when probing for HO-1, Hsp60, and Nrf2 or a horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulin G (IgG) in the case of iNOS and nitrotyrosine. Immunoreactive bands were scanned by a laser densitometer (LKB Ultroscan XL). Molecular weights of the proteins detected were determined by using a standard curve obtained with proteins of known molecular weight.

Preparation of Nuclear Extract and Western Blot for Nrf2

Astrocytes were washed twice with PBS, then harvested in 1 ml PBS and centrifuged at 3,000 rpm for 3 min at 4°C. The cell pellet was carefully resuspended in 200 μl cold buffer A, consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 μM dithiothreitol (DTT), and complete protease inhibitor cocktail (Roche, Mannheim, Germany). The pellet was then incubated on ice for 15 min to allow cells to swell. After this time, 15 μl of 10% NP-40 was added, and the tube was vortexed for 10 sec. The homogenate was then centrifuged at 3,000 rpm for 3 min at 4°C. The resulting nuclear pellet was resuspended in 30 μl cold buffer B consisting of 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 μM DTT, and protease inhibitors. The pellet was then incubated on ice for 15 min and vortexed for 10–15 sec every 2 min. The nuclear extract was finally centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant containing the nuclear proteins was loaded on an SDS-polyacrylamide gel, and Western blot analysis with Nrf2 antibodies (1:1,000 dilution) was performed as described above.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA from cell cultures was extracted with Trizol (Sigma) and treated with RNase-free DNase to remove any residual genomic DNA. Single-stranded cDNAs were synthesized by incubating total RNA (1 μg) with SuperScript II RNase H reverse transcriptase (RT; 200 U), oligo-(dT)12–18 primer (100 nM), dNTPs (1 mM), and RNase inhibitor (40 U) at 42°C for 1 hr in a final volume of 20 μl. The reaction was terminated by incubating at 70°C for 10 min. Forward and reverse primers used to amplify HO-1 were, respectively, HO-1 F: TGCTCGCATGAACACTCTG (GenBank accession No. NM 012580.1) and HO-1 R: TCCTCTGTCAGCAGTGCCT (GenBank accession No. NM 012580.1). The expected amplification product for HO-1 was 123 bp. Aliquots of cDNA (0.1 μg) and known amounts of external standard (purified PCR product, 102–108 copies) were amplified in parallel reactions with the forward and reverse primers. Each PCR (final volume, 20 μl) contained 0.5 μM of primers, 2.5 mM Mg^{2+}, and 1× Light Cycler DNA Master Sybr green (Roche Diagnostics, Indianapolis, IN). PCR amplifications were performed with a Light Cycler (Roche Molecular Biochemicals) by using the following four cycle programs: 1) denaturation of cDNA (one cycle: 95°C for 10 min); 2) amplification (40 cycles: 95°C for 0 sec, 58°C for 5 sec, 72°C for 10 sec); 3) melting curve analysis (one cycle: 95°C for 0 sec, 70°C for 10 sec, 95°C for 0 sec); 4) cooling (one cycle: 40°C for 3 min). The temperature transition rate was 20°C/sec except for the third segment of the melting curve analysis, where it was 0.2°C/sec. The fluorimeter gain value was 6. Real-time detection of fluorimetric intensity of Sybr green I, indicating the amount of PCR product formed, was measured at the end of each elongation phase. Quantification was performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards. For this analysis, fluorescence values measured in the log-linear phase of amplification were considered by using the second derivative maximum method of the Light Cycler Data Analysis software (Roche Molecular Biochemicals). The specificity of PCR products obtained was characterized by melting curve analysis, followed by gel electrophoresis, and visualized by ethidium bromide staining and DNA sequencing.

HO Activity Assay

HO activity was determined at the end of each treatment as described previously (Motterlini et al., 2000). In brief, microsomes from harvested cells were added to a reaction mixture containing NADPH, glucose-6-phosphate dehydrogenase, rat

liver cytosol as a source of biliverdin reductase, and the substrate hemin. The reaction mixture was incubated in the dark at 37°C for 1 hr and was terminated by the addition of 1 ml chloroform. After vigorous vortex mixing and centrifugation, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm ($\epsilon = 40\ mM^{-1}\ cm^{-1}$).

Mitochondrial Complex Activities

Cell homogenate was centrifuged at 1,000*g* for 10 min. The nuclear pellet was washed once, and the supernatants were centrifuged at 20,000*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 cytochrome c oxidase (complex IV), complex I, complexes II–III, and ATP synthase, the pellet was homogenized in 0.8 ml of 0.9% NaCl and sonicated for 10 sec 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) activity was determined according to the method of Warton and Tzagoloff (1967). NADH-CoQ_1 reductase (complex I; EC 1.6.99.3) and succinate-cytochrome c reductase (complex II–III; EC 1.8.3.1) activities were determined according to Schapira et al. (1990). ATP synthase (EC 3.6.1.34) was measured according to Buckle et al. (1986).

Protein SH Group Determination in Astrocyte Cultures

Protein SH groups were estimated by the method of Sedlak and Lindsey (1968). The content of SH groups was expressed in nanomoles per milligram of protein.

GSH and GSSG Assay

GSH and GSSG were measured by the NADPH-dependent GSSG reductase method as previously reported (Calabrese et al., 2000a). Samples of astrocytes were homogenized on ice for 10 sec 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 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 assay of GSSG, aliquots (0.5 ml) of homogenate were immediately added to 0.5 ml of a solution containing 10 mM N-ethylmaleimide (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 1 drop/sec 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. Aliquots (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. 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 nanomoles of GSH or GSSG, respectively, per milligram protein.

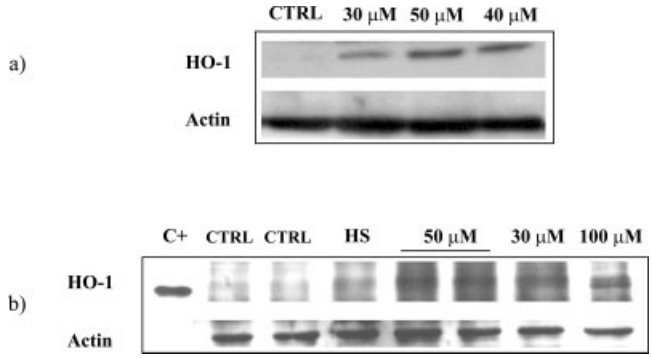

Fig. 1. **a:** Dose-dependent increase of HO-1 protein expression in astrocyte cultures after 6 hr of treatment with different concentrations of acetyl-L-carnitine (LAC). **b:** Increase in HO-1 protein expression after LAC treatment was compared with the effect of heat shock (HS).

Cell Viability Assay

Astrocytes were exposed to 50 mU/ml glucose oxidase (GOX) for 2 hr, in the absence or presence of 50 μM LAC or 10 μM ZnPP IX, and cell viability was assessed with the use of an Alamar blue assay according to the manufacturer's instructions (Serotec, Oxford, United Kingdom) as reported previously (Scapagnini et al., 2002). At the end of each treatment, cells were washed twice and incubated for an additional 4 hr in complete medium containing 10% Alamar blue solution. Optical density in each sample was measured by using a plate reader (Molecular Devices, Crawley, United Kingdom). The intensity of the color developed in the medium is proportional to the viability of cells, which is calculated as the difference in absorbance between 570 and 600 nm and expressed as percentage of control.

Determination of Protein

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

Statistical Analysis

Results were expressed as mean ± SEM of eight separate experiments (n = 8), each of which was performed, unless otherwise specified, in triplicate. Data were analyzed by one-way ANOVA, followed by inspection of all differences by Duncan's new multiple-range test. Differences were considered significant at $P < 0.05$.

