Oxidative and Nitrosative Mediators in Hepatic Injury Caused by Whole Body Hyperthermia in Rats

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Abstract

The involvement of oxidative and nitrosative mediators in liver injury caused by heat stress remains unclear. This study aimed to elucidate the role of endothelial nitric oxide synthase (eNOS), and inducible NOS (iNOS)-derived NO and nitrotyrosine in the whole-body hyperthermia (WBH)-induced liver injury. Rats were anesthetized with intraperitoneal pentobarbital, and were exposed to a heating lamp for 60 min to raise the core temperature to 42.5°C. The rats were maintained at the hyperthermic state for an additional 50 min. Blood urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, lactic dehydrogenase, creatine phosphokinase, amylase, lipase, nitrate/nitrite, methyl guanidine, and proinflammatory cytokines (tumor necrosis factor$_\alpha$, interleukin-1$_\beta$ and interleukin-10) were measured before and 14 h after hyperthermia. Immunohistochemical staining was employed to detect the eNOS, iNOS and nitrotyrosine levels. Western blotting was used to examine the expression of heatshock protein 70 (HSP 70). Histopathological examination of the liver tissue was performed. WBH caused liver injury accompanied with significant increases in biochemical factors, nitrate/nitrite, methyl guanidine, and proinflammatory cytokines. In addition, WBH enhanced the eNOS, iNOS, nitrotyrosine and HSP 70 levels. WBH caused hepatic injury. The pathogenetic mechanism is likely mediated through the NOS-derived NO, free radical, proinflammatory cytokines and nitrotyrosine. The enhanced expression of HSP 70 may play a protective role.

Key Words: whole body hyperthermia, heat stress, hepatic injury, nitric oxide, nitric oxide synthase, free radical, proinflammatory cytokines, nitrotyrosine, heat shock protein

Introduction

Heat stress or heatshock is a life-threatening disorder characterized by hyperpyrexia, multiple organ failure and neurological dysfunction (1, 4). Redistribution of blood flow under heat stress condition leads to low perfusion and ischemia in the splanchnic vascular beds, and subsequent injury as well as increased permeability in the mesenteric microcirculation (11, 17). The pathological changes induce release of endotoxin, nitric

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oxide (NO), proinflammatory cytokines, free radical and other mediators (2, 21, 27). Although oxidative and nitrosative stress has been implicated in the pathogenesis of cellular and organ injury in heatshock, the role of mediators in the heatshock-induced hepatic injury remains to be determined (1, 4, 11).

The present study was designed to investigate the role of NO, free radical, proinflammatory cytokines, nitrotyrosine, and heatshock protein in the hyperthermia-induced liver dysfunction and injury.

Materials and Methods

Animals

We used male rats of Spague-Dawley (SD) strain, 12-15 wk-old, weighing 330-360 g. The rats were obtained from the National Animal Center and housed in the University Laboratory Animal Center with adequate environmental control. The animal experiment was approved by the University Committee of Laboratory Animal Care and Use, and followed the guidelines of the National Animal Research Center. The room temperature was maintained at 21 ±1 °C under a 12/12 h light/dark regimen. Food and water were provided *ad libitum*.

Preparations

Acute experiment was carried out in the laboratory with controlled room temperature (21 ±1 °C) and relative humidity (60 ±4%). The rats were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg). During the experimental period, supplemental doses were given as needed. A femoral artery was catheterized and connected to pressure transducer (Gould Instruments, Cleveland, OH, USA) to record the arterial pressure and heart rate on a polygraph recorder (Power Lab, AD Instruments, Mountain View, CA, USA). A femoral vein was cannulated for intravenous (i.v.) administration of fluid or supplement of anesthetics.

Whole Body Hyperthermia

Whole body hyperthermia (WBH) was produced in 12 rats with a heating lamp. The core temperature was monitored with a thermistor placed at the rectum. Five min after anesthesia, the rats were exposed to conditioned room temperature (21 ± 1°C) for 60 min. The core temperature was 37.2 ± 0.4°C. Subsequently, the rats were subjected to heating lamp to raise the core temperature to 42.5 ± 0.6°C within 10 min and maintained for another 50 min. In addition to the increase in core temperature, WBH was evidenced by a decline in femoral arterial pressure from 118 ± 6 to 68 ± 4 mmHg. Control group (n = 12) was kept in room temperature for the same period of time.

