Journal of Thermal Biology 36 (2011) 49–56

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Heat-induced inhibition of superoxide dismutase and accumulation of reactive oxygen species leads to HT-22 neuronal cell death ☆

Naglaa F. El-Orabi [1], Colin B. Rogers, Heather Gray Edwards, Dean D. Schwartz *

Department of Anatomy, Physiology and Pharmacology, Auburn University College of Veterinary Medicine, Auburn, AL 36849, USA

ARTICLE INFO

Article history:
Received 10 June 2010
Accepted 1 November 2010

Keywords:
Superoxide dismutase
Heat stress
Reactive oxygen species
NADPH oxidase
HT-22 cells

ABSTRACT

Superoxide dismutase (SOD) is major cytosolic antioxidant enzyme responsible for dismutation of superoxide anion ($O_2^{\bullet -}$). Alterations in SOD expression and activity are associated with various neurological disorders. In the present study, we utilized neuronal HT-22 cells to investigate heat-stressed induced cytotoxicity. Heat stress at 43 °C for 30 min caused a decrease in SOD-1 mRNA levels, cytoplasmic SOD protein and enzyme activity and a corresponding decline in cell number during a 48 h recovery at 37°C. During the recovery phase, there was an increase in reactive oxygen species generation and an increase in NADPH oxidase activity with a corresponding increase in DNA fragmentation and release of cytochrome c from the mitochondria. The increase in ROS accumulation and cell death was abolished by pretreatment with the SOD mimetics EUK-134 and Mn(III)TBAP and the NADPH oxidase inhibitor apocynin. These data suggest that hyperthermia increases ROS generation by increasing NADPH activity and decreasing SOD activity leading to cytotoxicity in HT-22 cells.

1. Introduction

Oxidative stress is a condition characterized by an elevation in the cellular steady-state concentration of reactive oxygen species (ROS) due to an imbalance between the production of ROS and the destruction by antioxidant enzymes. ROS such as superoxide and hydrogen peroxide are produced at low levels during normal cellular metabolism (Boveris and Chance, 1973) and levels are kept low by the enzymatic activities of endogenous antioxidants such as superoxide dismutase, catalase and glutathione peroxidase (McCord and Fridovich, 1969). Manipulations that increase ROS generation or decrease the antioxidant capacity of cells lead to a state of oxidative stress. Accumulated ROS can react with lipids, proteins and DNA leading to lipid peroxidation, alterations in protein structure and enzymatic activities and DNA damage. The damage imparted by reaction with ROS has been reported to initiate an apoptotic program of cell death or necrosis (Galli et al., 2005b; Zhao et al., 2006).

Hyperthermia has been reported to enhance numerous forms of ROS including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($^{\bullet}NO$) and peroxynitrite ($OONO^-$) in various cell types (Zhao et al., 2006; Arnaud et al., 2002; Katschinski et al., 2000). This accumulation of ROS plays a pivotal role in heat-induced neuronal cell degeneration (Sreedhar et al., 2002) and apoptosis (Katschinski et al., 2000; Sugawara et al., 1999). The source of ROS generation in response to heat stress has traditionally been attributed to mitochondrial generation, but recent evidence suggests that other ROS generating systems, such as NADPH oxidase (NOX), may be involved (Starkie et al., 2005; Wartenberg et al., 2005; Zhang et al., 2003; Zuo et al., 2000). The superoxide radical has also been implicated in a broad range of human pathologic conditions including diabetes, cancer, inflammatory diseases, atherosclerosis, pulmonary fibrosis, diseases of ischemia and reperfusion injuries, neurodegenerative diseases and aging (McCord and Edeas, 2005; Thannickal and Fanburg, 2000).

Superoxide dismutases (SODs) are the front-line intracellular defense against ROS, particularly superoxide anion radicals (McCord and Edeas, 2005; Fridovich, 1997; Noor et al., 2002; Fattman et al., 2003). SODs are metalloenzymes that have been isolated and characterized from a wide variety of organisms. SODs have been reported to be expressed in the brain (Colombrita et al., 2003; Peluffo et al., 2005) and altered expression and/or mutation of SODs are associated with several degenerative neurological disorders including amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD) and Down's syndrome (Noor et al., 2002; Colombrita et al., 2003). The activity and the expression of brain SOD are also altered in response to many stressful conditions including hyperthermia. Total brain SOD was decreased in a heat stroke model in rats (Yang and Lin, 2002) whereas CuZn-SOD expressed at high levels in immature neurons is rapidly down

☆Grants: This work was supported in part by Grants from the Office of Naval Research (NAVY-N00014-03-10293) and Animal Health and Disease Research, Auburn University, AL.

* Corresponding author. Tel.: +1 334 844 5406; fax +1 334 844 5388.
E-mail address: schwadd@auburn.edu (D.D. Schwartz).
[1] Present Address: Department of Pharmacology and Toxicology, Faculty of Pharmacy, Suez Canal University, Egypt.

0306-4565/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jtherbio.2010.11.002

regulated in the hippocampus following excitotoxic insult (Peluffo et al., 2005). On the other hand, preconditioning of cortical neuronal cultures at 42.5 °C for 1 h increased SOD-1 protein levels (Meloni et al., 2005) whereas human neuronal SK-N-MC cells exposed to tetrachlorobiphenyl showed decrease SOD activity (Lee et al., 2005).

In a preliminary study, we observed a decrease in the gene expression of SOD-1 in the hippocampus of adult pigs that underwent a mild heat stress for 12 h (El-Orabi et al., 2005). In the present study, we extend these observations and report that a decrease in the activity and expression of SOD is responsible for the heat-induced increase in reactive oxygen species and cell death in the HT-22 cell line. The HT-22 cell line is an immortalized hippocampal cell line that has been used in a number of studies to study oxidative glutamate toxicity (Lewerenz et al., 2006; Xu et al., 2007).

2. Experimental procedures

2.1. Cell culture

A mouse hippocampal cell line, HT-22 cells, (gift from Dr. David Schubert, Salk Institute for Biological Studies, La Jolla, CA), was maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin G and 0.25 μg/ml amphotericin B and grown at 37 °C in 95% O_2/5% CO_2. Growth media was changed every other day. Cells were plated in 10 cm dishes and experiments were performed at 50–60% confluence.

2.2. Hyperthermic conditions

Hyperthermia was induced by incubation of HT-22 cells in either a 37 °C (control) or 43 °C (heat-stressed) water bath for 30 min. Cells were then allowed to recover in a cell culture incubator under normal growth conditions at 37 °C for 0–48 h. In experiments measuring SOD gene and protein expression and activity, medium was then aspirated and cells washed in ice-cold PBS before further analysis. This was performed to assure the removal of all dead cells floated in the culturing medium, or partially attached to the dish bottom.

2.3. SOD gene expression

Total RNA was isolated from both control and heated cells at various times using the RNeasy Plus Mini kit (Qiagen Inc., CA, USA). In each case, medium was aspirated from tissue culture dishes and cells were washed 2 × in ice-cold PBS. RNA was isolated following the manufacturer's protocol for total RNA isolation from animal cells. RNA quality and quantity were assessed using formaldehyde/agarose gel electrophoresis and UV spectrophotometry. All RNA samples were stored at −80 °C until gene expression analysis. First strand cDNA was synthesized using 1 μg total RNA and reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad laboratories, CA, USA).

