The protective role of nitric oxide and nitric oxide synthases in whole-body hyperthermia-induced hepatic injury in rats

Chao-Fuh Chen, David Wang, Fur-Jiang Leu & Hsing I. Chen


To link to this article: https://doi.org/10.3109/02656736.2012.677928

Published online: 12 Jun 2012.
The protective role of nitric oxide and nitric oxide synthases in whole-body hyperthermia-induced hepatic injury in rats

CHAO-FUH CHEN1,2, DAVID WANG3, FUR-JIANG LEU4, & HSING I. CHEN5

1Division of Gastroenterology, Department of Internal Medicine, Cheng Hsin General Hospital, Taipei, 2Department of Healthcare Information, School of Health, Ming Chuan University, Taipei, 3Department of Medicine, College of Medicine, Fu Jen Catholic University, Taipei, 4Department of Pathology, Catholic Cardinal Tien Hospital, Taipei, Taiwan, and 5Institute of Medical Sciences and of Physiological and Anatomical Medicine, Tzu Chi University, Hualien, Taiwan

(Received 30 June 2011; revised 20 February 2012; accepted 15 March 2012)

Abstract
Purpose: The present study was designed to elucidate the role of endothelial nitric oxide (NO) synthase (eNOS), inducible NOS (iNOS)-derived NO and heat-shock protein (Hsp70) in a rat model of whole-body hyperthermia (WBH)-induced liver injury.

Materials and methods: Real-time polymerase chain reaction, immunohistochemistry and western blot were used to observe the mRNA and protein expression of eNOS, iNOS and Hsp70. Rats were exposed to hyperthermia by immersion for 60 min at a conscious state in a water bath maintained at 41°C. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used to assess liver injury 15 h after the hyperthermia challenge. Nitrosative and oxidative mediators, particularly NO and hydroxyl radical were measured.

Results: Plasma AST, ALT, hydroxyl radical, and NO were significantly increased after WBH. There were 4.14 ± 0.42, 2.82 ± 0.34 and 2.91 ± 0.16-fold increases in the mRNA expression of eNOS, iNOS and Hsp70. Immunohistochemistry and western blot showed up-regulation of eNOS, iNOS and Hsp70 protein. An eNOS inhibitor (N^O-nitro-L-arginine methyl ester (L-NAME)), or an iNOS inhibitor (aminoguanidine (AG)), significantly aggravated the liver injury. On the contrary, administration of NO precursor, L-arginine (L-ARG), attenuated the liver injury. Hsp70 inhibitor quercetin reduced Hsp70, while aggravating the WBH-induced hepatic changes.

Conclusions: WBH induces increases in eNOS, iNOS and Hsp70 expression with increase in NO release. The deleterious effects of L-NAME and AG and the protective effects of L-ARG and Hsp70 inhibitor on the liver function and pathology suggest that NO and heat shock protein play a beneficial role in the WBH-induced hepatic injury.

Keywords: Hepatic injury, nitric oxide, NOS expression, rat liver, whole body hyperthermia

Introduction
Heat stress or heat shock is a life-threatening disorder characterised by hyperpyrexia, multiple organ failure and neurological dysfunction [1, 2]. Redistribution of blood flow under heat stress condition leads to low perfusion and ischaemia in the splanchnic vascular beds. Subsequently, injury and increased microvascular permeability ensue in the mesenteric beds [3, 4]. The pathological changes induce release of endotoxin, nitric oxide (NO), proinflammatory cytokines, free radical and other mediators [5–7]. We have reported that NO overproduction may be detrimental in the hepatic injury caused by ischaemia reperfusion [8].

Controversy exists with respect to the role of NO in liver injury. It appears that NO plays paradoxical or dual effects on the hepatic injury due to various causes, depending on the experimental conditions, amount of NO production and NO synthase isoforms [9–11]. In mice subjected to whole body...
hyperthermia (WBH), increase and decrease in NO production by pre-treatment with L-arginine and N\textsuperscript{2}-nitro-L-arginine methyl ester (L-NAME) affected the survival rate depending on the doses and plasma level of NO metabolites [12]. Administration of L-NAME after heat stress did not affect the core temperature and systemic hypotension [13].