RESULTS

A concentration-dependent increased expression of HO-1 was found in astrocytes exposed to LAC for 6 hr

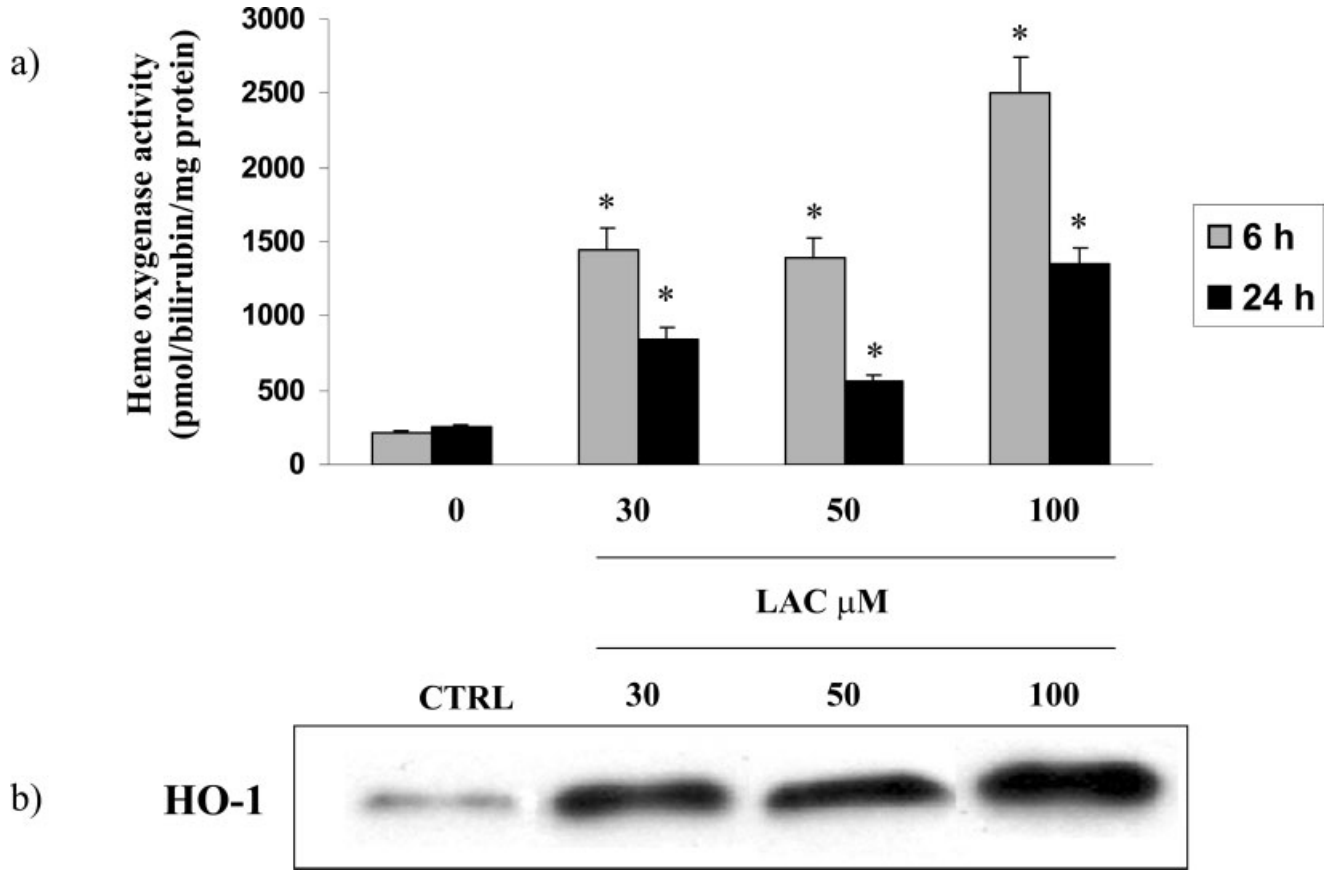

Fig. 2. Time course of HO-1 protein expression and activity in astrocyte cultures after treatment with LAC. Astrocytes were incubated with 30, 50, and 100 μM LAC for 6 and 24 hr, and activity was measured at the end of each treatment, as described in Materials and Methods (**a**). Values are means ± SEM of two to four determinations, each obtained from eight different cultures. *Significant vs. control ($P < 0.01$). **b:** Blots are shown that are representative of eight independent experiments on astrocytes treated for 6 hr with LAC at various concentrations.

(Fig. 1a). Expression of HO-1 induced by different concentrations of LAC was higher than that observed after HS (Fig. 1b). Consistently with increased protein, a concentration-dependent increased HO-1 activity was found in astrocytes exposed to LAC. Exposure of astrocytes to LAC for 6 hr led to greater activity than exposure of LAC for 24 hr (Fig. 2a). The increased protein expression of HO-1 in astrocytes exposed to LAC (a representative Western blot is additionally shown in Fig. 2b) was accompanied by increased message expression (Fig. 3a,b). The results shown in Figures 1–3 demonstrate that, even in the absence of other stimuli, exposure of astrocytes to LAC is sufficient to increase expression of HO-1 at the mRNA and protein levels and sufficient to increase activity of HO-1.

To determine whether this increased level and activity of HO-1 would be protective against neurotoxic stimuli, astrocytes were treated for 12 hr with LPS/INFγ (1 μg/ml and 100 U/ml), which is known to produce iNOS (Calabrese et al., 2000b). Figure 4 shows that LPS, INFγ, or the combination of LPS and INFγ increased iNOS protein expression significantly compared with control or LAC alone. However, addition of LAC, 1 hr prior to exposure of astrocytes to oxidative challenge with LPS and INFγ, markedly reduced iNOS protein expression, which is consistent with a protective response.

The mitochondrial-resident heat shock protein Hsp60 is known to be neuroprotective in brain aging and age-related neurodegenerative disorders (Poon et al., 2004a, b; Calabrese et al., 2004c). Treatment of astrocytes for 12 hr with LAC alone led to a dose-dependent increased expression of neuroprotective Hsp60 (Fig. 5a). Figure 5 shows that addition of LPS and INFγ resulted in a significant down-regulation of this Hsp member compared with control or LAC treatment. Notably, exposure of astrocytes to HS up-regulated Hsp60 (Fig. 5b). However, this effect was lost when HS was followed by treatment with LPS/INFγ. Treatment of astrocytes with LAC, added 1 hr prior to addition of LPS/INFγ, was able to prevent the decrease in Hsp60 protein promoted by LPS/