2.4. Real-time RT-PCR

Real-time PCR was carried out using an iCycler My iQ Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad). Real-time PCR reactions were carried out in a total volume of 25 μl containing: 12.5 μl iQ SYBR Green Supermix, 11 μl nuclease-free H_2O, 1 μl of 5 μM sense and antisense primer mix and 0.5 μl cDNA template. The thermal cycling conditions were set at 95 °C for 3 min, 45 repeats of two-step cycle; 10 s at 95 °C and 45 s at 65 °C. Melt curve analysis was carried out for each PCR analysis. Two housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and acidic ribosomal phosphoprotein (Arbp) were used as internal controls. The following oligonucleotide primers were used: NAD(P)H quinone oxidoreductase 1 (NQO1, Accession # NM_017000) 5′-CCT TGT ATT GGT TTG GGG TG (sense) and 5′-GCA TAC GTG TAG GCG AAT CCT GCT (antisense); Heme oxygenase-1 (HO1, Accession # NM_012580) 5′-CAG GTG TCC AGG GAA GGC TTT AAG C (sense) and 5′-TTT CGC TCT ATC TCC TCT TCC AGG G (antisense); catalase (Accession # NM_ 012520) 5′-ACC CAG CCA GCG ACC AGA TGA A (sense) and 5′-CAC GAG GTC CCA GTT ACC ATC TTC A; SOD-1 (Accession # NM_000454) 5′-ACA GCA GGC TGT ACC AGT GCA GGT CCT (sense) and 5′-CAT TGC CCA AGT CTC CAA CAT GCC TCT (antisense); GAPDH (accession number AF141959) 5′-TGC ACC ACC AAC TGT TAG C (sense) and 5′-GGC ATG GAC TGT GGT CAT GAG (antisense); Arbp (Accession # NM_007475), 5′-AAG CGC GTC CTG GCA TTG TCT (sense) and 5′-CCG CAG GGG GCA GCA GTG GT (antisense). The change in gene expression in heat stressed HT-22 cells was calculated relative to the gene expression in non-heated control samples according to the modified ΔΔCt method (Vandesompele et al., 2002).

2.5. SOD protein expression

SOD protein expression was determined by Western blotting in whole cell lysates. To prepare cell lysates, HT-22 cells were washed twice in phosphate-buffered saline, scraped and pelleted at 2000*g* for 5 min at 4 °C. Cells were solubilized in 200 μl cell lysis buffer containing: Tris–HCl, (50 mM pH 7.5) NaCl (100 mM), EDTA (2 mM), 1% Nonidet P-40, $NaVO_4$ (100 μM), NaF (100 μM) and 5 μl/ml protease inhibitor cocktail (Sigma, MI). The lysate was then centrifuged at 14,000*g* for 10 min at 4 °C. Supernatant protein concentration was determined using the DC protein assay kit (Bio-Rad) using BSA as the standard and all samples were normalized per microgram total protein.

Equivalent amounts of cell lysate total protein (15 μg) were boiled in an equal volume of Laemmli sample buffer containing 5% β-mercaptoethanol for 5 min. Cell lysates were separated by SDS-PAGE on 12% Tris–HCl precast gels (Bio-Rad) and transferred to nitrocellulose membranes (Amersham Biosciences, UK). Membranes were blocked for 1 h at room temperature with TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl and 0.1% Tween 20) containing 5% non-fat dry milk. Membranes were then incubated with a rabbit anti-Cu/Zn SOD polyclonal antibody (1:1000 dilution in blocking buffer, Stressgen Bioreagents, Victoria, Canada) overnight at 4 °C. Beta tubulin expression was determined as a background control and was detected using a rabbit anti-tubulin polyclonal antibody. Membranes were washed 3 × 5 min with TBS-T at room temperature and then exposed to a goat anti-rabbit IgG HRP-coupled antibody (1:3000 dilution in blocking buffer) for 1 h at room temperature. Membranes were washed 5 × 5 min with TBS-T and immunoreactive bands were detected by the ECL Plus Western Blotting Detection System (Amersham Biosciences, UK). Blots were scanned with a Flour-S Multi Imaging System (Bio-Rad) to determine the density of the resulting bands and analyzed with Quantity One software.

2.6. Measurement of SOD enzyme activity

A superoxide dismutase assay kit (Cayman Chemicals, Ann Arbor, Michigan) was used to measure SOD activity (Peskin and Winterbourn, 2000). Cells from two 10-cm plates were washed, scraped and collected in ice-cold PBS at 2000g, for 10 min at 4 °C. Pellets were homogenized in 0.1 ml HEPES buffer (20 mM, pH 7.5) containing mannitol (210 mM), sucrose (70 mM) and EDTA (1 mM)

with a syringe and a narrow-gauge (No. 27) needle. The homogenate was centrifuged at 1500*g* for 5 min at 4 °C. The resulting supernatant was then centrifuged twice at 10,000*g* for 15 min at 4 °C and the supernatant was collected as the cytosolic fraction. The protein concentration was adjusted to 2 μg/ml using HEPES buffer. SOD activity was carried out following the manufacturer instructions and color change was measured in 96-well plates at 450 nm using a Fusion-Alpha Universal microplate analyzer (Perkin Elmer Inc, Boston, MA). SOD standard curves were performed using serial dilutions of the SOD standard stock solution (0.25–0.05 U/ml). Assays were performed in duplicate and the average absorbance of each standard and test sample was calculated.

2.7. Cytotoxicity studies

The effect of hyperthermia on cell number was estimated using the CyQUANT Cell Proliferation Assay Kit (Molecular Probes Inc, Eugene, OR). HT-22 cells were heat stressed as described above. At the end of each time point, cells were trypsinized, suspended in culture media and collected by centrifugation for 5 min at 2000*g*. The supernatants were removed and the cell pellets were frozen at −80 °C. Frozen cells were thawed and lysed by addition of 200 μl 1 × cell lysis buffer containing the CyQUANT GR dye. The entire 200 μl corresponding to each sample was transferred to a 96-well microplate and fluorescence was measured directly at Ex/Em 480/520 nm using a Fusion-Alpha Universal microplate analyzer (Beckman Coulter). A calibration standard curve was created for converting the observed fluorescence values into cell numbers (Jones et al., 2001) using known concentrations of HT-22 cells.

2.8. Intracellular reactive oxygen species (ROS):

Intracellular ROS levels were determined by fluorescence microscopy using the Image-iT Live Green ROS detection kit (Molecular Probes Inc., Eugene, OR) using the fluorescent probe 6-carboxy-2′,7′-dichlorodihydroflourescein diacetate (carboxy-H_2DCFDA) (Bass et al., 1983). HT-22 cells were cultured on glass coverslips and heated as described above. Cells were washed with warm HBSS with Ca^{+2} and Mg^{+2} and labeled with 25 μM carboxy-H_2DCFDA in HBSS for 30 min at 37 °C in the dark and with 1 μM Hoeschst 33342 stain for the last 5 min. Cells were then washed three times with warm HBSS, mounted on glass slides and visualized using a Nikon Eclipse E 800 Fluorescence microscope.

2.9. NADPH oxidase activity

NADPH-dependent superoxide production was measured using lucigenin-enhanced chemiluminescence (Li et al., 1999). HT-22 cells were pretreated for 1 h with apocynin (300 μM; NADPH oxidase inhibitor) or its vehicle and then incubated in a water bath at either 37 °C (control) or 43 °C for 30 min followed by 15 min at 37 °C in a cell culture incubator. The cells were washed once with 5 ml PBS and dislodged using 1 ml of trypsin (0.25%) for 5 min at 37 °C. Cells were washed twice in a Krebs–Hepes buffer (KHB; containing 115 mM NaCl, 47 mM KCl, 19 mM $CaCl_2$, 12 mM $MgSO_4$, 1 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, 20 mM HEPES and 0.1% BSA, pH 7.4) and resuspended in 500 μl KHB and divided into two aliquots containing 200 μl each for duplicate determination of NADPH oxidase activity. Aliquots of cell suspensions were added to KHB containing 5 μM lucigenin (Sigma-Aldrich; St. Louis, MO) in luminometer cuvettes and luminescence was determined for 5 min in a TD-20/20 Luminometer (Turner Designs) to determine background luminescence. To determine stimulated activity, NADPH (100 μM final concentration) was added and luminescence measured again for 5 min. NADPH oxidase activity was expressed as relative light units (RLUs) in the presence of NADPH minus that in the absence of NADPH. Cell number was counted for each sample and NADPH activity was expressed as RLUs/2×10^5 cells.