On the other hand, the formation of peroxynitrite as a consequence of NO combined with superoxide anion is detrimental to cells and organs [14–16]. The presence of abundant nitrotyrosine in liver tissue may contribute to the hepatic injury induced by whole body hyperthermia. An in vitro study has indicated the NO increases hydrogen peroxide toxicity against rat liver endothelial cells and hepatocytes through inhibition of hydrogen peroxide degradation [17].

The present study was designed to test whether heat stress-induced eNOS and iNOS mRNA and protein expression in liver tissue lead to increased NO production. In addition, the effects of eNOS and iNOS inhibitors N\textsuperscript{2}-nitro-L-arginine methyl ester (L-NAME) and aminoguanine (AG) and NO precursor L-arginine (L-ARG) on hyperthermia-induced liver injury were evaluated in order to elucidate the beneficial or detrimental role of NO in this type of liver injury. Hsp70 mRNA and protein expression were also examined to determine the role of heat shock protein in the hyperthermia-induced hepatic pathology. We used a Hsp70 inhibitor, quercetin to define the role of HSP.

Materials and methods

Preparation of animals

Male Sprague-Dawley rats (300 to 350 g, 12–16 wks old, pathogen-free) were purchased from the National Animal Centre. They were housed in a controlled environment at a temperature of 22°C ± 1°C under a 12 h/12 h light/dark cycle. Food and water were available ad libitum. Care and use of the animals followed the National Animal Center guidelines. Rats were fasted overnight prior to the operation but had access to water. Conscious rats state were exposed to hyperthermia. They were immersed in a water bath with their heads above the water. The temperature of the water was maintained at 41°C, and the rat remained in the 41°C water for 1 h. The rats were then taken out of the water bath and allowed to recover for 15 h. Blood was collected at 15 h after hyperthermia challenge and plasma samples were analysed to assess levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), methyl guanidine (MG) and NO metabolites (nitrate/nitrite).

Quantification of liver injury by measuring AST and ALT activities in plasma

Blood samples were immediately centrifuged. Plasma levels of AST and ALT were measured using a Kodak Ektachem DT60 analyser (Rochester, NY, USA) and expressed in IU/L [8].

Measurement of methyl guanidine by spectrofluorometry

Because the formation of MG is an index of hydroxyl radical production in the blood [18], we measured MG levels as a reflection of hyperthermia-induced hydroxyl radical production. A spectrofluorometer (Jusco 821-FP, Hachioji, Japan) was used, and fluorescence spectra were obtained with an emission maximum at 500 nm and excitation maximum at 395 nm. Blood samples were diluted 1:100 with distilled water. Liver tissue samples were homogenised by Kontes sonicator (KT 50, Ultrasonicator, Atlantic City, NJ, USA) with ice-cold phosphate buffer in a ratio of 1:10. Following 10 min of incubation at 4°C, cell debris was removed by centrifugation at 12,000 rpm for 20 min, and the supernatant was diluted by 1:40 to measure the MG. The assay was calibrated with authentic MG (Sigma M0377, St Louis, MO, USA).

Measurement of nitrate/nitrite by HPLC

Plasma levels of nitrate/nitrite were determined using a high-performance liquid chromatographic (HPLC) method. This method has a sensitivity of 30 pmol for both anions. As little as 0.05–0.1 mL of sample volume is required, and linearity is observed up to 60 nmol for each anion. At concentrations above 60 nmol, the detection curve was essentially linear. The non-linearity of the curve in pmol/L concentration may result in some variations in data collection. Before injection into the chromatographic system (ENO-20, Eicom Nox analyser, Kyoto, Japan), the samples were diluted and subjected to suitable clean-up procedures, and serum samples were deproteinated by ultrafiltration through membranes with a molecular mass cut-off of 3000. The samples were separated on a strong anion-exchange column (Spherisorb SAX, 250 × 4.6 mm internal diameter, 5 μm) and this separation was followed by two online post-column reactions. The first involved nitrate reduction to nitrite on a copper-plated cadmium-filled column. The second reaction involved a diazotisation-coupling reaction between nitrite and the Griess reagent (0.05% naphthylendiamine dihydrochloride plus 0.5% sulphanilamide in 5% phosphoric acid). The absorbance of the chromophore was read at 540 nm.
RNA isolation