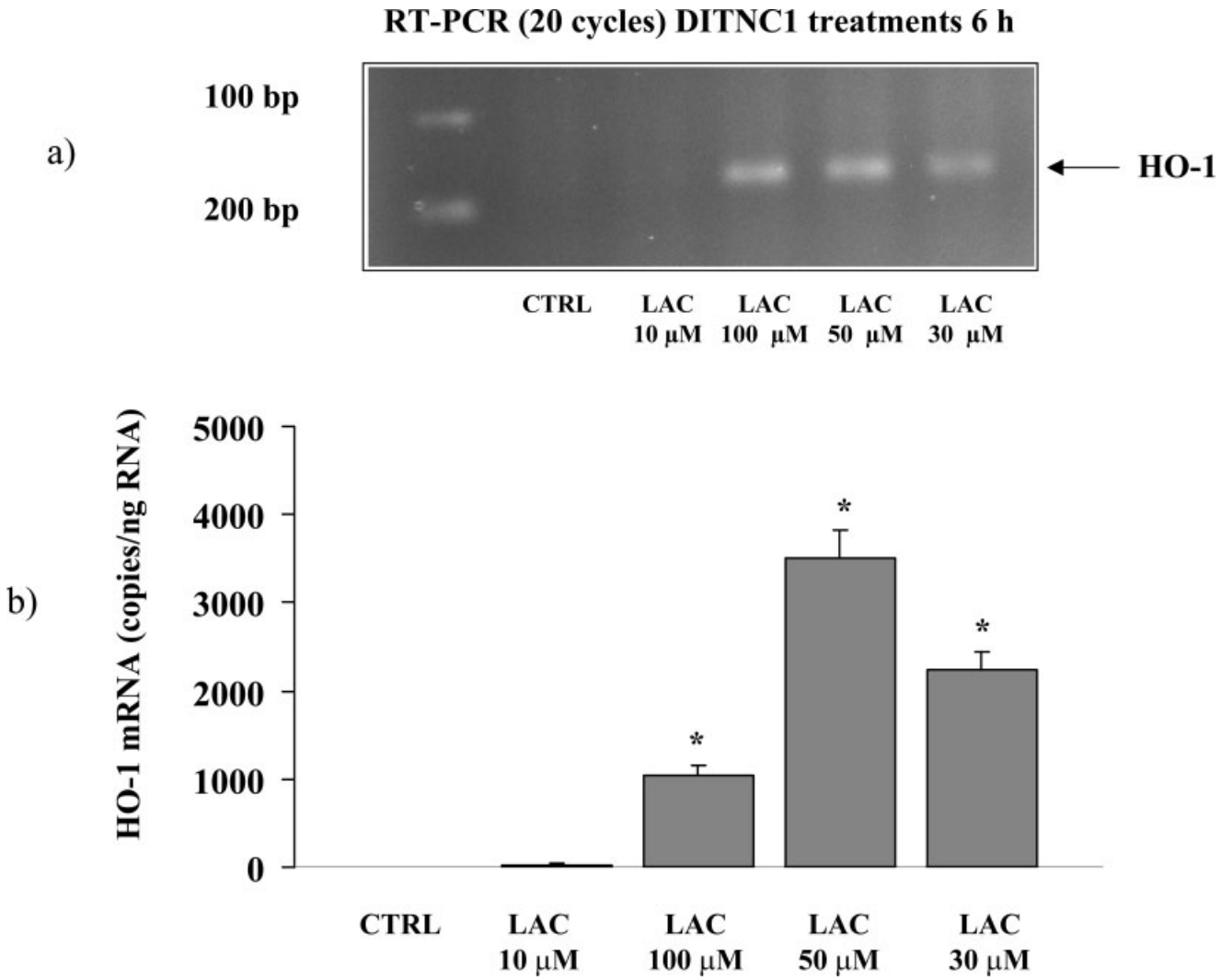

Fig. 3. Effect of LAC on HO-1 mRNA expression. **a:** Astrocytes were incubated with 0, 30, 50, and 100 μM LAC for 6 hr, and HO-1 mRNA expression was examined by quantitative RT-PCR. Forward (FP) and reverse (RP) primers used to amplify HO-1 are reported in Materials and Methods. PCR amplifications were performed with a Light-Cycler (Roche) as reported in the text. **b:** Quantification of results; n = 8; $*P < 0.05$.

INFγ treatment, whereas LAC alone did induce a significant increment in Hsp60 protein measured after 12 hr (Fig. 5b).

According to our previous studies (Calabrese et al., 2000b, 2001), neurotoxicity elicited by excess of reactive nitrogen species may be mediated by mitochondrial dysfunction. Therefore, we analyzed the activity of respiratory chain enzymes in astrocyte culture subjected to LPS/INFγ treatment in the absence and presence of LAC. Table I shows that the enzymatic activity of cytochrome c oxidase decreased drastically and in a dose-dependent manner after LPS/INFγ treatment. Addition of L-NMMA (a nonisoform-specific NOS inhibitor) to the culture medium abolished this effect, whereas addition of LAC produced a significant restoration of the cytochrome oxidase activity. Unlike the activity of complex IV, the activities of complex I, complex II–III, and ATP synthase were unmodified either by LPS/INFγ alone or by LPS/INFγ plus LAC.

Cytotoxicity of astrocytes was also induced by oxidative damage initiated by the action of glucose oxidase (Fig. 6). However, cotreatment of astrocytes with 50 μM LAC and glucose oxidase was significantly protective. Inhibition of the activity of HO-1 by ZnPP IX abrogated the protective effect of LAC, demonstrating that HO-1 was involved in the LAC-mediated cytoprotection against the oxidative damage caused by glucose oxidase. Consistently with the results in Figure 4, LPS/INFγ together or LPS alone caused increased production of 3-nitrotyrosine (Fig. 7), a marker of nitrosative stress (Butterfield and

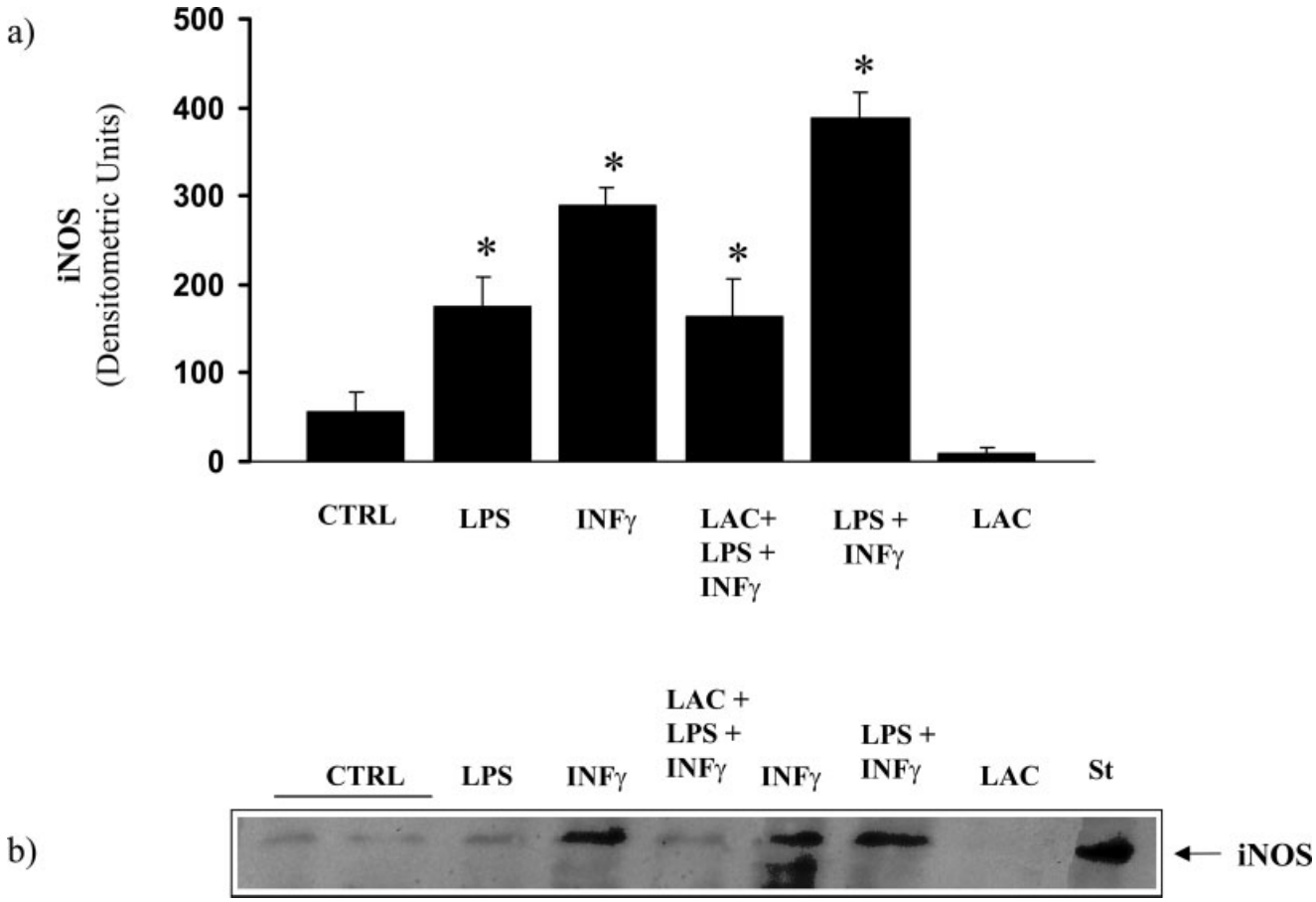

Fig. 4. Effect of LAC on LPS/INFγ-induced expression of iNOS in astrocytes. Cells were incubated with 1 μg/ml LPS, 100 U/ml INFγ, alone or in combination for 12 hr, in the absence and presence of 50 μM LAC, and protein expression was examined by Western blot analysis. Thirty micrograms of protein extract were loaded onto 8% SDS-PAGE gels, and the blot was probed with the polyclonal anti-iNOS antibody (1:500) for 1 hr. The iNOS protein was visualized by chemiluminescence. Values in **a**, expressed as arbitrary densitometric units, are the mean ± SEM of four determinations, each obtained from eight different culture wells. **b:** Representative Western blot of iNOS; $^{*}P < 0.05$.