2.10. Cytoplasmic cytochrome c detection

Cytochrome c was detected in the cytoplasm of HT-22 cells using western blotting. HT-22 cells were incubated in a water bath at 37 °C or 43 °C for 30 min and allowed to recover at 37 °C in a cell culture incubator for 12 h. Adherent and floating cells were collected and centrifuged at 500*g* for 5 min at 4 °C, washed with ice-cold PBS and resuspended in 100 μl mitochondria isolation buffer (320 mM sucrose, 1 mM potassium-EDTA, 10 mM Tris–HCl, (pH 7.4) 1 mM phenylmethanesulfonylfluoride (PMSF), 1 μg/ml leupeptin, 20 μg/ml pepstatin, 30 μg/ml aprotinin). Cells were triturated with a syringe and a narrow-gauge (No. 27) needle. The homogenate was centrifuged at 500*g* for 15 min at 4 °C. The resulting supernatant was centrifuged at 17,000*g* for 15 min at 4 °C, and the supernatant was collected as the cytosol fraction. The pellet was resuspended in isolation buffer and centrifuged again at 17,000*g* for 15 min at 4 °C. The supernatant was collected and the cytosol fractions combined. The resultant pellet was incubated in 50 μl of RIPA lysis buffer supplemented with 1 mM PMSF for 15 min on ice and then centrifuges at 13,000*g* for 4 min and the supernatant collected as the mitochondrial fraction. Sun and Leaman (2005). Equal amounts of protein (15 μg/lane) were analyzed using SDS-PAGE as described above with a mouse anti-cytochrome c monoclonal antibody (1:500 dilution, Stressgen) and a goat anti-mouse IgG HRP-coupled antibody (1:3000 dilution in blocking buffer). Purified cytochrome c protein was used as a positive control and β-actin was used as a cytosolic marker. Immunoreactive bands were detected by ECL Plus Western Blotting Detection System (Amersham Biosciences, UK). Blots were scanned with a Flour-S Multi Imaging System (Bio-Rad) to determine the density of the resulting bands and analyzed with Quantity One software.

2.11. Analysis of DNA fragmentation

HT-22 cells were heated to 43 °C for 30 min and allowed to recover at 37 °C for 24 h in a cell culture incubator. Adherent and floating cells were collected and total genomic DNA was isolated from cell pellets by use of DNeasy tissue kit (Qiagen). DNA concentration was determined spectrophotometrically. DNA (5 μg) samples were separated on 1.5% horizontal agarose gels and stained with ethidium bromide. DNA fragmentation was visualized using UV illumination on Flour-S imager (Bio-Rad).

2.12. Statistical analysis

Data represent the mean ± SEM of the indicated number of samples. Statistical analysis between groups was made using ANOVA single factor. Student's *t*-test and *F*-test were used to analyze between time points.

3. Results

3.1. Cytotoxic effects of heat stress

HT-22 cells were incubated in a water bath at 37 or 43 °C for 30 min and then allowed to recover at 37 °C in a cell culture incubator for up to 48 h. Exposure of HT-22 cells to 43 °C for 30 min caused a continuous decrease in adherent cell number during the recovery period (Fig. 1). A significant decrease in adherent cell number was seen as early as 2 h after heat stress and the decrease in cell number continued up to the 12 h time point. Cell number remained constant

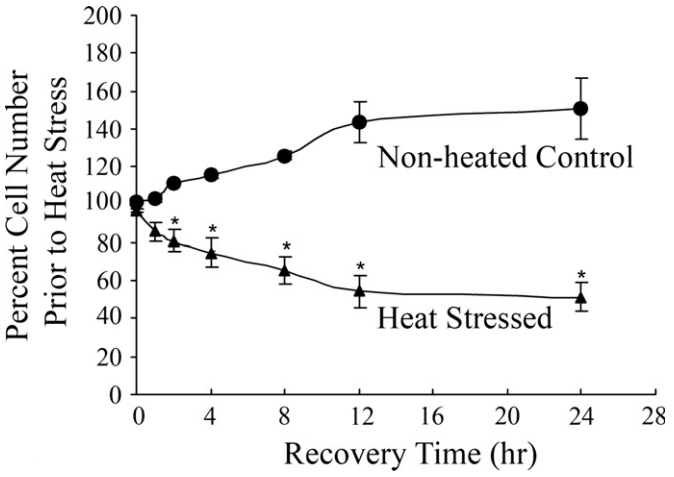

Fig. 1. Effect of heat stress on HT-22 cell survival. HT-22 cells were incubated in either a 37 °C (control) or 43 °C (heat-stressed) water bath for 30 min. Cells were allowed to recover in a cell culture incubator under normal growth conditions at 37 °C for 0–48 h and cell number measured. Values are means ± standard error of three independent experiments. * Indicates significantly different from unheated control cells ($P \leq 0.05$).

from 12 to 48 h of recovery. Non-heated cells continued to grow and their number reached 147 ± 10% of time 0 by 24 h at which point cells were confluent and cell number did not increase further.

3.2. Superoxide dismutase expression and activity

Hyperthermia also caused a time dependent decrease in gene expression, protein expression and activity of SOD in HT-22 cells. Cu/Zn-SOD gene expression declined shortly into the recovery period and reached a maximum decrease of 82% at 12 h before expression started to increase back towards baseline (Fig. 2). Gene expression of Cu/Zn-SOD was not different from non-heated cells at 24 h of recovery. There was no change in the expression of the housekeeping genes GAPDH or Arbp in response to hyperthermia, nor was there any change in Cu/Zn-SOD expression in control non-heated cells over the same time period (Fig. 2A). Furthermore, when we looked at the expression of HO1, NQO1 and catalase we observed an increase in HO1 gene expression at 4 and 8 h into the recovery phase but no significant change in either NQO1 or catalase (Fig. 2B). Hyperthermia also decreased the cytoplasmic protein expression of SOD during the recovery period (Fig. 3). This decrease in SOD protein expression was significant from control at 4 h of recovery and continued to decrease up to 48 h after hyperthermia. At 48 h there was a 54% decrease in SOD protein content compared to non-heated control cells. There was no change in the expression of beta-tubulin over the same time-period nor was there a change in SOD expression in control non-heated cells. Similar to what was seen for gene and protein expression, lysate SOD enzyme activity was also decreased in response to hyperthermia in HT-22 cells (Fig. 3). The effect of hyperthermia on SOD enzyme activity was apparent immediately following the 30 min heat stress. There was approximately a 35% inhibition in total SOD activity at the end of the hyperthermia incubation (0 h) when compared with unheated control cells. The decrease in activity was maximal at 8 h of recovery and remained at this level for the duration of the experiment. SOD activity after 8 h was 16.2 ± 1.3% of that measured in non-heated control cells.

3.3. Cytochrome c release and DNA fragmentation

To determine the characteristics of heat-induced HT-22 cell death, mitochondrial release of cytochrome c and genomic DNA