Blood Sampling and Biochemical Determination

Our pilot experiments revealed that overt liver injury occurred at 12-16 h after WBH. Blood samples (0.5 ml) were collected at two time points: the first time point was after anesthesia, but before WBH; the second time point was at 14 h after WBH. In control group, blood sample was collected after anesthesia. After addition of heparin (100 U), the blood samples were centrifuged at 3,000 g for 10 min. The plasma was used for biochemical determination. Blood urea nitrogen (BUN), creatinine (Cr), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase (CPK), lactic dehydrogenase (LDH), amylase and lipase were measured with an autoanalyzer (Vitros 750, Johnson & Johnson, Rochester, NY, USA) (28).

Nitrate/nitrite as NO metabolites was determined with high-performance liquid chromatography (ENO-20, AD Instruments, Mountain View, CA, USA) (18, 25). The formation of methyl guanidine has been identified as an index of hydroxyl radical production (26). It was determined with its fluorescence spectrum (Jasco 821-FP, Spectroscopic CO., Tokyo, Japan). The emission maximum was set at 500 nm and the excitation maximum at 398 nm. The assay was calibrated with authentic methyl guanidine (Sigma M0377). Tumor necrosis factor$_{\alpha}$ (TNF$_{\alpha}$), interleukin-1$_{\beta}$ (IL-1$_{\beta}$) and interleukin-10 (IL-10) were measured with antibody enzyme-linked immunosorbent assays (ELISAs) with a commercial antibody pair, recombinant standards, and a biotin-streptavidin-peroxidase detection system (Endogen, Rockford, IL, USA) (14). All reagents, samples and working standards were prepared in room temperature according to the manufacture's directions. The optical density was measured at 450/540 nm wavelengths by an automated ELISA readers.

Immunohistochemical Detection of Endothelial and Inducible NO Synthases (eNOS and iNOS) and Nitrotyrosine in Liver Tissue

The rats were euthanized with an overdose of pentobarbital (100 mg/kg, i.v.). The liver was removed. Immunohistochemical stain with labeled streptavidin and antibodies was employed for the detection of eNOS, iNOS and nitrotyrosine (37). Liver tissues were dissected and fixed in fixation buffer. The tissue sections (5 μm) were placed in poly-L-lysine, silane-coats slides and incubated at 70°C for 20 min, then rehydrated in water and digested with antigen retrieval solution (DAKO, Carpinteria, CA, USA) for 20 min at 99°C. The slides were incubated in 30 % aqueous hydrogen peroxide for 30 min to quench endogenous peroxidase activity and rinsed in deionized water. Nonspecific bending of antibody was blocked by incubating specimens with a mixture of goat serum and avidin blocking reagent (Vector

Laboratories, Burlingame, CA, USA) for 30 min. Primary antibodies (mouse anti-rat eNOS, iNOS and nitrotyrosine monoclonal antibodies) (DAKO, Carpinteria, CA, USA) were placed on specimens at a dilution of 1:100 with DAKO diluent composed biotin blocking reagent and incubated at 4°C overnight. The specimens were then incubated with biotinylated anti-mouse IgG (1:800) and streptavidin reagent. Color development with the antigen retrieval technique was obtained by incubation with 3-amino-9-ethylcarbazole (34).

Western Blotting for the Detection of Heatshock Protein (HSP)

Liver tissue sections were subjected for the detection of heatshock protein. Primary anti-HSP70 antibody (SPA-810, Stress Gen, Victoria, BC, Canada) and secondary antibody (goat anti-mouse IgG-horseredish-peroxidase) were used for the detection of HSP70 with western blotting. β-actin was used for internal standard.

Pathological Examinations

Specimens of liver tissue were fixed in formaldehyde, embedded in paraffin. Tissue blocks were sectioned at 5 μm in thickness. The tissue sections were stained with hematoxylin and eosin.

Assessments and Comparisons

The levels of eNOS, iNOS, nitrotyrosine and HSP 70 in hepatic tissue were assessed in a blind fashion by several laboratory assistants. Each one gave a score (percent increase over the pre-WBH levels). The average was used for an individual data. Biochemical variables at 14 h after WBH were compared with the levels before WBH (pre-WBH) and control groups.

Statistical Evaluation

The data were expressed as mean ± SEM. The measured variables were compared before and after WBH and between control and WBH group using one way analysis of variance with repeated measures, followed by a *post hoc* comparison with Newman-Keul's test. Differences were considered to be statistically significant at $P < 0.05$.