Stadtman, 1997; Castegna et al., 2003). However, significantly depressed levels of 3-nitrotyrosine were found when LAC was added as well.

Thiols, particularly GSH, serve an important role in maintenance of redox balance of cells (Butterfield et al., 2002a; Calabrese et al., 2004a, c). Astrocytes exposed to LPS/INFγ had, 6 hr post-treatment, a concentration-dependent loss of protein thiol content and GSH levels and a concomitant increase in GSSG and total GSSG released in the medium (Table II). However, LAC and NMMA treatment of astrocytes significantly restored levels of all these thiol markers to nearly control concentrations in astrocytes exposed to the inflammatory agents LPS/INFγ.

The transcription factor Nrf2 is activated when the GSH/GSSH ratio decreases below a critical threshold (Motterlini, 2003; Alam, 2004). Nrf2 binds to the ARE of DNA, leading to the transcription of cytoprotective genes, including HO-1 and Hsp60 (Calabrese et al., 2004c). In keeping with the notion that pharmacologically derived elevation of cytoprotective enzymes is possible (Calabrese et al., 2004b, c; Poon et al., 2004a, b), Figure 8 shows that, at 6 hr after addition of LAC to astrocytes, the time for significant expression of HO-1 at the message and protein levels (Figs 2, 3), the expression of Nrf2 is maximal. Thus, not only does Nrf2 respond to a decreased redox status of the cell (Naughton, 2002), but Figure 8 shows that LAC alone is able to induce expression of Nrf2 as well.

DISCUSSION

Mitochondrial dysfunction is characteristic of several neurodegenerative disorders, and evidence for mitochondria being a site of damage in neurodegenerative disorders is partially based on decreases in respiratory chain complex activities in Parkinson's disease, Alzheimer's disease, and Huntington's disease (Beal, 2003; Calabrese et al., 2004a–c). Such defects in respiratory complex activities, possibly associated with oxidant/antioxidant balance per-

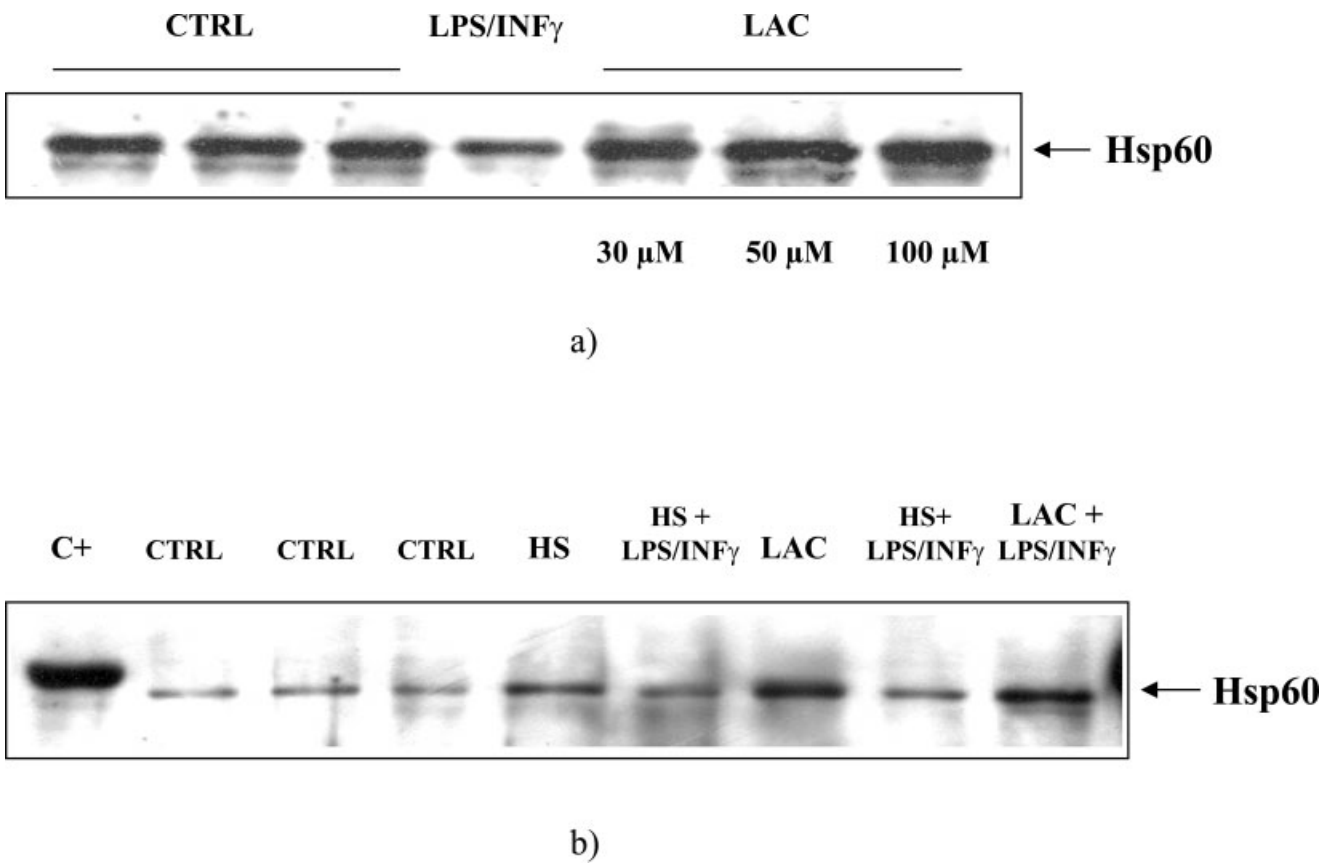

Fig. 5. **a:** Effects of LAC (50 μM) or LPS/INFγ (LPS 1 μg/ml plus INFγ 100 U/ml) on expression of Hsp60 in astrocytes. **b:** Hsp60 expression was examined after heat shock and compared with the effect of LPS/INFγ alone or in combination with 50 μM LAC. Representative Western blots are shown.

TABLE I. Effect of LPS/INFγ and LAC Treatments on the Specific Activity of Complex I, Complex II–III, Complex IV, and ATP Synthase†

	NADH -CoQ_1 reductase (complex I) (nmol/min/mg prot)	Succinate-cyt. C reductase (complex II–III) (nmol/min/mg prot)	Cytochrome c oxidase (complex IV) (K/min/mg prot)	ATP synthase (nmol/min/mg prot)
Control	68.6 ± 3.6	14.2 ± 2.6	3.42 ± 0.32	310 ± 50
LPS (0.1 μg/ml) + INFγ (10 U/ml)	62.2 ± 4.53	13.6 ± 1.5	2.6 ± 0.3★	303 ± 61
LPS (1.0 μg/ml) + INFγ (100 U/ml)	60.2 ± 2.2	12.9 ± 1.4	1.03 ± 0.2★	272 ± 55
LPS (1.0 μg/ml) + INFγ (100 U/ml) + NMMA (1 mM)	60.1 ± 6.7	13.8 ± 1.4	2.93 ± 0.4★★	320 ± 23
LPS (1.0 μg/ml) + INFγ (100 U/ml) + LAC 50 μM	64.0 ± 3.3	13.0 ± 2.8	2.78 ± 0.35★★	298 ± 19

†Results are mean ± SEM of eight different experiments each performed in triplicate. Astrocytes were treated with LPS/INFγ in the absence and presence of LAC for 12 hr and then mitochondrial complex activities were analyzed as described in Materials and Methods.
★Significant vs. control ($P < 0.05$).
★★Significant vs. LPS/INFγ alone ($P < 0.05$).