Isolation of mRNA from liver tissues was performed using an mRNA isolation kit (QIAGEN RNeasy kits, Valencia, CA, USA). The mRNA isolated from each liver tissue sample was reverse-transcribed to cDNA following the manufacturer’s recommended procedures. The integrity of mRNA was validated by RNA gel electrophoresis. The resolution of 18S and 28S RNA would indicate the quality of mRNA isolation.

Real-time PCR

PCR primers and TaqMan-MGB probes (Table I) were designed using Primer Express V.2.0 software (Applied Biosystems, Foster, CA, USA) based on the sequences from GenBank. TaqMan-MGB probes were labelled with 6-carboxy-fluorescein (FAM) as the reporter dye. Real-time PCR was performed in a two-step process: In the first step, sample RNA (100 ng) was reverse-transcribed with 50 ng random hexamers in a volume of 20 μL using 200 U of SuperScript III reverse transcriptase and 40 U of RNaseOUT recombinant RNase inhibitor (both from Invitrogen, Carlsbad, CA, USA). In the second step, real-time PCR was carried out in a MicroAmp Optical 96-well plate using TaqMan Master Mix (Applied Biosystems), with 5 μL cDNA in each well. PCR reactions were monitored in real-time using the ABI PRISM 7000 Sequence Detector (Applied Biosystems, Foster, CA, USA). The thermal cycling conditions for real-time PCR were 1) 50°C for 2 min, 2) 95°C for 10 min, and 3) 40 cycles of melting (95°C, 15 s) and annealing/extension (60°C, 60 s). The relationship between the initial amount A of target present and the amount Xn of DNA produced after n PCR cycle can be expressed as $X_n = A \times (1 + E)^n$, where E is the amplification efficiency of one PCR step. Threshold cycle (Ct) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The variation in gene expression of candidate genes A and B is shown by $\Delta$Ct. The relative gene expression of target, normalised to an endogenous reference (18s rRNA; supplied by Applied Biosystems) and relative to a calibrator, was determined by $2^{-\Delta\text{Ct}}$ in various tissues. Less $\Delta$Ct means higher target mRNA expression before amplification.

Immunohistochemistry

Liver tissues were dissected 15 h after hyperthermia challenge for immunochemical analysis of the protein expression of eNOS and iNOS.

1) Liver tissues from hyperthermia-treated and sham-operated rats were fixed in tissue fix buffer, embedded in Super-Tek OCT compound (PS0001 and PS0002 Gene Research Laboratory, Taipei, Taiwan), and frozen in liquid nitrogen. Sections (5-μm thickness) were cut on a cryostat (Leica CM1900), then thawed and mounted onto gelatin-coated slides. All 5-μm frozen liver sections from the WBH and sham groups were used for immunohistochemical staining.

2) Liver sections were first incubated with blocking reagent, then with the appropriate dilution of primary antibody (mouse anti-rat eNOS or anti-rat iNOS monoclonal antibody at a titer of 1:50; Chemicon MAb, 13421, Temecula, CA, USA), and finally with an anti-mouse IgG-horseradish peroxidase (HRP) secondary antibody at a titre of 1:100. Sections were labelled and developed with HRP substrate solution and counterstained with a haematoxylin stain kit (PS003, Gene Research Laboratory, Taiwan).

3) To quantify immunohistochemical differences in rat liver sections without relying on subjective assessments, we used digital imaging and the Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) microimaging package. Data were collected and analysed by the method described in the user guide in the counting, measuring and classifying sections. The overall fields from each section of each tissue were digitally captured with a high-resolution cooled CCD camera (ProgRes C14, Jenoptik Laser, Optik, System, Jena, Germany) and stored as 8-bit colour images. The immunostained tissue cells were automatically highlighted by Image-Pro Plus, and the area covered by immunohistochemically positive cells (with red colour) was scored as positive and divided by the total area.