Results

Blood Urea Nitrogen (BUN), Creatinine (Cr), Asparate Aminotransferase (AST), and Alanine Aminotransferase (ALT)

The plasma levels of BUN, Cr, AST and ALT were significantly elevated 14 h after WBH (Fig. 1). The levels in Control group and before WBH (Pre-WBH) were 9.2 ± 1.4 and 9.8 ± 1.6 mg/dl for BUN; 0.20 ± 0.04 and 0.24 ± 0.06 mg/dl for Cr; 234 ± 32 and 248 ± 34 unit/l for ALT. The control values were not different from those obtained before WBH. Accordingly, comparisons were made between the values of Pre-WBH and 14 h Post-WBH. BUN increased from 9.8 ± 1.6 (pre-WBH level) to 41.2 ± 3.6 mg/dl, Cr from 0.24 ± 0.06 to 1.27 ± 0.14 mg/dl, AST from 248 ± 34 to 2469 ± 126 unit/l, and ALT from 204 ± 29 to 1031 ± 116 unit/l.

Lactic Dehydrogenase (LDH), Creatine Phosphokinase (CPK), Amylase, and Lipase

Fig. 2 shows the increases in LDH, CPK, amylase and lipase following WBH for 14 h. Similarly, the values in Control group and those obtained before WBH (Pre-WBH) were not different. The increases from pre-WBH to 14 h post-WBH were LDH 868 ± 109 to 5262 ± 249 unit/l; CPK 198 ± 36 to 4031 ± 196 unit/l; amylase 866 ± 48 to 5628 ± 188 unit/l; and lipase 741 ± 41 to 4236 ± 160 unit/l.

Nitrate/nitrite and Methyl Guanidine (MG)

The values in Control group and those obtained at Pre-WBH were not different. The plasma levels of nitrate/nitrite and MG were similarly elevated following WBH (Fig. 3). The increases in nitrate/nitrite and MG were 344 ± 26 to 2546 ± 174 pM and 1.68 ± 0.14 to 5.79 ± 0.49 mM, respectively.

Tumor Necrosis Factor$_\alpha$ (TNF$_\alpha$), Interleukin-1$_\beta$ (IL-1$_\beta$) and Interleukin-10 (IL-10)

The Control values did not differ from those at Pre-WBH. WBH caused significant increases in TNF$_\alpha$, IL-1$_\beta$ and IL-10 (Fig. 4). The increases in these proinflammatory cytokines from pre-WBH level to 14 h post-WBH were 143 ± 34 to 2987 ± 201 pg/ml for TNF$_\alpha$, 128 ± 26 to 5569 ± 249 pg/ml for IL-1$_\beta$, and 38 ± 12 to 578 ± 87 pg/ml for IL-10.

Immunohistochemical Detection of eNOS, iNOS and Nitrotyrosine in Liver Tissue

Following WBH, immunohistochemical stain revealed that the eNOS, iNOS and nitrotyrosine levels were obviously enhanced over the pre-WBH levels (Fig. 5). Table 1 summarizes the percent changes of eNOS, iNOS, and nitrotyrosine over the pre-WBH levels.

Western Blotting for HSP 70 Expression

HSP70 expression was also enhanced 14 h after WBH. The increase was 8.7 ±1.3-fold the pre-WBH level (Fig. 6).