turbation, are thought to underlie defects in energy metabolism and to induce cellular degeneration. Evidence that mitochondrial dysfunction may be a mechanism for NO-mediated neurotoxicity arises from different studies that indicate excessive production of NO, a free radical that has several important messenger functions within the CNS, leads to formation of peroxynitrite anion ($ONOO^-$) by reacting with the superoxide anion. This extremely potent oxidizing agent can interact at the binuclear centre of cytochrome oxidase, leading to inhibition of respiratory

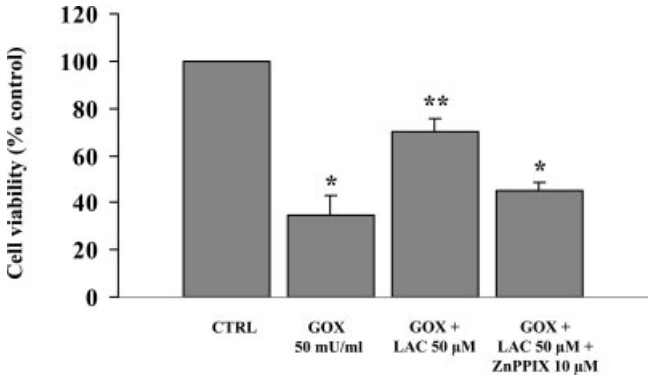

Fig. 6. Protective effect of LAC against oxidative damage induced by glucose oxidase. Astrocytes were incubated with LAC for 24 hr in the presence or absence of 10 μM of zinc protoporphyrin IX (ZnPP IX), a specific inhibitor of heme oxygenase enzyme activity. After these pretreatments, cells were incubated for 2 hr with 50 mU/ml glucose oxidase (GOX) to induce oxidative stress, and then the cells were washed and viability was assessed by Alamar blue assay. ⋆$P < 0.05$ vs. control; ⋆⋆$P < 0.05$ vs. GOX alone.

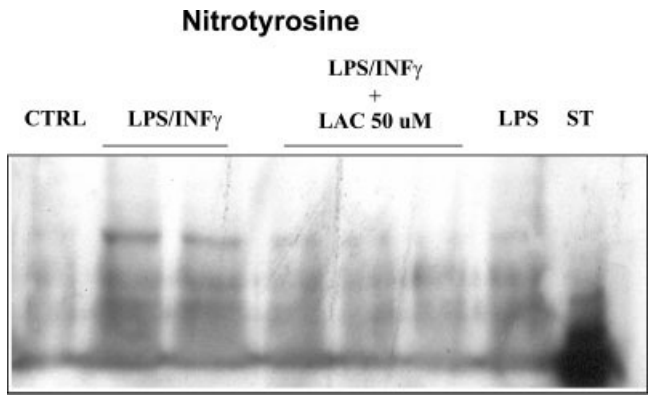

Fig. 7. Typical Western blot for protein nitrotyrosine in astrocytes exposed to nitrosative stress induced wih LPS/INFγ (LPS 1 μg/ml plus INFγ 100 U/ml) in the absence and presence of LAC. LAC was added at the concentration of 50 μM 1 hr prior to treatment. St, protein nitrotyrosine standards of various molecular weights. Blot shows protein nitrotyrosine immunoreactivity over a molecular weight range of approximately 50–215 kD.

rate and ATP stores (Calabrese et al., 2001). NO· can also stimulate the S-nitrosylation of numerous proteins to modify these proteins and also binds to the nonheme iron of ribonucleotide reductase to inhibit DNA synthesis (Gegg et al., 2002).

Efficient functioning of maintenance and repair process seems to be crucial for both survival and physical quality of life. This is accomplished by a complex network of the so-called longevity assurance processes, which are composed of several genes termed *vitagenes* (Calabrese et al., 2004b; Poon et al., 2004a). Among these, chaperones are highly conserved proteins responsible for the preservation and repair of the correct conformation of cellular macromolecules, such as proteins, RNAs, and DNA. Hsps and molecular chaperones have been known to protect cells against a wide variety of toxic conditions, including extreme temperatures, oxidative stress, virus infection, and exposure to heavy metals or cytotoxic drugs (Calabrese et al., 1998, 2000b; Poon et al., 2004b). Chaperone-buffered silent mutations may be activated during the aging process and lead to the phenotypic exposure of previously hidden features and contribute to the onset of polygenic diseases, such as age-related disorders, atherosclerosis, and cancer (Soti and Csermely, 2003). Hence, Hsp induction not only is a signal for detection of physiological stress but is utilized by the cells in the repair process following a wide range of injuries, to prevent damage resulting from the accumulation of nonnative proteins (Kelly and Yenari, 2002).

The involvement of the HO pathway in antidegenerative mechanisms, expecially those operating in AD, has been demonstrated: The expression of HO is closely related to that of amyloid precursor protein (APP; Dore, 2002; Ghanbari et al., 2004). HO induction, which occurs together with the induction of other Hsps during various physiopathological conditions, by generating the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin, represents a protective system potentially active against brain oxidative injury (Calabrese et al., 2003a). The HO-1 gene is redox regulated; this is supported by the fact that HO-1 gene has an HS consensus sequence as well as AP1, AP2, and NFκB binding sites in its promoter region. In addition, HO-1 is rapidly up-

TABLE II. Effect of LPS/INFγ Treatment on the Content of Protein SH Groups, GSH, and GSSG in Astrocytes[†]

	Protein SH groups (nmol/mg prot)	GSH (nmol/mg prot)	GSSG (nmol/mg prot)	Total GSSG released in the medium (nmol)
Control	78.48 ± 5.5	19.2 ± 3.3	0.266 ± 0.03	0.340 ± 0.05
LPS (0.1 μg/ml) + INFγ (10 U/ml)	63.6 ± 7.5	14.4 ± 5.5	0.302 ± 0.06	0.396 ± 0.03
LPS (1.0 μg/ml) + INFγ (100 U/ml)	38.4 ± 5.5⋆	11.4 ± 2.2⋆	0.588 ± 0.06⋆	0.704 ± 0.07⋆
LPS (1.0 μg/ml) + INFγ (100 U/ml) + NMMA (1 mM)	56.8 ± 8.8	14.7 ± 2.5	0.344 ± 0.05	0.401 ± 0.03
LPS (1.0 μg/ml) + INFγ (100 U/ml) + LAC 50 μM	68.2 ± 8.1	14.4 ± 2.7	0.303 ± 0.05	0.387 ± 0.04

[†]Results are mean ± SEM of six different experiments each performed in triplicate. Astrocytes were treated with LPS/INFγ in the absence and presence of LAC for 6 hr and then sulfhydryl groups were analyzed as described in Materials and Methods.
⋆Significant vs. control ($P < 0.01$).