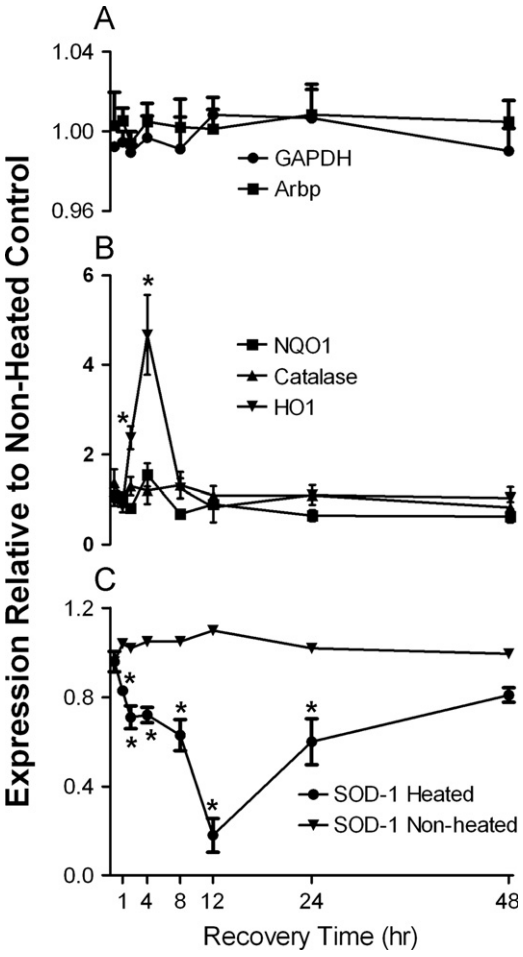

Fig. 2. Effect of heat-stress on antioxidant gene expression. HT-22 cells were incubated in a water bath at 37 °C (control) or 43 °C (heat-stressed) for 30 min. Cells were allowed to recover in a cell culture incubator under normal growth conditions at 37 °C for 0–48 h and gene expression was measured as described in Methods. (A) Expression of the housekeeping genes, GAPDH and Arbp in heat-treated cells showing no change in expression of the housekeeping genes in response to hyperthermia. (B) Expression of NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO1) and catalase. (C) expression of SOD-1 in heated and non-heated control HT-22 cells. Values are means ± standard error of three independent experiments performed in duplicate. Values represent the expression of the indicated gene in response to hyperthermia relative to the same gene in non-heated cells at the same time point. * Indicates significantly different from non-heated control for corresponding expression ($P \leq 0.05$).

fragmentation were determined to assess apoptotic cell death (Fig. 4). For these experiments, cells for analysis were composed of both floating and attached cells in control and hyperthermia-stressed groups. The proportion of floating cells was much higher in the heated groups compared to the control group. Cytochrome c immunoreactivity was undetectable in the cytosol of non-heated control cells but was dramatically increased in the cytoplasm of heat-stressed cells (Fig. 4A). A similar scenario was observed for DNA fragmentation. DNA laddering was seen in cells that were exposed to hyperthermia but not in non-heated control cells (Fig. 4B).

3.4. SOD mimetics

In light of the decrease in SOD expression and activity, we next examined the contribution of SOD to the heat induced cytotoxic effects. HT-22 cells were treated with the SOD mimetics, EUK-134

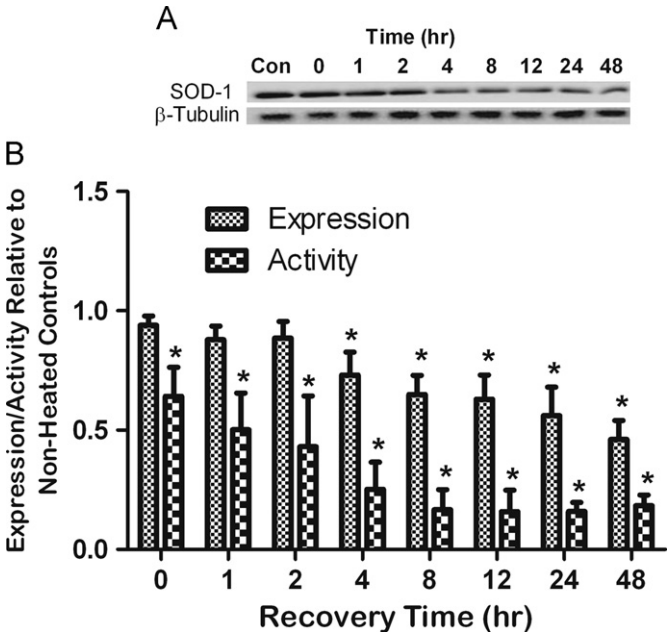

Fig. 3. Effect of heat-stress on SOD protein expression and cytosolic SOD activity. HT-22 cells were incubated in a water bath at 37 °C (control) or 43 °C (heat-stressed) for 30 min. Cells were allowed to recover in a cell culture incubator under normal growth conditions at 37 °C for 0–48 h and protein expression and activity were measured as described in Methods. (A) Representative Western blot of SOD-1 and beta-tubulin expression in heated cells. Con refers to non-heated control cells. (B) SOD-1 expression and cytosolic SOD activity expressed relative to non-heated control cells. Values are means ± standard error of three independent experiments performed in duplicate. * Indicates significantly different from non-heated control for corresponding expression or activity ($P \leq 0.05$).

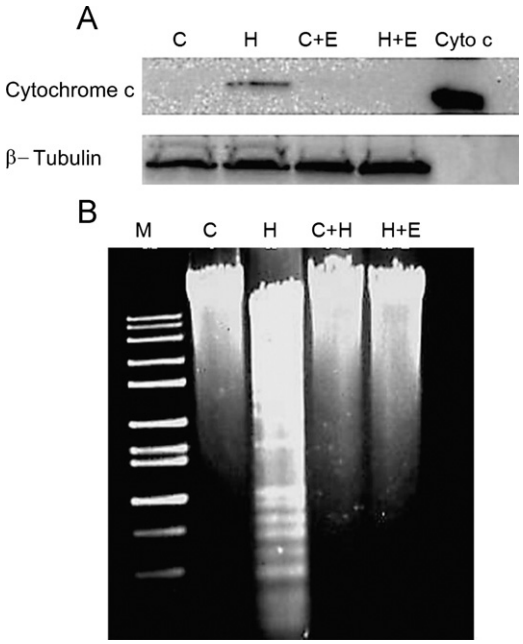

Fig. 4. Effect of heat stress on cytochrome c release and DNA fragmentation in HT-22 cells. (A) Western blot analysis for cytochrome c in cytosolic fraction prepared from HT 22 cells. Cells were treated with EUK-134 (10 μM) or it's vehicle for 1 h and incubated in either a 37 or 43 °C water bath for 30 min and allowed to recover at 37 °C for 12 h. C, vehicle-treated, non-heated control; H, vehicle-treated, heat stressed; C+E, EUK-134-treated, non-heated control; H+E, EUK-134-treated, heat stressed; Cyto c, purified cytochrome c protein as a positive control. β-tubulin was used as internal control. Blot is a representative of three independent experiments. (B) Effect of heat stress on DNA fragmentation in HT-22 cells. HT-22 cells were incubated at 37 or 43 °C for 30 min and allowed to recover at 37 °C for 24 h. Genomic DNA was isolated and separated by agarose gel electrophoresis. DNA fragmentation was visualized using UV illumination. M, DNA ladder; C, unheated control; H, heat stressed cells, C+E, EUK-134-treated, non-heated control; H+E, EUK-134-treated, heat stressed cells. Gel is a representative of three independent experiments.

(10 μM), Mn(III)TBAP (100 μM) or their vehicle for 1 h and then exposed to hyperthermia. EUK-134 (10 μM) had no effect on growth in non-heated control cells but completely reversed the cytotoxic effect of hyperthermia in HT-22 cells (Fig. 5). The growth of heated HT-22 cells pretreated with EUK-134 was indistinguishable from control-non-heated cells. Similar to EUK-134, Mn(III)TBAP (100 μM) pretreatment also protected HT-22 cells from hyperthermia-induced cell death (Fig. 5). Along similar lines, pretreatment of HT-22 cells with EUK-134 (10 μM) had no effect on cytosolic cytochrome c immunoreactivity or DNA laddering in control cells, but completely blocked the increase in the cytoplasmic appearance of cytochrome c and DNA laddering in HT-22 cells exposed to heat (Fig. 5).