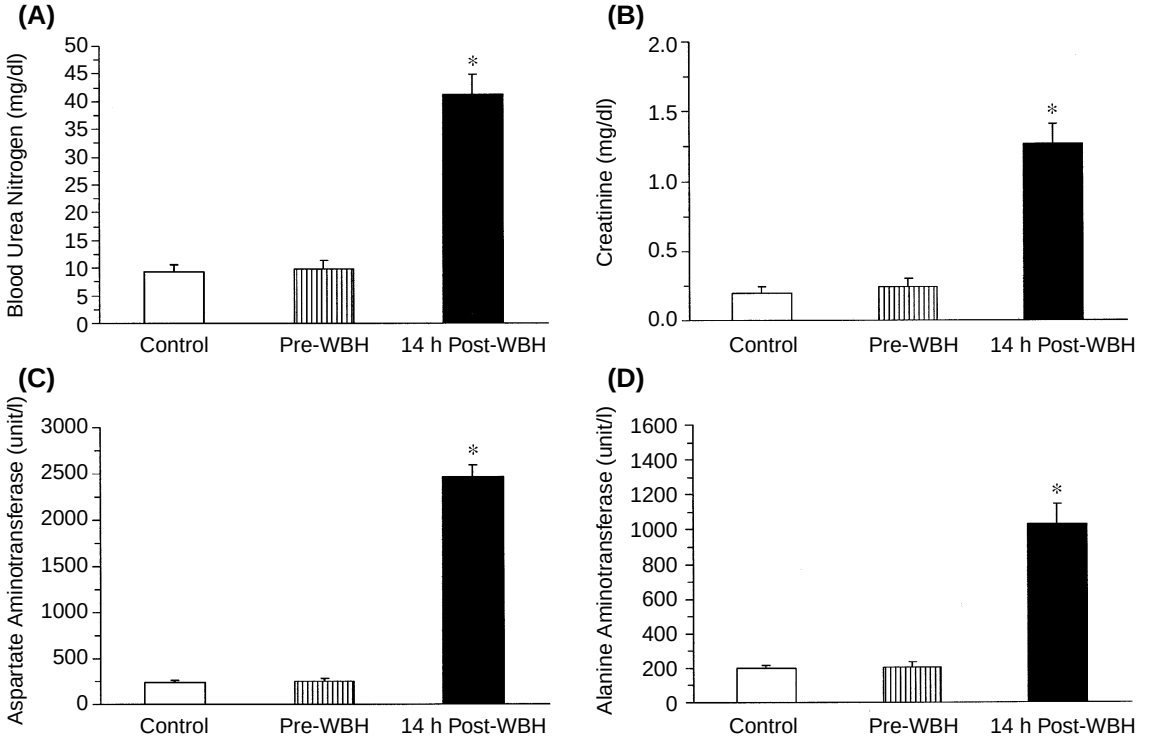

Fig. 1. Blood urea nitrogen (A), creatinine (B), aspartate aminotransferase (C), and alanine aminotransferase (D) of Control group, before and 14 after whole body hyperthermia (WBH). WBH significantly elevated these biochemical factors. In figures 1-4, Pre-WBH and 14 h Post-WBH denote before and 14 h after whole body hyperthermia. *$P < 0.05$ compared to Control or Pre-WBH values. The statistical symbol in this figure also applies to figures 2-4.

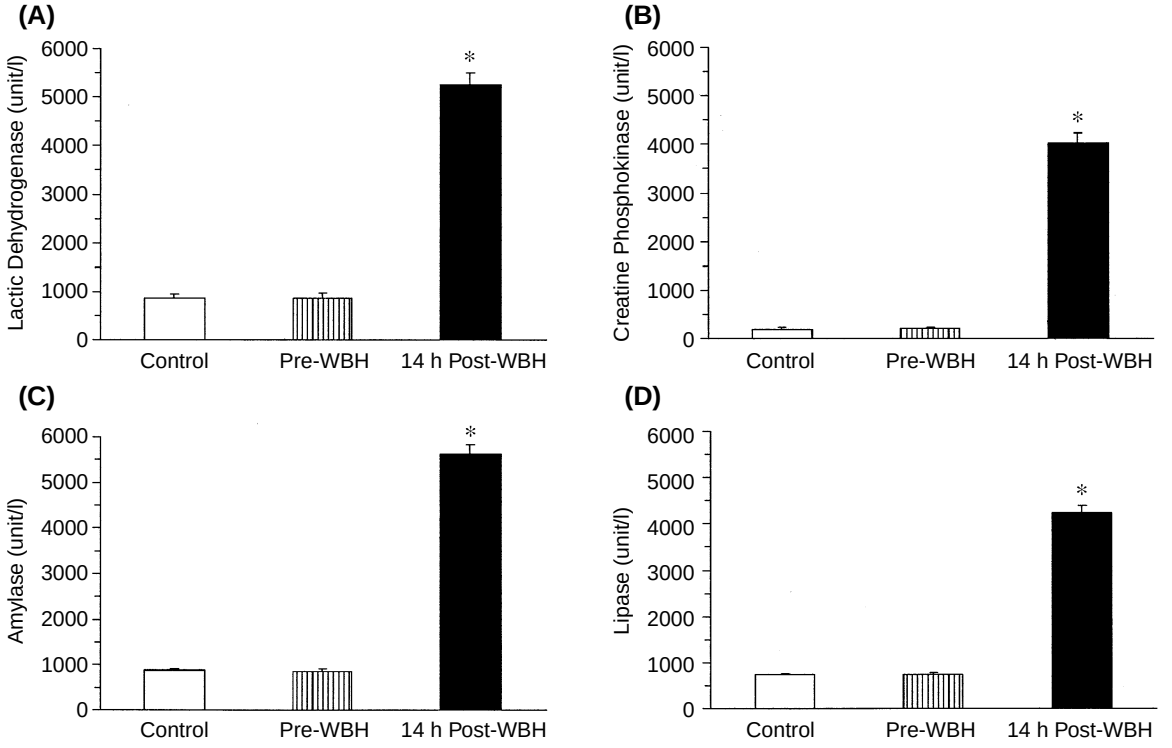

Fig. 2. Lactic dehydrogenase (A), creatine phosphokinase (B), amylase (C), and lipase (D). WBH significantly increased these enzymes measured at 14 h after WBH.