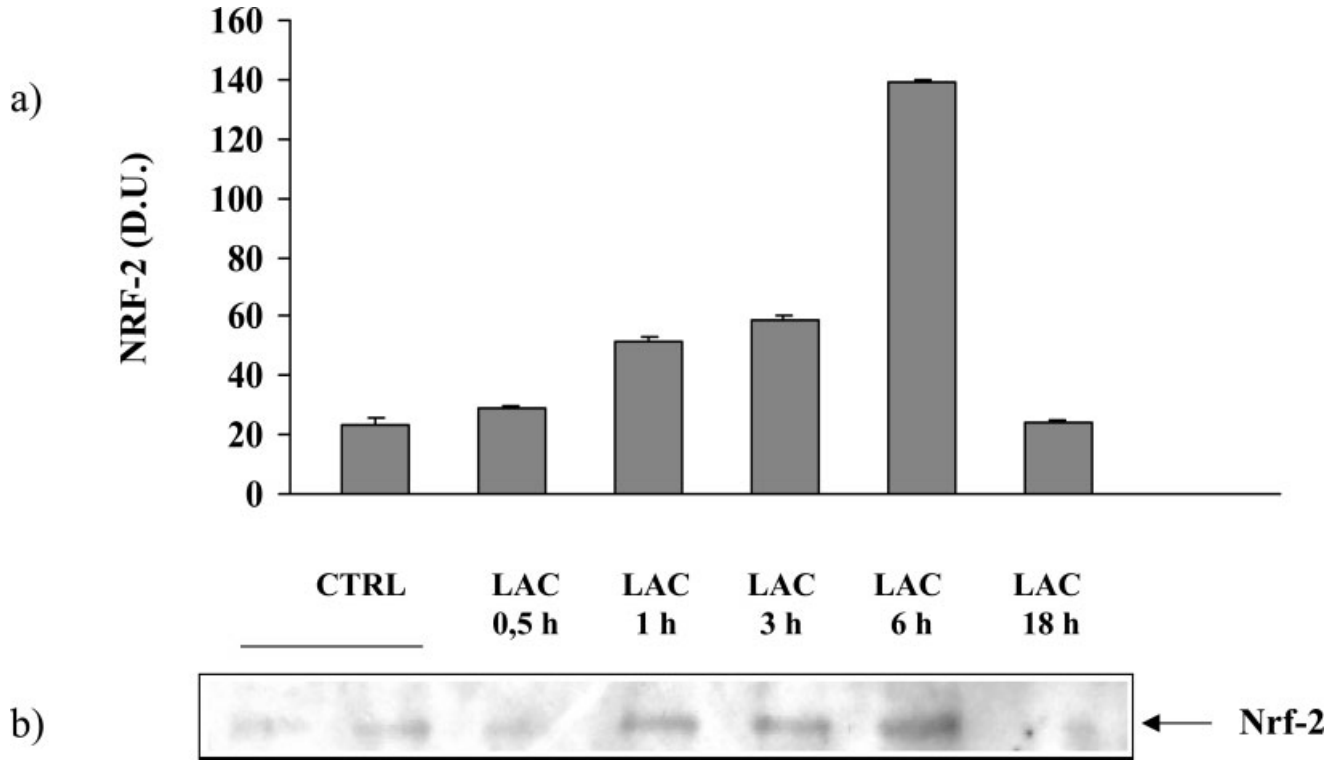

Fig. 8. **a,b:** Time course of expression of Nrf-2 transcription factor in DITNC1 astrocytes treated with LAC. Cells were incubated with 50 μM LAC for different times, and protein expression was examined by Western blot analysis.

regulated by oxidative and nitrosative stresses as well as by glutathione depletion.

All this evidence emphasizes the well-established concept of cellular stress response to oxidative insults (Calabrese et al., 2004a, b), as a crucial mechanism operating against neurodegenerative damage (Poon et al., 2004c). However, relatively new is the notion that pharmacological or nutritional intervention can lead to the same cytoprotective cellular responses (for review see Butterfield et al., 2002b, c; Calabrese et al., 2004c). LAC has been proposed as a therapeutic agent for several neurodegenerative disorders as well as an agent protective in numerous disease paradigms (Beal, 2003; Pisano et al., 2003); however, the mechanism of protection in brain disorders remains elusive. In the current study, we show that LAC is cytoprotective against inflammatory and oxidative insults in astrocytes in part by being able to up-regulate cytoprotective cellular stress responses, particularly induction of HO-1 and Hsp60, while inhibiting induction of NOS. LAC, an ester of L-carnitine, is normally synthesized in several organs, particularly in brain and liver, by the enzyme acetyl-L-carnitine transferase. There is now evidence to suggest that the carnitine system is involved in membrane stabilization and repair processes and in the metabolism of disruptive acyl CoAs such as acylcarnitines (Calabrese and Rizza, 1999a, b; Calabrese et al., 2004d). Carnitine and LAC attenuate neuronal damage produced by 3-nitroproprionic acid, rotenone, and MPTP (Beal, 2003; Loots et al., 2004). Moreover, LAC induced, after ischemia-reperfusion in rats, a more rapid recovery of ATP, PCr, and lactate levels (Aureli et al., 1994; Virmani et al., 1995). The results from our study show for the first time that LAC treatment of astrocytes induces HO-1 and Hsp60. Hsp60 is encoded in the nucleus and resides mainly in the mitochondria (Calabrese et al., 2002b). Hsp60 form the chaperonin complex, which is implicated in protein folding and assembly within the mitochondria under normal conditions (Izaki et al., 2001). Most mitochondrial proteins are synthesized in the cytosol and must be imported into the organelles in an unfolded state (Izaki et al., 2001). During translocation, the proteins interact with Hsp70. ATP-dependent binding and release of Hsp70 provide the major driving force for complete transport of polypeptides into the matrix. Most imported polypeptides are released from soluble Hsp70; however, a subset of aggregation-sensitive polypeptides must be transferred from Hsp70 to Hsp60 for folding (Okubo et al., 2000). Because of the close functional interaction between this chaperonin system and the Hsp70 system, it is likely that up-regulation of Hsp60 is a fundamental site targeted by LAC action, with consequent restoration of complex IV function. Accordingly, under conditions of nitrosative stress, accumulation of covalently modified proteins occurs, which compete with the heat shock factor (HSF).

This transcription factor in unstressed cells is maintained in a monomeric, non-DNA binding form through interaction with constitutive Hsp70 (Morimoto and Santoro, 1998). Upon stress and dissociation from Hsp70, HSF assembles into a trimer, which in the nucleus binds with specific heat shock sequence elements (HSE) in HS gene promoters, and hence induces mRNA transcription. Although this is speculative, it is conceivable that acetylcarnitine alone, in unstressed conditions, by promoting acetylation of DNA-binding proteins, can elicit similar effects.

We also provide experimental evidence that up-regulation of HO-1 (and Hsp60) might involve the transcription factor Nrf2, which was highly expressed in the nuclear fraction of cells exposed to this test compound. The transcription factor Nrf2 is a member of the cap'n'collar family of basic leucine transcription factors and plays an essential role in the ARE-mediated expression of phase II detoxifying enzymes and stress-inducible genes. The activity of Nrf2 is normally suppressed in the cytosol by specific binding to the chaperone Keap1 (Martin et al., 2004; Nguyen et al., 2004). However, upon stimulation by electrophilic agents or compounds that possess the ability to modify thiol groups (Li et al., 2004; Kobayashi et al., 2004), Keap 1 repression of Nrf2 is lost, allowing Nrf2 protein to translocate into the nucleus and potentiate the ARE response. This mechanism of gene activation leads to the synthesis of highly specialized proteins that efficiently protect mammalian cells from various forms of stress and, consequently, reduce the susceptibility of target tissues to oxidative damage. Among others, inducible proteins that require transcription via Nrf2 activation include γ-glutamylcysteine synthetase (Shih et al., 2003), glutathione S-transferase (Balogun et al., 2003), NADP(H):quinone oxidoreductase, and HO-1 (Alam, 2002). The HO-1 gene is, in fact, redox regulated depending on the presence in the promoter region of two upstream enhancers, E1 and E2 (Naughton, 2002). Both enhancer regions contain multiple stress (or antioxidant)-responsive elements (StRE; also called ARE) that conform to the sequence of the Maf-recognition element (MARE) and are recognized by Nrf2 (Martin et al., 2004). The direct implication of Nrf2 in the regulation of the HO-1 gene via the ARE has been recently reported with the use of Nrf2 dominant negative mutants (Nrf2M). In fibroblasts overexpressing Nrf2M, HO-1 mRNA was almost completely suppressed in response to arsenite, cadmium, and hemin, a known group of HO-1 inducers. That this mechanism might underly the associated increase in Hsp60 observed in our study is a conceivable possibility that deserves further elucidation. In the present study, we also demonstrate that a cytokine-dependent mitochondrial complex IV dysfunction occurs together with significant changes in the thiol pool, both of which are partially restored after treatment with LAC. Particularly, the restored GSH content may result from the Nrf2-dependent activation of γ-glutamylcysteine synthetase gene, which leads to up-regulation of glutathione synthesis. In addition, up-regulation of mitochondrial Hsp60, as discussed above, and down-regulation of iNOS might both contribute to an increased resistance of mitochondrial respiratory chain complexes to nitrosative insult.