3.5. Reactive oxygen species generation

The decrease in SOD activity also led us to investigate the generation of intracellular reactive oxygen species (ROS) in response to hyperthermia in HT-22 cells. Cells were labeled with the Image-iT Live Green ROS detection kit 2 h after heat exposure, at which time there was a 25% decrease in cell number compared to control non-heated cells. The intensity of green fluorescence corresponding to oxidized DCF is directly proportional to the level of intracellular ROS. In non-heated control cells, little to no green fluorescence was detected and nuclei stained blue and appeared rounded (Fig. 6A). In heated cells, there was enhanced generation and accumulation of intracellular ROS as determined by an increase in the number and intensity of green fluorescence inside the cells (Fig. 6B). Cells pretreated with EUK-134 showed reduced intensity of green fluorescence as well as the number of DCF-labeled cells when compared to vehicle-treated, heat stressed HT-22 cells (Fig. 6C). This increase in ROS generation was further examined by examining the effects of hyperthermia on NADPH oxidase (NOX)

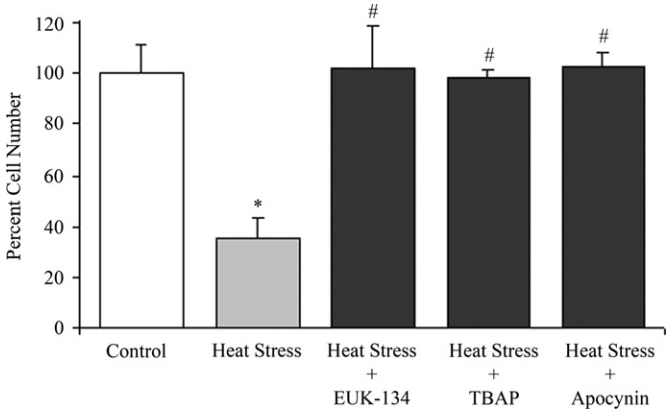

Fig. 5. Effect of heat stress on HT-22 cell survival in presence of the SOD mimetics EUK-134 and TBAP and the NOX inhibitor Apocynin. HT-22 cells were pretreated with EUK-134 (10 μM), TBAP (100 μM), apocynin (300 μM) or their vehicles for 1 h and then incubated at either 37 °C (control) or 43 °C (heat-stressed) for 30 min and allowed to recover under normal growth conditions for 12 h and cell number was determined. Values are means ± standard error of three independent experiments performed in duplicates. * Indicates significantly different from control ($P \leq 0.05$).

activity (Fig. 6D). Heat increased NOX activity from 14.3±2.3 RLU/2 × 10^5 cells to 35.8±5.1 RLU/2 × 10^5 cells. The effect of heat on the increase in NOX activity was blocked by pretreatment of HT-22 cells with the NOX inhibitor, apocynin (300 μM). Apocynin also blocked the cytotoxic effects of hyperthermia on cell survival (Fig. 3).

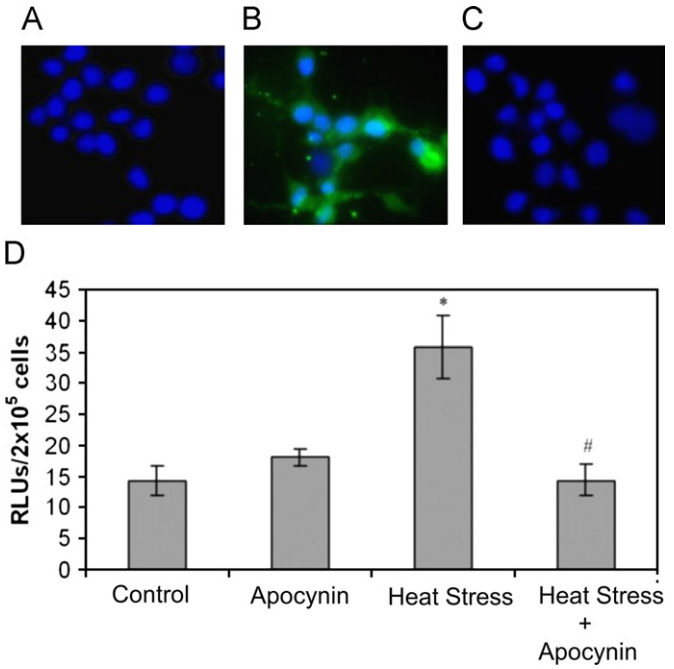

Fig. 6. Effect of heat stress on ROS generation and NOX activity in HT-22 cells. HT-22 cells were exposed to heat stress at 43 °C for 30 min and allowed to recover at 37 °C for 2 h. Unheated control cells were maintained at 37 °C for the same period. Cells were labeled with 25 μM carboxy-H_2DCFDA and 1 μM Hoeschst and visualized under fluorescence microscope. (A) unheated control cells; (B) heat stressed cells; (C) heat-stressed cells pretreated with EUK-134 (10 μM) for 1 h before heat stress. Pictures are representative of three independent experiments. (D) NOX activity in response to heat stress. NOX activity is expressed in relative light units (RLU)/2×10^5 cells. The control bar represents NOX activity in absence of heat stress. The apocynin bar represents control cells treated for 1 h with apocynin (300 μM). The heat stress bar represents NOX activity in cells following heat stress. The apocynin+heat stress bar represents the effect of apocynin (300 μM) on NOX activity in heat stress cells. Values are means ± standard error of three independent experiments. * Indicates significantly different from control ($P < 0.05$).

4. Discussion

Exposure of HT-22 cells to 43 °C for 30 min followed by recovery at 37 °C caused a decrease in Cu/Zn-SOD mRNA levels, cytoplasmic SOD protein and enzyme activity and a corresponding decline in cell number. During the recovery phase after heating, there was an increase in reactive oxygen species generation and an increase in NADPH oxidase activity that was associated with an increase in DNA fragmentation and release of cytochrome c from the mitochondria. The increase in ROS accumulation and cell death was abolished by pretreatment with the SOD mimetics EUK-134 and Mn(III)TBAP as well as the NADPH oxidase inhibitor apocynin. These data suggest that hyperthermia increased ROS generation by increasing NADPH activity and decreasing SOD activity leading to cytotoxicity in HT-22 cells.

In many cell types, hyperthermia-induced cell death appears to be both time and temperature dependent. In rat embryonic striatal neurons in culture, a heat load of 43 °C for 2 h was necessary to produce delayed cell death (White et al., 2003) whereas increasing the temperature to 45 °C for only 30 min caused delayed death in cortical neurons cultured for 10–15 days (White et al., 2007). In the present study, heating cells to 43 °C for 30 min lead to significant cell death. The time and temperature load is lower than has been reported for cells in culture. However, our experiments were performed at 50% confluence in what would be considered a rapidly growing population of cells. The developing nervous system appears to be especially sensitive to hyperthermia and actively dividing population of cells are highly susceptible to hyperthermia-induced apoptosis (Khan and Brown, 2002). In our study, if the hyperthermic insult was carried out at 70–80% confluence, there was a considerable decrease in the ability of our heating regimen to produce its cytotoxic effects (data not shown). Therefore, it appears that rapidly dividing HT-22 hippocampal cells in culture are more susceptible to hyperthermia.

Hyperthermia is associated with accumulation of ROS such as superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) (Zhao et al., 2006; Arnaud et al., 2002; Perez-Crespo et al., 2005; Niu et al., 2003; Shin et al., 2008). This increase in reactive oxygen species generation has been shown to be critical in both the apoptotic as well as anti-apoptotic pathways induced by hyperthermia (Katschinski et al., 2000). In the present study, heating cells to 43 °C for 30 min increased ROS generation estimated by the fluorescent indicator carboxy-H_2DCFDA and well as by the superoxide detector lucigenin. Both methods suggest that there was an increase in ROS generation. The source of ROS generation in response to hyperthermia has traditionally been attributed to mitochondrial generation, but recent evidence suggests that other ROS generating systems, such as NADPH oxidase (NOX), may be involved (Starkie et al., 2005; Wartenberg et al., 2005; Zhang et al., 2003; Zuo et al., 2000). HT-22 cells contained mRNA for NOX 1 and NOX4 (data not shown) and exposure of HT-22 cells to hyperthermia caused a significant increase in chemiluminescence in the presence of NADPH compared to control cells. The hyperthermia-induced increase in chemiluminescence was attenuated by pretreatment with the NOX inhibitors apocynin (300 μM) and DPI (1 μM, data not shown). Apocynin, at the concentration that blocked NOX activity also blocked hyperthermia induced apoptosis. Taken together, these data suggest that the hyperthermia-induced increase in ROS generation was mediated by NADPH oxidase. The mechanism for the hyperthermia-induced activation of NADPH oxidase however, is unclear. Protein kinase C has been reported to activate NOX in various cell types including neuronal (Chan et al., 2007), endothelial (Min et al., 2005), cardiac (Rude et al., 2005) and vascular smooth muscle (Lavrentyev and Malik, 2009) whereas phosphatidylinositol 3-kinases (PI3 kinase) have also been reported to activate NOX in phagocytes (Yamamori et al., 2004) and endothelial cells (Frey et al., 2006; El-Assal and Besner, 2005). However, whether PKC or PI3K is involved in hyperthermia-induced increase in NADPH oxidase activity is unknown.