Table 1. The activities of endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS) and nitrotyrosine

eNOS	iNOS	Nitrotyrosine
148 ± 19%	236 ± 22%	179 ± 18%

The data are mean ± SEM (n = 12) and expressed as percent of pre-WBH levels.

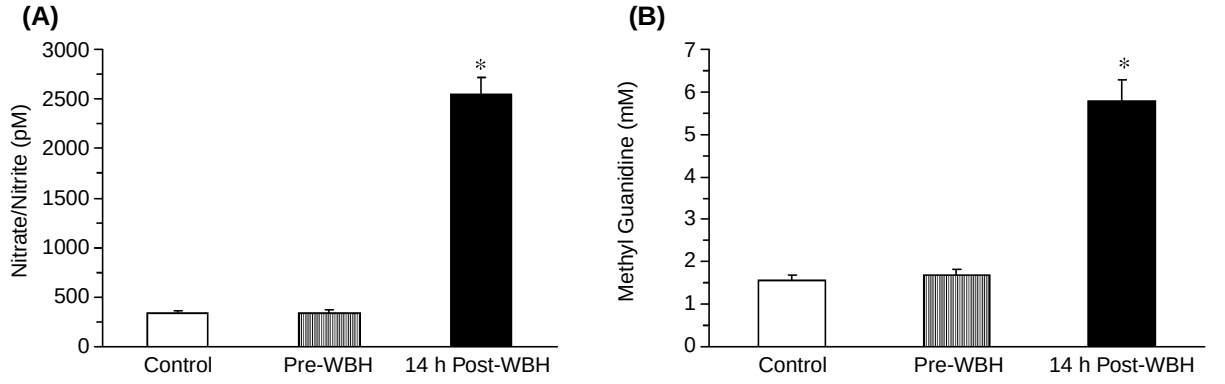

Fig. 3. Nitrate/nitrite (A) and methyl guanidine (B). WBH caused increases in the nitric oxide metabolites and hydroxyl radical determined at 14 h after WBH.