Western blot analysis

Pieces of liver tissue (5 g) were washed twice with ice-cold phosphate-buffered saline and resuspended in

---

Table I. Sequence of PCR primers and TaqMan probes for putative NOS target genes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer</th>
<th>Probe</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat eNOS</td>
<td>CCGGGACCTTTCAATCAGTACTAT</td>
<td>FAM-AGGCTGCGGAGACC-MGB</td>
<td>CCTGAAAGCGCCTGCTCAT</td>
</tr>
<tr>
<td>Rat iNOS</td>
<td>GAGAGAGATCCGGTTTCACAGTCTT</td>
<td>FAM-ACAGCGCTTTCACC-MGB</td>
<td>CGCATTAGCACAGAAGCAA</td>
</tr>
</tbody>
</table>
1 mL of ice-cold tissue lysis buffer (containing 15 mM NaCl, 100 µM ethylene diamine tetraacetic acid (EDTA), 0.1% Triton X-100, 250 µM sodium pyrophosphate, 100 µM β-glycerolphosphate, 100 µM Na3VO4, 0.1 µg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride in 2 mM Tris-HCl buffer, pH 7.5). After 5-min incubation on ice, tissues were homogenised to liquid form and transferred to microcentrifuge tubes. Following 20 to 30 min incubation at 4°C, cell debris was removed by centrifugation at 12,000 rpm for 10 min, and the supernatant was used as cell lysate and stored at −80°C when necessary. An aliquot was used to determine protein concentration using the protein quantitation kit (Gene Research, Taiwan) with bovine serum albumin as standard. A total of 30 µg of each extract was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis with a 10% polyacrylamide gel (SDS-PAGE) and transferred to PVDF membrane (0.2-µm polyvinylidine fluoride, Amersham Life Science, Arlington Heights, IL, USA). Membranes were blocked in TTBS (10% non-fat milk, 0.1% Tween-20 in Tris buffer solution). Immunodetection of eNOS, iNOS and Hsp70 were done using Gene-RL western blot assay kit (Gene Research, Taiwan). Primary antibody was used at a 1:500 dilution at 4°C overnight. Blots were washed with TTBS and incubated with horseradish peroxidase- anti-IgG antibody for 45 min. Immunocomplexes were viewed by chemiluminescence using Gene-RL western blot assay kit (Gene Research, Taiwan) and Biomax film (Kodak, Rochester, NY). Relative expressions of eNOS, iNOS and Hsp70 were normalised by an internal standard of β-actin.

Liver histology

At 15 h after WBH challenge, the animal was euthanised by an overdose of pentobarbital. A lobe of liver was taken for histological examination. The tissues were immersed in a 10% formaldehyde fixative for 24 h. The liver lobe was then washed for 8 h with tap water to remove the formaldehyde. For light microscopy the liver tissues were dehydrated with graded alcohol (70, 80, 90, 95% or absolute alcohol, each concentration for 45 min) and put into xylene for 1 h and then embedded in paraffin at 60°C. A series of 5-µm sections was cut and stained with haematoxylin and eosin (H&E). The histological changes were observed under an Axioplan microscope (Zeiss, Oberkochen, Germany). The liver injury was scored in a blind fashion. Each observer gave a grade from 0 to 6 according to the severity of hepatic changes. There were six pathologists and students. The score was blind to the observers and the average value was used for the hepatic injury.
Experimental design

The animals were randomly divided into five groups. In the WBH group \((n=12)\), rats were given saline only prior to induction of hyperthermia. In the L-NAME group \((n=12)\), rats received 5 mg/kg L-NAME (Sigma) by intraperitoneal injection 30 min prior to the induction of hyperthermia. In the AG group \((n=12)\), rats received 5 mg/kg AG...
Intraperitoneal injection of L-arginine (Sigma) by intraperitoneal injection 30 min before hyperthermia. In the L-ARG group \((n = 12)\), rats received 2 mg/kg L-ARG (Sigma) by intraperitoneal injection 30 min before hyperthermia. The doses for NO inhibitors and precursor were based on the dose range described previously \([16, 18, 19]\). Rats in the pre-WBH group \((n = 12)\) were prepared in the same manner as the control group without hyperthermia challenge, but were exposed to 37°C water in a water bath.