In addition to the hyperthermia-induced increase in reactive oxygen species generation and apoptosis, we also observed a decrease in SOD-1 expression and activity that contributed to its pro-apoptotic effects. Under normal physiological conditions, cellular levels of the reactive oxygen species superoxide are kept low by several antioxidant mechanisms including the enzyme copper/zinc superoxide dismutase (Cu/Zn SOD) (McCord and Fridovich, 1969), which catalyzes the dismutation of superoxide to hydrogen peroxide (H_2O_2) in the cytosol of various cell types. Hydrogen peroxide is reduced to water by the actions of catalase and glutathione peroxidase. In the CNS, SOD-1 plays an important role in neuroprotection and has been reported to be rapidly down regulated after several types of CNS injury (Peluffo et al., 2005). Overexpression of SOD-1 is protective in neuronal cells and delays apoptosis triggered by many oxidative insults (Schwartz et al., 1998). On the contrary, downregulation of SOD-1 promotes apoptosis (Troy and Shelanski, 1994). Many stressors and pathological conditions associated with accumulation of reactive oxygen species and development of oxidative stress decrease the level of SOD-1 mRNA with or without a decline in protein content and/or enzymatic activity in different tissues and species. Such conditions include immobilization stress (Oishi and Machida, 2002), septic shock (Ghosh et al., 1996), hypoxia/ischemia (Martin et al., 2002) (Rawal et al., 2004), chronic fatigue syndrome (Steinau et al., 2004), inflammatory bowel disease (Segui et al., 2004), type-II diabetes

(Sakuraba et al., 2002) and chemical induced excitotoxic injury (Peluffo et al., 2005). Inhibition of SOD-1 activity following exposure to elevated temperature have also been reported in rat brain (Yang and Lin, 2002), gold fish brain (Lushchak and Bagnyukova, 2006), rat liver (Morrison et al., 2005; Zhang et al., 2003) and in PC-3 cells (Moriyama-Gonda et al., 2002).

In our study, the decrease in SOD activity occurred immediately after hyperthermia. SOD activity in heated cells decreased by 35% compared to non-heated cells at time 0 suggesting that there was an immediate disruption of SOD activity despite no change in protein content. Lushchak and Bagnyukova (2006) have also reported that in goldfish brain, a 1 h exposure to 35 °C decreased SOD activity by 40% in brain whereas further heat treatment increased SOD activity. In our study, a rebound effect on SOD activity was not observed and lysate SOD activity never recovered. We did not investigate the cause of the immediate decrease in SOD activity but others have attributed this to thermal/oxidative inactivation of the enzyme (Lushchak and Bagnyukova, 2006).

The pretreatment of HT-22 cells with the salen manganese antioxidant SOD-mimetic compound (EUK-134) rescued HT-22 cells from heat-induced death. EUK-134 is a synthetic SOD-mimetic drug that has the ability to scavenge superoxide anion and its dismutation product hydrogen peroxide (Pong et al., 2000). EUK-134 has been shown to be protective against oxidative injury in many types of cells including rat cortical neurons (Pong et al., 2001) and motorneurons (Sanchez-Carbente et al., 2005). In our study, EUK-134 protected HT-22 cells against hyperthermia-induced accumulation of intracellular ROS, mitochondrial cytochrome c release, DNA fragmentation and most importantly cell death. These data suggest that oxidative stress plays a major role in hyperthermia-neuronal cell damage and EUK-134 is protective via an antioxidant mechanism. Interestingly, salen manganese SOD-mimetic drugs (EUKs) have been used successfully to abrogate oxidative stress-induced cellular damage and apoptosis in different cell types and tissues associated with many pathologic conditions such as neurodegenerative disorders like Alzheimer's disease (Anderson et al., 2001), Parkinson's disease (Peng et al., 2005) and amyotrophic lateral sclerosis (Jung et al., 2001). They have been used also in the treatment of cell injuries in many other pathological conditions such as diabetes (Olcott et al., 2004), inflammatory immune diseases (Decraene et al., 2004), ischemia-reperfusion injuries (Li et al., 2005), heat stress-induced liver injury (Zhang et al., 2006, 2004), septic shock (Wayman et al., 2002) and heavy metal-induced toxicity (Yang et al., 2005).

In conclusion, exposure of HT-22 cell to 43 °C for 30 min followed by recovery at 37 °C decreased in SOD-1 mRNA levels, cytoplasmic SOD protein and enzyme activity and a corresponding decline in cell number. During the recovery phase, there was an increase in reactive oxygen species generation and an increase in NADPH oxidase activity that was associated with an increase in DNA fragmentation and an increase in the release of cytochrome c from the mitochondria. The increase in ROS accumulation and cell death was abolished by pretreatment with the SOD mimetics EUK-134 and Mn(III)TBAP and the NADPH oxidase inhibitor apocynin. These data suggest that hyperthermia increases ROS generation by increasing NADPH activity and decreasing SOD activity leading to cytotoxicity in HT-22 hippocampus cells.

References

Arnaud, C, Joyeux, M, Garrel, C, Godin-Ribuot, D, Demenge, P, Ribuot, C, 2002. Free-radical production triggered by hyperthermia contributes to heat stress-induced cardioprotection in isolated rat hearts. Br. J. Pharmacol. 135 (7), 1776–1782.

Anderson, I, Adinolfi, C, Doctrow, S, Huffman, K, Joy, KA, Malfroy, B, Soden, P, Rupniak, HT, Barnes, JC, 2001. Oxidative signalling and inflammatory pathways in Alzheimer's disease. Biochem. Soc. Symp. 67, 141–149.

Boveris, A, Chance, B, 1973. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem. J. 134 (3), 707–716.

Bass, DA, Parce, JW, Dechatelet, LR, Szejda, P, Seeds, MC, Thomas, M, 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J. Immunol. 130 (4), 1910–1917.

Bianca, RV, Wayman, NS, McDonald, MC, Pinto, A, Shape, MA, Chatterjee, PK, Thiemermann, C, 2002. Superoxide dismutase mimetic with catalase activity, euk-134, attenuates the multiple organ injury and dysfunction caused by endotoxin in the rat. Med. Sci. Monit. 8 (1), BR1–7.

Colombrita, C, Calabrese, V, Stella, AM, Mattei, F, Alkon, DL, Scapagnini, G, 2003. Regional rat brain distribution of heme oxygenase-1 and manganese superoxide dismutase mrna: relevance of redox homeostasis in the aging processes. Exp. Biol. Med. (Maywood, NJ) 228 (5), 517–524.

Chan, SH, Wang, LL, Tseng, HL, Chan, JY, 2007. Upregulation of at1 receptor gene on activation of protein kinase cbeta/nicotinamide adenine dinucleotide diphosphate oxidase/erk1/2/c-fos signaling cascade mediates long-term pressor effect of angiotensin ii in rostral ventrolateral medulla. J. Hypertension 25 (9), 1845–1861.

Decraene, D, Smaers, K, Gan, D, Mammone, T, Matsui, M, Maes, D, Declercq, L, Garmyn, M, 2004. A synthetic superoxide dismutase/catalase mimetic (euk-134) inhibits membrane-damage-induced activation of mitogen-activated protein kinase pathways and reduces p53 accumulation in ultraviolet b-exposed primary human keratinocytes. J. Invest. Dermatol. 122 (2), 484–491.