Collectively, the data presented in this paper are consistent with the activation of protective genes, such as HO and, conceivably, other Hsps (i.e., Hsp60), in response to activation of Nrf2/ARE complex and sustain the emerging notion that the pharmacological activity attributed to several well-known or newly discovered drugs could rely on their intrinsic ability to activate the HO system (Schillinger et al., 2004). This highly inducible system, therefore, should be seriously considered as a target for novel therapeutic interventions focussing on the capability that compounds such as antioxidant polyphenols or acetyl-L-carnitine have to up-regulate the vitagene system as a mean to limit deleterious consequences of oxidative and nitrosative stress associated with aging and age-related disorders.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the Wellcome Trust and MIUR FIRB RBNE01ZK8F and by grants from the National Institute of Health [AG-10836, AG-05119]. The authors acknowledge helpful discussions with Dr. Roberto Motterlini (Northwick Park Institute for Medical Research, Harrow, United Kingdom) and Dr. Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA).

REFERENCES

Alam J. 2002. Heme oxygenase-1: past, present, and future. Antiox Redox Signal 4:559–562.

Alam J, Cook JL. 2003. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 9:2499–2511.

Aureli T, Miccheli A, Di Cocco ME. 1994. Effect of acetyl-L-carnitine on recovery of brain phosphorus metabolites and lactic acid level during reperfusion after cerebral ischemia in the rat—study by ^{13}P- and ^{1}H-NMR spectroscopy. Brain Res 643:92–99.

Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, Alam J, Motterlini R. 2003. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. Biochem J 371:887–895.

Beal MF. 2003. Bioenergetic approaches for neuroprotection in Parkinson's disease. Ann Neurol 53:S39–S47.

Buckle M, Guerrieri F, Pazienza A, Papa S. 1986. Studies on polypeptide composition, hydrolitic activity and proton conduction of mitochondrial F_oF_1 H^+ ATPase in regenerating rat liver. Eur J Biochem 155:439–455.

Butterfield DA, Stadtman ER. 1997. Protein oxidation processes in brain aging. Adv Cell Aging Gerontol 2:161–191.

Butterfield DA, Pocernich CB, Drake J. 2002a. Elevated gutathione as a therapeutic strategy in Alzheimer's disease. Drug Disc Res 56:428–437.

Butterfield DA, Castegna A, Pocernich CB, Drake J, Scapagnini G, Calabrese V. 2002b. Nutritional approaches to combat oxidative stress in Alzheimer's disease. J Nutr Biochem 13:444–461.

Butterfield DA, Castegna A, Drake J, Scapagnini G, Calabrese V. 2002c. Vitamin E and neurodegenerative disorders associated with oxidative stress. Nutr Neurosci 5:229–239.

Calabrese V, Rizza V. 1999a. Effects of l-carnitine on the formation of fatty acid ethyl esters in brain and peripheral organs after short-term ethanol administration in rat. Neurochem Res 24:79–84.

Calabrese V, Rizza V. 1999b. Formation of propionate after short-term ethanol treatment and its interaction with the carnitine pool in rat. Alcohol 19:169–176.

Calabrese V, Renis M, Calderone A, Russo A, Reale S, Barcellona ML, Rizza V. 1998. Stress proteins and SH-groups in oxidant-induced cell injury after chronic ethanol administration in rat. Free Rad Biol Med 24:1159–1167.

Calabrese V, Testa D, Ravagna A, Bates TE, Giuffrida Stella AM. 2000a. Hsp70 induction in the brain following ethanol administration in the rat: regulation by glutathione redox state. Biochem Biophys Res Commun 269:397–400.

Calabrese V, Copani A, Testa D, Ravagna A, Spadaro F, Tendi E, Nicoletti V, Giuffrida Stella AM. 2000b. Nitric oxide synthase induction in astroglial cell cultures: Effect on heat shock protein 70 synthesis and oxidant/antioxidant balance. J Neurosci Res 60:613–622.

Calabrese V, Scapagnini G, Giuffrida Stella AM, Bates TE, Clark JB. 2001. Mitochondrial involvement in brain function and dysfunction: relevance to aging, neurodegenerative disorders and longevity. Neurochem Res 26:739–764.

Calabrese V, Scapagnini G, Ravagna A, Fariello RG, Giuffrida Stella AM, Abraham N. 2002a. Regional distribution of heme oxygenase, hsp70, and glutathione in brain: relevance for endogenous oxidant/antioxidant balance and stress tolerance. J Neurosci Res 67:612–623.

Calabrese V, Scapagnini G, Ravagna A, Giuffrida Stella AM, Butterfield DA. 2002b. Molecular chaperones and their roles in neural cell differentiation. Dev Neurosci 24:1–13.

Calabrese V, Butterfield DA, Giuffrida Stella AM. 2003a. Nutritional antioxidants and the heme oxygenase pathway of stress tolerance: novel targets for neuroprotection in Alzheimer's disease. Ital J Biochem 52:177–181.

Calabrese V, Scapagnini G, Colombrita C, Ravagna A, Pennisi G, Giuffrida Stella AM, Galli F, and Butterfield DA. 2003b. Redox regulation of heat shock protein expression in aging and neurodegenerative disorders associated with oxidative stress: a nutritional approach. Amino Acids 27:15–23.

Calabrese V, Scapagnini G, Ravagna A, Bella R, Butterfield DA, Calvani M, Pennisi G, Giuffrida Stella AM. 2003c. Disruption of thiol homeostasis and nitrosative stress in the cerebrospinal fluid of patients with active multiple sclerosis: evidence for a protective role of acetylcarnitine. Neurochem Res 28:1321–1328.

Calabrese V, Scapagnini G, Ravagna A, Colombrita C, Spadaro F, Butterfield DA, Giuffrida Stella AM. 2004a. Increased expression of heat shock proteins in rat brain during aging: relationship with mitochondrial function and glutathione redox state. Mech Aging Dev 125:325–335.

Calabrese V, Boyd-Kimball D, Scapagnini G, Butterfield DA. 2004b. Nitric oxide and cellular stress response in brain aging and neurodegenerative disorders: the role of vitagenes. In Vivo 18:245–268.

Calabrese V, Giuffrida Stella AM, Butterfield DA, Scapagnini G. 2004c. Redox regulation in neurodegeneration and longevity: role of the heme oxygenase and Hsp70 systems in brain stress tolerance. Antiox Redox Signal 5:895–913.

Calabrese V, Calvani M, Butterfield DA. 2004d. Increased formation of short-chain organic acids after chronic ethanol administration and their interaction with the carnitine pool in rat. Arch Biochem Biophys 431: 271-278.

Castegna A, Throngboonkerd V, Klein JB, Lynn B, Markesbery WR, Butterfield DA. 2003. Proteomic identification of nitrated proteins in Alzheimer's disease brain. J Neurochem 85:1394–1401.

Chen J, Regan RF. 2004. Heme oxygenase-2 gene deletion increases astrocyte vulnerability to hemin. Biochem Biophys Res Commun 318: 88–94.

Dore S. 2002. Decreased activity of the antioxidant heme oxygenase enzyme: implications in ischemia and in Alzheimer's disease. Free Rad Biol Med 32:1276–1282.

Foresti R, Hoque M, Bains S, Green CJ, Motterlini R. 2003. Haem and nitric oxide: synergism in the modulation of the endothrlial haem oxygenase pathway. Biochem J 372:381–390.

Galeotti N, Bartolini A, Calvani M, Nicolai R, Ghelardini C. 2004. Acetyl-L-carnitine requires phospholipase C-IP3 pathway activation to induce antinociception. Neuropharmacology 47:286–294.

Gegg ME, Beltran B, Salas-Pino S, Bolanos JP, Clark JB, Moncada S, Heales SJ. 2002. Differential effect of nitric oxide on glutathione metabolism and mitochondrial function in astrocytes and neurones: implications for neuroprotection/neurodegeneration? J Neurochem 86:228–237.

Ghanbari HA, Ghanbari K, Harris PL, Jones PK, Kubat Z, Castellani RJ, Wolozin BL, Smith MA, Perry G. 2004. Oxidative damage in cultured human olfactory neurons from Alzheimer's disease patients. Aging Cell 3:41–44.

Halliwell B. 2002. Hypothesis: proteasomal dysfunction: a primary event in neurogeneration that leads to nitrative and oxidative stress and subsequent cell death. Ann N Y Acad Sci 962:182–194.