In the second series of experiments, we tested the effects of quercetin (QUE) on the WBH liver injury. Quercetin is an agent of Hsp70 inhibitor or antisense. QUE was given intraperitoneally at a dose of 50 mg/kg before and after WBH. The drug was kindly provided by Y.L. Yang at Chiayi University, Taiwan. In the pre-WBH group \((n = 12)\), saline was administered, and in the QUE group \((n = 12)\), QUE was introduced. The experimental protocol basically followed those described previously \([20]\).
Data analysis

Data were expressed as means ± SE. Comparisons within and among groups were made using one-way analysis of variance with repeated measures followed by a post hoc comparison with the Newman–Keuls test. Values of \( p < 0.05 \) were considered statistically significant.

Results

Real-time PCR analysis revealed marked increases in the expression of eNOS (2.8 ± 0.4-fold increase), iNOS (3.1 ± 0.5-fold increase) and Hsp70 (3.2 ± 0.8-fold increase) in the WBH-challenged group compared with the pre-WBH group (*\( p < 0.05 \), Figure 1). Western blot analysis disclosed the increases in eNOS, iNOS and Hsp70 expression. The eNOS, iNOS and HSP expression was increased 2.1 ± 0.6, 2.3 ± 0.9, and 2.2 ± 0.7-fold for eNOS, iNOS and Hsp70, respectively (Figure 2). Immunohistochemical examination of eNOS, iNOS and Hsp70 expression in liver tissue disclosed marked increases in iNOS and Hsp70 with a moderate increase in eNOS in the WBH group. There was essentially no immunostaining for eNOS, iNOS and Hsp70 in the pre-WBH group (Figure 3).

Figure 4 showed that nitrate/nitrite, the NO metabolites increased significantly after hyperthermia (**\( p < 0.001 \), significantly different between WBH and pre-WBH group). Inhibitors of eNOS and iNOS (L-NAME and AG) significantly attenuated the hyperthermia-induced NO release. NO precursor (L-arginine) increased the NO release (*\( p < 0.05 \), WBH versus pre-WBH). The changes of plasma methyl guanidine (representing hydroxyl radical) were similar to those for nitrite/nitrate. NOS inhibitors potentiated hydroxyl radical release, while NO precursor attenuated the hydroxyl radical production (*\( p < 0.05 \), WBH versus pre-WBH, Figure 4).

Figure 5 showed that there were significant increases in plasma AST and ALT after exposure to hyperthermia (**\( p < 0.001 \)). Administration of eNOS and iNOS inhibitors (L-NAME and AG) significantly increased the levels of AST and ALT. In contrast, administration of NO precursor, L-ARG reduced the levels of AST and ALT (*\( p < 0.05 \), WBH versus pre-WBH). Quantitation of the liver injury score indicated that WBH induced hepatic injury. L-NAME, AG and L-ARG did not affect the liver injury score in control rats without WBH (pre-WBH or sham group). On the contrary, L-NANE, AG and QUE aggravated the WBH-induced hepatic injury, while L-ARG reduced the liver injury score (Table II).

Western blot analysis of Hsp70 activity revealed that WBH increased the Hsp70 (*\( p < 0.05 \)). Administration of Hsp70 inhibitor, quercetin significantly enhanced the WBH-induced liver injury. This agent also attenuated the WBH-induced increase in Hsp70 activity (Figure 6). Plasma nitrate/nitrite, methyl guanidine, AST, ALT, the hepatic pathological change and white cell infiltration were greatly elevated (Table III). The results strongly support the protective effects of Hsp70 in the hyperthermia hepatic injury.