El-Orabi, N, Rogers, C, Lay, D, Cheng, H, Sartin, J, Schwartz, D, 2005. Effect of heat stress on gene expression in porcine hippocampus. FASEB J. 19, A1106.

El-Assal, ON, Besner, GE, 2005. Hb-egf enhances restitution after intestinal ischemia/reperfusion via pi3k/akt and mek/erk1/2 activation. Gastroenterology 129 (2), 609–625.

Fridovich, I, 1997. Superoxide anion radical ($O_2^{\bullet -}$), superoxide dismutases, and related matters. J. Biol. Chem. 272 (30), 18515–18517.

Fattman, CL, Schaefer, LM, Oury, TD, 2003. Extracellular superoxide dismutase in biology and medicine. Free Radical Biol. Med. 35 (3), 236–256.

Frey, RS, Gao, X, Javaid, K, Siddiqui, SS, Rahman, A, Malik, AB, 2006. Phosphatidylinositol 3-kinase gamma signaling through protein kinase czeta induces nadph oxidase-mediated oxidant generation and nf-kappab activation in endothelial cells. J. Biol. Chem. 281 (23), 16128–16138.

Galli, F, Piroddi, M, Annetti, C, Aisa, C, Floridi, E, Floridi, A, 2005b. Oxidative stress and reactive oxygen species. Contrib. Nephrol. 149, 240–260.

Ghosh, B, Hanevold, CD, Dobashi, K, Orak, JK, Singh, I, 1996. Tissue differences in antioxidant enzyme gene expression in response to endotoxin. Free Radical Biol. Med. 21 (4), 533–540.

Jones, LJ, Gray, M, Yue, ST, Haugland, RP, Singer, VL, 2001. Sensitive determination of cell number using the cyquant cell proliferation assay. J. Immunol. Methods 254 (1-2), 85–98.

Jung, C, Rong, Y, Doctrow, S, Baudry, M, Malfroy, B, Xu, Z, 2001. Synthetic superoxide dismutase/catalase mimetics reduce oxidative stress and prolong survival in a mouse amyotrophic lateral sclerosis model. Neurosci. Lett. 304 (3), 157–160.

Katschinski, DM, Boos, K, Schindler, SG, Fandrey, J, 2000. Pivotal role of reactive oxygen species as intracellular mediators of hyperthermia-induced apoptosis. J. Biol. Chem. 275 (28), 21094–21098.

Khan, VR, Brown, IR, 2002. The effect of hyperthermia on the induction of cell death in brain, testis, and thymus of the adult and developing rat. Cell Stress Chaperones 7 (1), 73–90.

Lee, JY, Kim, JW, Lim, HS, Joo, WH, Cho, YK, Moon, JY, 2005. Changes in antioxidant defense systems by 2,2′,5,5′-tetrachlorobiphenyl exposure in neuronal sk-n-mc cells. Toxicol. Lett. 157 (2), 139–149.

Lewerenz, J, Klein, M, Methner, A, 2006. Cooperative action of glutamate transporters and cystine/glutamate antiporter system xc-protects from oxidative glutamate toxicity. J. Neurochem. 98 (3), 916–925.

Li, Y, Zhu, H, Trush, MA, 1999. Detection of mitochondria-derived reactive oxygen species production by the chemilumigenic probes lucigenin and luminol. Biochim. Biophys. Acta 1428 (1), 1–12.

Lavrentyev, EN, Malik, KU, 2009. High glucose-induced nox1-derived superoxides downregulate pkc-betaii, which subsequently decreases ace2 expression and ang(1–7) formation in rat vsmcs. Am. J. Physiol. 296 (1), H106–118.

Lushchak, VI, Bagnyukova, TV, 2006. Temperature increase results in oxidative stress in goldfish tissues. 2. Antioxidant and associated enzymes. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 143 (1), 36–41.

Li, L, Zhou, QX, Shi, JS, 2005. Protective effects of icariin on neurons injured by cerebral ischemia/reperfusion. Chin. Med. J. 118 (19), 1637–1643.

McCord, JM, Fridovich, I, 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244 (22), 6049–6055.

McCord, JM, Edeas, MA, 2005. Sod, oxidative stress and human pathologies: a brief history and a future vision. Biomed. Pharmacother. 59 (4), 139–142.

Meloni, BP, Van Dyk, D, Cole, R, Knuckey, NW, 2005. Proteome analysis of cortical neuronal cultures following cycloheximide, heat stress and mk801 preconditioning. Proteomics 5 (18), 4743–4753.

Min, JK, Kim, YM, Kim, SW, Kwon, MC, Kong, YY, Hwang, IK, Won, MH, Rho, J, Kwon, YG, 2005. Tnf-related activation-induced cytokine enhances leukocyte adhesiveness: induction of icam-1 and vcam-1 via tnf receptor-associated factor and protein kinase c-dependent nf-kappab activation in endothelial cells. J. Immunol. 175 (1), 531–540.

Martin, R, Fitzl, G, Mozet, C, Martin, H, Welt, K, Wieland, E, 2002. Effect of age and hypoxia/reoxygenation on mrna expression of antioxidative enzymes in rat liver and kidneys. Exp. Gerontol. 37 (12), 1481–1487.

Morrison, JP, Coleman, MC, Aunan, ES, Walsh, SA, Spitz, DR, Kregel, KC, 2005. Aging reduces responsiveness to bso- and heat stress-induced perturbations of glutathione and antioxidant enzymes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289 (4), R1035–1041.

Moriyama-Gonda, N, Igawa, M, Shiina, H, Urakami, S, Shigeno, K, Terashima, M, 2002. Modulation of heat-induced cell death in pc-3 prostate cancer cells by the antioxidant inhibitor diethyldithiocarbamate. BJU Int. 90 (3), 317–325.

Noor, R, Mittal, S, Iqbal, J, 2002. Superoxide dismutase—applications and relevance to human diseases. Med. Sci. Monit. 8 (9), RA210–215.

Niu, KC, Lin, KC, Yang, CY, Lin, MT, 2003. Protective effects of alpha-tocopherol and mannitol in both circulatory shock and cerebral ischaemia injury in rat heatstroke. Clin. Exp. Pharmacol. Physiol. 30 (10), 745–751.

Oishi, K, Machida, K, 2002. Different effects of immobilization stress on the mrna expression of antioxidant enzymes in rat peripheral organs. Scand. J. Clin. Lab. Invest. 62 (2), 115–121.

Olcott, AP, Tocco, G, Tian, J, Zekzer, D, Fukuto, J, Ignarro, L, Kaufman, DL, 2004. A salen-manganese catalytic free radical scavenger inhibits type 1 diabetes and islet allograft rejection. Diabetes 53 (10), 2574–2580.

Peluffo, H, Acarin, L, Faiz, M, Castellano, B, Gonzalez, B, 2005. Cu/zn superoxide dismutase expression in the postnatal rat brain following an excitotoxic injury. J. Neuroinflammation 2 (1), 12.

Peskin, AV, Winterbourn, CC, 2000. A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (wst-1). Clin. Chim. Acta; Int. J. Clin. Chem. 293 (1-2), 157–166.

Perez-Crespo, M, Ramirez, MA, Fernandez-Gonzalez, R, Rizos, D, Lonergan, P, Pintado, B, Gutierrez-Adan, A, 2005. Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. Mol. Reprod. Dev. 72 (4), 502–510.

Pong, K, Doctrow, SR, Baudry, M, 2000. Prevention of 1-methyl-4-phenylpyridinium- and 6-hydroxydopamine-induced nitration of tyrosine hydroxylase and neurotoxicity by euk-134, a superoxide dismutase and catalase mimetic, in cultured dopaminergic neurons. Brain Res. 881 (2), 182–189.