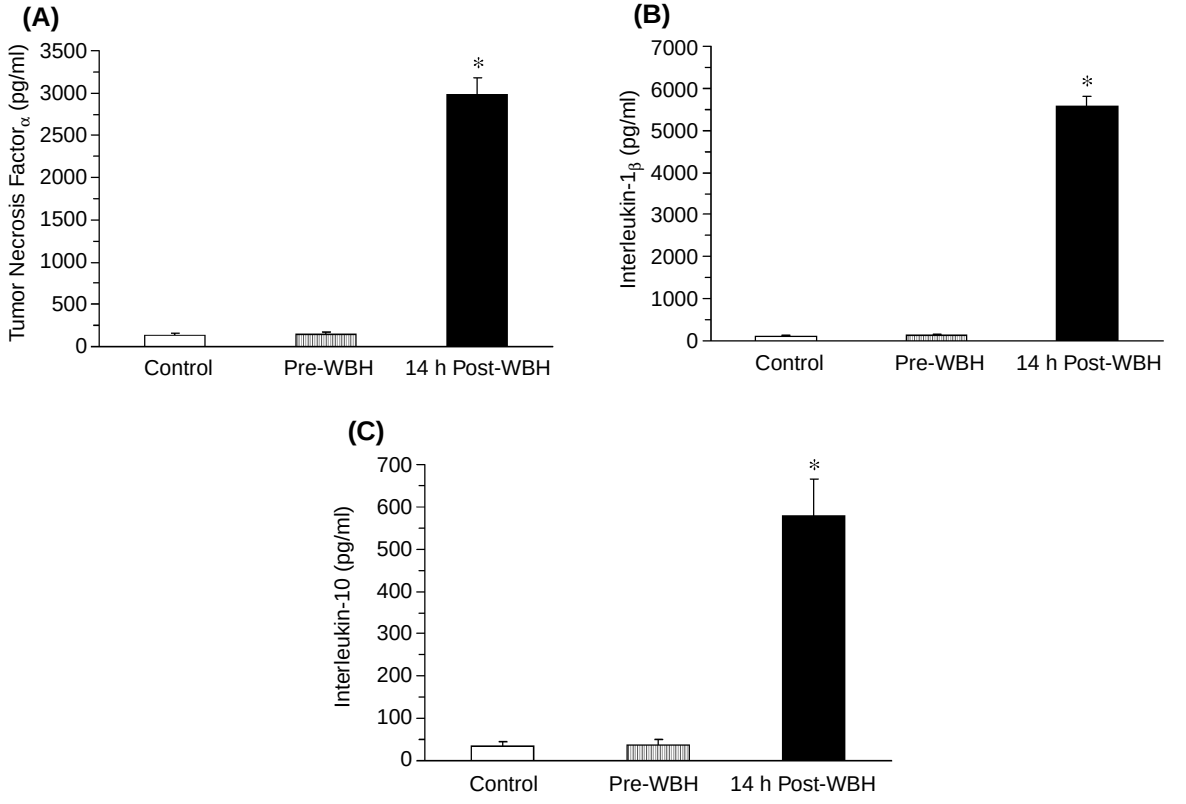

Fig. 4. Tumor necrosis factor$_\alpha$ (A), interleukin-1$_\beta$ (B) and interleukin-10 (C). The proinflammatory cytokines at 14 h Post-WBH were remarkably increased over the Control and Pre-WBH levels.

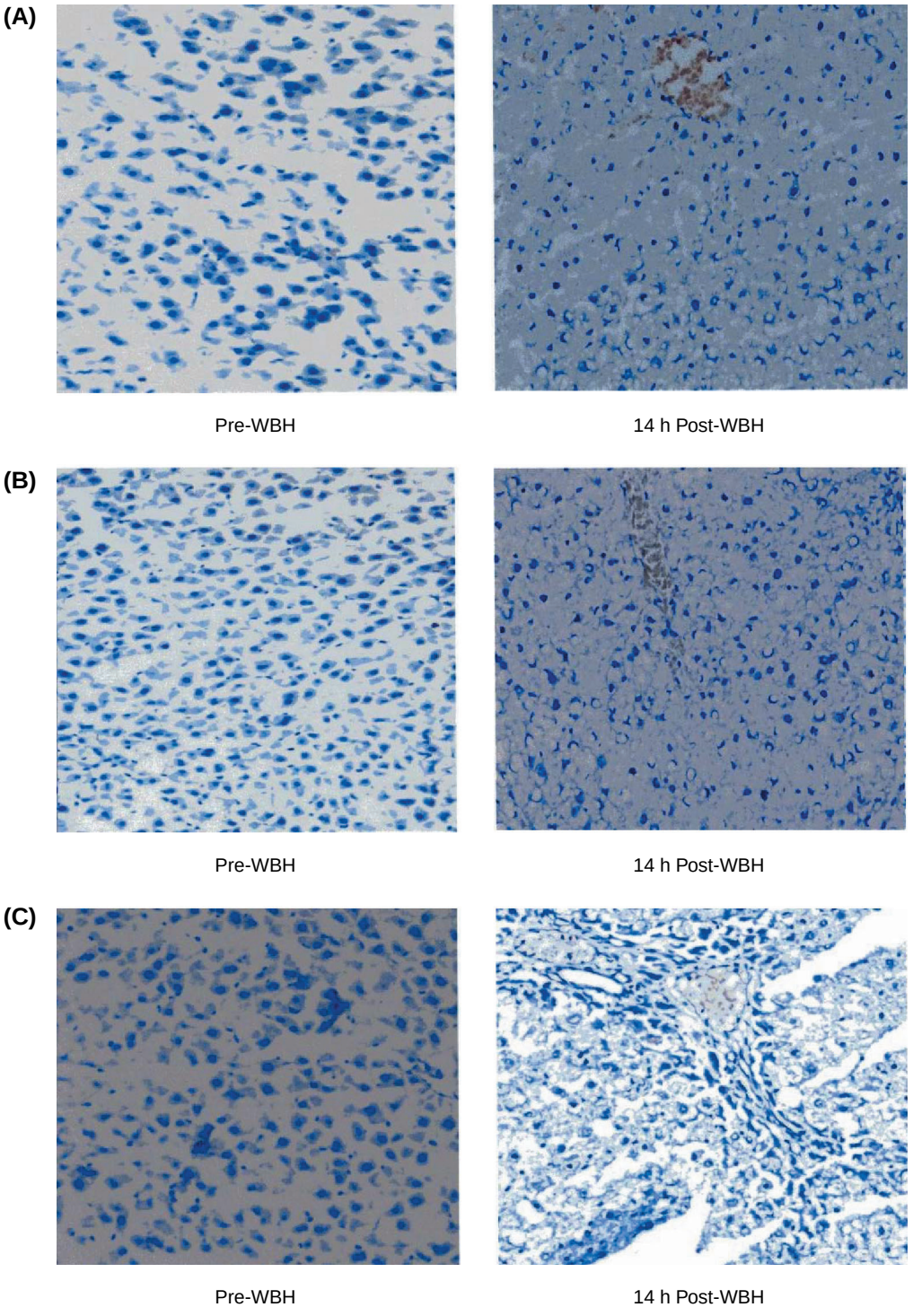

Fig. 5. Immunohischemical staining of the endothelial nitric oxide synthase (eNOS, A), inducible NOS (iNOS, B), and nitrotyrosine (C) in liver tissue before and 14 h after WBH. The eNOS, iNOS and nitrotyrosine levels were obviously enhanced after WBH (original magnification ×200).