Izaki K, Kinouchi H, Watanabe K, Owada Y, Okubo A, Itoh H, Kondo H, Tashima Y, Tamura S, Yoshimoto T, Mizoi K. 2001. Induction of mitochondrial heat shock protein 60 and 10 mRNAs following transient focal cerebral ischemia in the rat. Brain Res Mol Brain Res 88:14–25.

Kelly S, Yenari MA. 2002. Neuroprotection: heat shock proteins. Curr Med Res Opin 18:55–60.

Kobayashi A, Kang MI, Okawa H, Ohtsuji9 M, Zenke Y, Chiba T, Igarashi K, Yamamoto M. 2004. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol 24:7130–7139.

Kravets A, Hu Z, Miralem T, Torno MD, Maines MD. 2004. Biliverdin reductase, a novel regulator for induction of activating transcription factor-2 and heme oxygenase-1. J Biol Chem 279:19916–19923.

Li N, Alam J, Venkatesan MI, Eiguren-Fernandez A, Schmitz D, Di Stefano E, Slaughter N, Killeen E, Wang X, Huang A, Wang M, Miguel AH, Cho A, Sioutas C, Nel AE. 2004. Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals. J Immunol 173:3467–3481.

Liu J, Head E, Gharib AM, Yuan W, Ingersoll RT, Hagen TM, Cotman CW, Ames BN. 2002. Memory loss in old rats is associated with brain mitochondrial decay and RNA/DNA oxidation: partial reversal by feeding acetyl-L-carnitine and/or R-alpha-lipoic acid. Proc Natl Acad Sci U S A 99:7184–7185.

Loots du T, Mienie LJ, Bergh JJ, Van der Schyf CJ. 2004. Acetyl-L-carnitine prevents total body hydroxyl free radical and uric acid production induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the rat. Life Sci 75:1243–1253.

Maines MD. 2002. Heme oxygenase transgenic mice as a model to study neuroprotection. Methods Enzymol 353:374–388.

Martin D, Rojo AI, Salinas M, Diaz R, Gallardo G, Alam J, De Galarreta CM, Cuadrado A. 2004. Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. J Biol Chem 279:8919–8929.

Morimoto RI, Santoro MG. 1998. Stress-inducible response and heat shock proteins: new pharmacologic targets for cytoprotection. Nat Biotechnol 16:833–838.

Mosser DD, Morimoto RI. 2004. Molecular chaperones and the stress of oncogenesis. Oncogene 23:2907–2918.

Motterlini R, Foresti R, Bassi R, Calabrese V, Clark JE, Green CJ. 2000. Endothelial heme oxygenase-1 induction by hypoxia: modulation by inducible nitric oxide synthase (iNOS) and S-nitrosothiols. J Biol Chem 275:13613–13620.

Naughton P, Hoque M, Green CJ, Foresti R, Motterlini R. 2002. Interaction of heme with nitroxyl or nitric oxide amplifies heme oxygenase-1 induction: involvement of the transcription factor Nrf2. Cell Mol Biol 48:885–894.

Nguyen T, Yang CS, Pickett CB. 2004. The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. Free Rad Biol Med 37:433–441.

Okubo A, Kinouchi H, Owada Y, Kunizuka H, Itoh H, Izaki K, Kondo H, Tashima Y, Yoshimoto T, Mizoi K. 2000. Simultaneous induction of mitochondrial heat shock protein mRNAs in rat forebrain ischemia. Brain Res Mol Brain Res 84:127–134.

Pisano C, Pratesi G, Laccabue D, Zunino F, Lo Giudice P, Bellucci A, Pacifici L, Camerini B, Vesci L, Castorina M, Cicuzza S, Tredici G, Marmiroli P, Nicolini G, Galbiati S, Calvani M, Carminati P, Cavaletti G. 2003. Paclitaxel and cisplatin-induced neurotoxicity: a protective role of acetyl-carnitine. Clin Cancer Res 9:5756–5767.

Poon HF, Calabrese V, Scapagnini G, Butterfield DA. 2004a. Free radicals: key to brain aging and heme oxygenase as a cellular stress response to oxidative stress. J Gerontol 59(Part A):478–493.

Poon HF, Calabrese V, Scapagnini G, Butterfield DA. 2004b. Free radicals and brain aging. Clin Geriatr Med 20:329–359.

Poon HF, Joshi G, Sultana R, Farr SA, Banks WA, Morley JE, Calabrese V, Butterfield DA. 2004c. Antisense directed at the Abeta region of APP decreases brain oxidative markers in aged senescence accelerated mice. Brain Res 1018: 86–96.

Scapagnini G, Foresti R, Calabrese V, Giuffirida Stella AM, Green CJ, Motterlini R. 2002. Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. Mol Pharmacol 61:554–561.

Schapira AHV, Mann VM, Cooper JM, Dexter D, Daniel SE, Jenner P, Clark JB, Marsden CD. 1990. Anatomic and disease specificity of NADH CoQ_1 reductase (complex I) deficiency in Parkinson's disease. J Neurochem 55:2142–2145.

Schillinger M, Exner M, Minar E, Mlekusch W, Mullner M, Mannhalter C, Bach FH, Wagner O. 2004. Heme oxygenase-1 genotype and restenosis after ballon angioplasty: a novel vascular protective factor. J Am Coll Cardiol 43:950–957.

Schipper HM. 2000. Heme oxygenase-1: role in brain aging and neurodegeneration. Exp Gerontol 35:821–830.

Schipper HM. 2004a. Brain iron deposition and the free radical-mitochondrial theory of ageing. Ageing Res Rev 3:265–301.

Schipper HM. 2004b. Heme oxygenase-1: transducer of pathological brain iron sequestration under oxidative stress. Ann N Y Acad Sci 1012:84–93.

Sedlak J, Lindsey RH. 1968. Estimation of total protein-bound and nonprotein sulfhydryl groups in tissues with Ellman's reagent. Anal Biochem 25:192–205.

Shih AY, Johnson DA, Wong G, Kraft AD, Jiang L, Erb H, Johnson JA, Murphy TH. 2003. Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J Neurosci 23:3394–3406.

Smith P, Krohn R, Hermanson G, Mallia A, Gartner F, Provenzano M, Fujimoto E, Goeke N, Olson B, Klenk D. 1985. Measurement of proteins using bicinchoninic acid. Anal Biochem 150:76–85.

Soti C, Csermely P. 2003. Aging and molecular chaperones. Exp Gerontol 38:1037–1040.

Takeda A, Smith MA, Avila J, Nunomura A, Siedlak SL, Zhu X, Perry G, Sayre LM. 2000a. In Alzheimer's disease, heme oxygenase is coincident with Alz50, an epitope of tau induced by 4-hydroxy-2-nonenal modification. J Neurochem 75:1234–1241.

Takeda A, Perry G, Abraham NG, Dwyer BE, Kutty RK, Laitinen JT, Petersen RB, Smith RB. 2000b. Overexpression of heme oxygenase in neuronal cells, the possible interaction with tau. J Biol Chem 275:5395–5399.

Virmani MA, Biselli R, Spadoni A. 1995. Protective actions of L-carnitine and acetyl-L-carnitine on the neurotoxicity evoked by mitochondrial uncoupling or inhibitors. Pharmacol Res 32:383–389.

Warton DC, Tzagoloff A. 1967. Cytochrome oxidase from bovine heart mitochondria. Methods Enzymol 10:245–257.

Wutzke KD, Lorenz H. 2004. The effect of L-carnitine on fat oxidation, protein turnover, and body composition in slightly overweight subjects. Metabolism 53:1002–1006.

Yoo MS, Chun HS, Son JJ, DeGiorgio LA, Kin DJ, Peng C, Son JH. 2003. Oxidative stress regulated genes in nigral dopaminergic neuronal cells: correlation with the known pathology in Parkinson's disease. Brain Res Mol Brain Res 110:76–84.

Zhang Y, Gordon GB. 2004. A strategy for cancer prevention: stimulation of the Nrf2-ARE signaling pathway. Mol Cancer Ther 3:885–893.