Pong, K, Doctrow, SR, Huffman, K, Adinolfi, CA, Baudry, M, 2001. Attenuation of staurosporine-induced apoptosis, oxidative stress, and mitochondrial dysfunction by synthetic superoxide dismutase and catalase mimetics, in cultured cortical neurons. Exp. Neurol. 171 (1), 84–97.

Peng, J, Stevenson, FF, Doctrow, SR, Andersen, JK, 2005. Superoxide dismutase/catalase mimetics are neuroprotective against selective paraquat-mediated dopaminergic neuron death in the substantial nigra: implications for parkinson disease. J. Biol. Chem. 280 (32), 29194–29198.

Rude, MK, Duhaney, TA, Kuster, GM, Judge, S, Heo, J, Colucci, WS, Siwik, DA, Sam, F, 2005. Aldosterone stimulates matrix metalloproteinases and reactive oxygen species in adult rat ventricular cardiomyocytes. Hypertension 46 (3), 555–561.

Rawal, AK, Muddeshwar, MG, Biswas, SK, 2004. Rubia cordifolia, fagonia cretica linn and tinospora cordifolia exert neuroprotection by modulating the antioxidant system in rat hippocampal slices subjected to oxygen glucose deprivation. BMC Complement Altern. Med. 4, 11.

Sreedhar, AS, Pardhasaradhi, BV, Khar, A, Srinivas, UK, 2002. A cross talk between cellular signalling and cellular redox state during heat-induced apoptosis in a rat histiocytoma. Free Radical Biol. Med. 32 (3), 221–227.

Sugawara, T, Fujimura, M, Morita-Fujimura, Y, Kawase, M, Chan, PH, 1999. Mitochondrial release of cytochrome c corresponds to the selective vulnerability of hippocampal ca1 neurons in rats after transient global cerebral ischemia. J. Neurosci. 19 (22), RC39.

Starkie, RL, Hargreaves, M, Rolland, J, Febbraio, MA, 2005. Heat stress, cytokines, and the immune response to exercise. Brain Behav. Immun. 19 (5), 404–412.

Sun, Y, Leaman, DW, 2005. Involvement of noxa in cellular apoptotic responses to interferon, double-stranded rna, and virus infection. J. Biol. Chem. 280 (16), 15561–15568.

Shin, MH, Moon, YJ, Seo, JE, Lee, Y, Kim, KH, Chung, JH, 2008. Reactive oxygen species produced by nadph oxidase, xanthine oxidase, and mitochondrial electron transport system mediate heat shock-induced mmp-1 and mmp-9 expression. Free Radical Biol. Med. 44 (4), 635–645.

Schwartz, PJ, Reaume, A, Scott, R, Coyle, JT, 1998. Effects of over- and under-expression of cu,zn-superoxide dismutase on the toxicity of glutamate analogs in transgenic mouse striatum. Brain Res. 789 (1), 32–39.

Steinau, M, Unger, ER, Vernon, SD, Jones, JF, Rajeevan, MS, 2004. Differential-display pcr of peripheral blood for biomarker discovery in chronic fatigue syndrome. J. Mol. Med. 82 (11), 750–755.

Segui, J, Gironella, M, Sans, M, Granell, S, Gil, F, Gimeno, M, Coronel, P, Pique, JM, Panes, J, 2004. Superoxide dismutase ameliorates tnbs-induced colitis by reducing oxidative stress, adhesion molecule expression, and leukocyte recruitment into the inflamed intestine. J. Leukoc. Biol. 76 (3), 537–544.

Sakuraba, H, Mizukami, H, Yagihashi, N, Wada, R, Hanyu, C, Yagihashi, S, 2002. Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese type ii diabetic patients. Diabetologia 45 (1), 85–96.

Sanchez-Carbente, MR, Castro-Obregon, S, Covarrubias, L, Narvaez, V, 2005. Motoneuronal death during spinal cord development is mediated by oxidative stress. Cell Death Differ. 12 (3), 279–291.

Thannickal, VJ, Fanburg, BL, 2000. Reactive oxygen species in cell signaling. Am. J. Physiol. Lung Cell Mol. Physiol. 279 (6), L1005–1028.

Troy, CM, Shelanski, ML, 1994. Down-regulation of copper/zinc superoxide dismutase causes apoptotic death in pc12 neuronal cells. Proc. Natl. Acad. Sci. USA 91 (14), 6384–6387.

Vandesompele, J, De Preter, K, Pattyn, F, Poppe, B, Van Roy, N, De Paepe, A, Speleman, F, 2002. Accurate normalization of real-time quantitative rt-pcr data by geometric averaging of multiple internal control genes. Genome Biol. 3 (7) RESEARCH0034.

Wartenberg, M, Gronczynska, S, Bekhite, MM, Saric, T, Niedermeier, W, Hescheler, J, Sauer, H, 2005. Regulation of the multidrug resistance transporter p-glycoprotein in multicellular prostate tumor spheroids by hyperthermia and reactive oxygen species. Int. J. Cancer 113 (2), 229–240.

White, MG, Emery, M, Nonner, D, Barrett, JN, 2003. Caspase activation contributes to delayed death of heat-stressed striatal neurons. J. Neurochem. 87 (4), 958–968.

White, MG, Luca, LE, Nonner, D, Saleh, O, Hu, B, Barrett, EF, Barrett, JN, 2007. Cellular mechanisms of neuronal damage from hyperthermia. Prog. Brain Res. 162, 347–371.

Xu, X, Chua, CC, Kong, J, Kostrzewa, RM, Kumaraguru, U, Hamdy, RC, Chua, BH, 2007. Necrostatin-1 protects against glutamate-induced glutathione depletion and caspase-independent cell death in ht-22 cells. J. Neurochem. 103 (5), 2004–2014.

Yang, CY, Lin, MT, 2002. Oxidative stress in rats with heatstroke-induced cerebral ischemia. Stroke: J. Cereb. Circ. 33 (3), 790–794.

Yamamori, T, Inanami, O, Nagahata, H, Kuwabara, M, 2004. Phosphoinositide 3-kinase regulates the phosphorylation of nadph oxidase component p47(phox) by controlling cpkc/pkcdelta but not akt. Biochem. Biophys. Res. Commun. 316 (3), 720–730.

Yang, PM, Chiu, SJ, Lin, LY, 2005. Differential effects of salen and manganese-salen complex (euk-8) on the regulation of cellular cadmium uptake and toxicity. Toxicol. Sci. 85 (1), 551–559.

Zhao, QL, Fujiwara, Y, Kondo, T, 2006. Mechanism of cell death induction by nitroxide and hyperthermia. Free Radical Biol. Med. 40 (7), 1131–1143.

Zhang, HJ, Xu, L, Drake, VJ, Xie, L, Oberley, LW, Kregel, KC, 2003. Heat-induced liver injury in old rats is associated with exaggerated oxidative stress and altered transcription factor activation. FASEB J. 17 (15), 2293–2295.

Zuo, L, Christofi, FL, Wright, VP, Liu, CY, Merola, AJ, Berliner, LJ, Clanton, TL, 2000. Intra- and extracellular measurement of reactive oxygen species produced during heat stress in diaphragm muscle. Am. J. Physiol. Cell Physiol. 279 (4), C1058–1066.

Zhang, Z, Ferraris, JD, Brooks, HL, Brisc, I, Burg, MB, 2003. Expression of osmotic stress-related genes in tissues of normal and hyposmotic rats. Am. J. Physiol. Renal Physiol. 285 (4), F688–693.

Zhang, HJ, Doctrow, SR, Oberley, LW, Kregel, KC, 2006. Chronic antioxidant enzyme mimetic treatment differentially modulates hyperthermia-induced liver hsp70 expression with aging. J. Appl. Physiol. 100 (4), 1385–1391.

Zhang, HJ, Doctrow, SR, Xu, L, Oberley, LW, Beecher, B, Morrison, J, Oberley, TD, Kregel, KC, 2004. Redox modulation of the liver with chronic antioxidant enzyme mimetic treatment prevents age-related oxidative damage associated with environmental stress. FASEB J. 18 (13), 1547–1549.