Pathology

WBH caused inflammatory lesions including increase in Kupffer cells and leukocyte infiltration in the liver (Fig. 7).

Discussion

In our pilot study and the present experiment, we found that overt hepatic injury occurred at 14 h after whole body hyperthermia. The hepatic injury

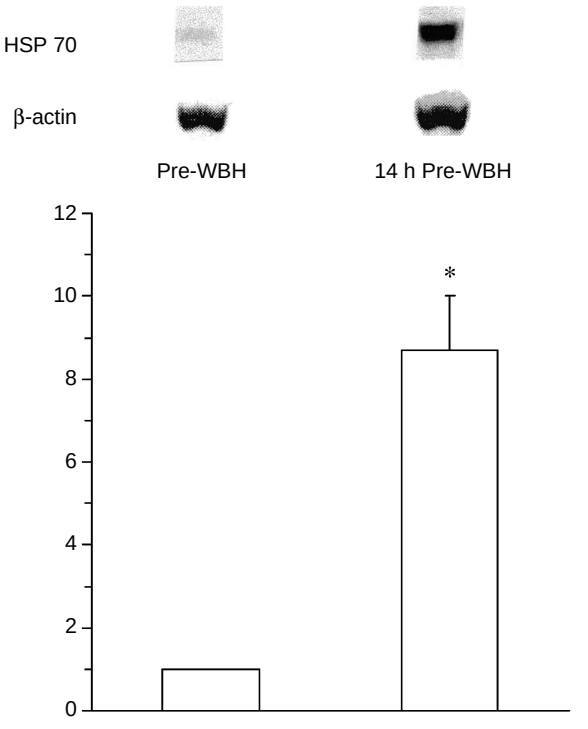

Fig. 6. Western blotting for the heashock protein 70 (HSP 70) before and 14 h after WBH. The HSP 70 was increased to 8.7 ±1.3-fold the pre-WBH level. β-actin was used for contrast.

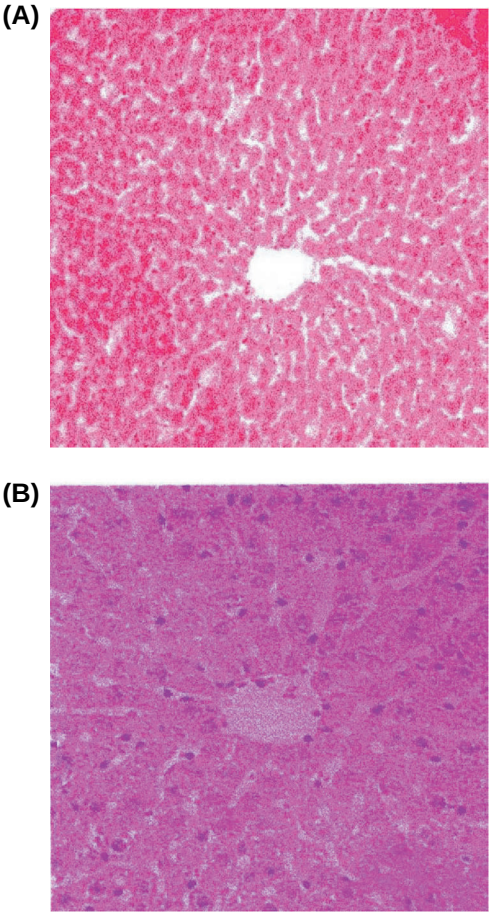

Fig. 7. Histopathological micrographs of the liver tissue section. (A) pre-WBH, and (B) 14 h post-WBH. There were inflammatory changes as evidenced by increased Kupffer cells and leukocyte infiltration after WBH (B). Hematoxylin and eosin stain, original magnification ×200.

was evidenced by the pathological lesions and increases in AST, ALT and LDH. In addition, heat challenge caused elevations of biochemical factors such as BUN, Cr, CPK, amylase, and lipase. The plasma nitrate/nitrite, methyl guanidine, TNF_{α}, $IL\text{-}1_{\beta}$ and IL-10 were significantly augmented. Immunohistochemical stain revealed increased levels of eNOS, iNOS and nitrotyrosine in hepatic tissue. In particular, the iNOS and nitrotyrosine were 2.36- and 1.79-fold the pre-WBH levels. Western blotting disclosed a rise in heatshock protein 70 to 8.7-fold the pre-WBH level.

Heatshock or heat stress has been implicated to be a systemic inflammatory response similar to endotoxemia (4, 9, 21). Our laboratory has demonstrated that the increases in amylase and lipase at 12-18 h following endotoxin administration in conscious rats account for the multiple organ injury (12, 18). In the present experiment, amylase and lipase were elevated to 5.5 to 6.4-fold the pre-WBH levels. These two enzymes are toxic to the liver and other organs. The marked increases in other biochemical factor such as LDH and CPK suggest the involvement of other organs.

The liver and gastrointestinal tract is one of the major target organs in hyperthermia-induced organ damage (10, 29). The gastrointestinal tract fuels the inflammatory response during or after hyperthermia (7, 10, 11, 33). Release or activation of inflammatory cytokines plays a pivotal role in the heatshock organ failure including the liver (5, 6, 20, 38). In the present study, the proinflammatory cytokines (TNF_{α}, $IL\text{-}1_{\beta}$ and IL-10) increased 15.2 to 43.5-fold the pre-WBH levels.

Several studies have reported that heat-induced organ injury was associated with increased oxidative stress such as reactive oxygen species, stress-response transcription factors, free radical and other factors (2, 10, 39, 42). In the present study, we demonstrated increases in nitrate/nitrite (7.4-fold) and methyl guanidine (3.5-fold) at 14 h after WBH. Immunohistochemical staining revealed enhancement of eNOS, iNOS and nitrotyrosine. The extent of increases in eNOS, iNOS and nitrotyrosine averaged 148, 236 and 179% of the pre-WBH levels. Controversy exists with respect to the effects of NO on the liver injury. It appears that NO plays a paradoxical or dual role in hepatic injury due to various causes, depending on the experimental conditions, amount of NO production and NOS isoforms (13, 15, 19). In mice subjected to WBH, increase and

decrease in NO production by pre-treatment with L-arginine and N-nitro-L-arginine methyl ester (L-NAME) affected the survival rate depending on the doses and plasma level of NO metabolites (30). Administration of L-NAME after heat stress did not affect the core temperature and the systemic hypotension (32). On the other hand, the formation of peroxynitrite as a consequence of combination of NO with superoxide anion is detrimental to cells and organs (17, 35). The presence of abundant nitrotyrosine in liver tissue may contribute to the WBH-induced hepatic injury. A recent *in vitro* study indicated that NO increased hydrogen peroxide toxicity against rat liver endothelial cells and hepatocytes through inhibition of hydrogen peroxide degradation (31).

Although excessive heatstress induces hepatic injury, preconditioning with relatively mild hyperthermia has been employed to reduce liver damage. The mechanism of protective effects of hyperthermia preconditioning has been proposed to be mediated by the induction of cytoprotective proteins and/or molecules such as heatshock proteins, heme oxygenase-1 and other factors. In addition, suppression of cytotoxic molecules such as reactive oxygen species and cytokines are also involved (22, 24, 41). We observed that there was a 8.7-fold increase in HSP 70 at 14 h after WBH. Heatshock proteins has been considered to be beneficial to heat-induced organ injury. Yang *et al*. found that induction of HSP 72 with arsenite reduced the cerebral ischemia, neural damage, and systemic hypotension, and increased the survival rate in rats exposed to heatstress (40). HSP 70 also exerted protective effects on the hepatic injury caused by ischemia-reperfusion in mice (3, 16) and acted to stimulate the immunological system under condition of oxidative stress (23). In HSP knockout mice, the hepatotoxicity of acetaminophen was enhanced (36). Furthermore, induction of heat shock response *in vivo* resulted in expression of heatshock proteins, inhibition of NF-κB, and proinflammatory cytokines with reduction in liver injury and mortality in rats following endotoxemia (8). These studies have provided evidence for the protective role of heat shock proteins in organ injury induced by different challenges.

In summary, WBH caused liver damage accompanied with significant increases in biochemical factors (BUN, Cr, AST, ALT, LDH, CPK, amylase and lipase), nitrate/nitrite, methyl guanidine, and proinflammatory cytokines (TNF_{α}, $IL\text{-}1_{\beta}$ and IL-10). Immunohistochemical staining disclosed upregulation of eNOS, iNOS and nitrotyrosine levels in liver tissue. Western blotting revealed enhancement of HSP 70. Our study suggest the association between these variables and WBH-induced hepatic damage. Excessive NO may be damaging, but NO induced by eNOS could be beneficial to the host. In addition, eNOS and HSP 70 may play a beneficial role.

Acknowledgments

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