Discussion

In the present study, rats in a conscious state were subjected to whole body hyperthermia. Examinations taken 15h after WBH revealed a significant up-regulation of inducible NO synthase (iNOS)
with a significant albeit slight increase in endothelial NO synthase (eNOS) by real-time polymerase chain reaction and immunobiochemical staining. Western blot analysis disclosed that WBH significantly elevated the protein of iNOS, eNOS and Hsp70 in liver tissue. WBH significantly increased the plasma nitrate/nitrite, methyl guanidine (MG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). These biochemical changes were aggravated by pretreatment with L-NAME and AG, while attenuated by L-ARG. Histopathological examination observed liver cell injury with sequestration of white blood cells. The hepatic pathology with inflammatory cell sequestration was enhanced by L-NAME and AG, while attenuated by L-ARG. The findings that inhibition of HSP enhanced the hepatic injury is associated with white cell infiltration (WBC) in the sham (pre-WBH) and quercetin (QUE) groups (n = 12 in each group).

Table III. The plasma NOx (nitrate/nitrite), MG (methyl guanidine), aspartate aminotransferase (AST), alanine aminotransferase (ALT), liver injury score (LIS) and white cell infiltration (WBC) in the sham (pre-WBH) and quercetin (QUE) groups (n = 12 in each group).

<table>
<thead>
<tr>
<th></th>
<th>NOx</th>
<th>MG</th>
<th>AST</th>
<th>ALT</th>
<th>LIS</th>
<th>WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (pre-WBH)</td>
<td>23.82 ± 4.66</td>
<td>151.62 ± 12.86</td>
<td>34.64 ± 5.68</td>
<td>21.62 ± 4.34</td>
<td>0.28 ± 0.11</td>
<td>4.38 ± 0.19</td>
</tr>
<tr>
<td>WBH</td>
<td>124.68 ± 10.22*</td>
<td>716.02 ± 22.33*</td>
<td>81.36 ± 7.01*</td>
<td>96.20 ± 8.14*</td>
<td>3.02 ± 0.34*</td>
<td>10.89 ± 0.22*</td>
</tr>
<tr>
<td>Pre-WBH + QUE</td>
<td>34.73 ± 5.18*</td>
<td>142.48 ± 11.94</td>
<td>31.68 ± 4.24*</td>
<td>20.16 ± 3.69</td>
<td>0.34 ± 0.13</td>
<td>4.56 ± 0.4</td>
</tr>
<tr>
<td>WBH + QUE</td>
<td>349.12 ± 20.19**</td>
<td>1046.82 ± 30.24**</td>
<td>138.46 ± 8.12**</td>
<td>158.41 ± 10.21**</td>
<td>4.76 ± 0.88**</td>
<td>24.36 ± 0.84**</td>
</tr>
</tbody>
</table>

WBH, whole body hyperthermia; LAME, N°-nitro-L-arginine methyl ester; AG, aminoarginine; L-ARG, L-arginine; QUE, quercetin. *p < 0.01 versus sham (pre-WBH) group; **p < 0.05 versus WBH group.

428 C.-F. Chen et al.

In summary, the present study has demonstrated that WBH increases NO production, iNOS, eNOS and Hsp70 expression. It elevates the plasma nitrate/nitrite, methyl guanidine, AST and ALT. The WBH-hepatic injury is associated with white cell infiltrations of inflammatory factors such as cytokines [19, 22, 23]. Our and other laboratories have demonstrated the detrimental role of NO through the iNOS isoform in the lung injury caused by various causes [24–26]. Further investigations are required to elucidate the mechanisms by which NO and NO isoforms exert different effects on the organ injury. WBH induces a 6.4-fold increase in heat shock protein expression in liver tissue, a similar observation in our previous study [16, 25]. HSPs have been considered to be beneficial to heat-induced organ injury. Yang et al. found that induction of Hsp72 with arenite reduced the cerebral ischaemia, neural damage, and systemic hypotension, and increased the survival rate in rats exported to heat stress [23]. Hsp70 also exerted protective effects on the hepatic injury caused by ischaemia-reperfusion in mice [23, 26, 27] and acted to stimulate the immunological system under conditions of oxidative stress. In HSP knockout mice, the hepatic toxicity of acetaminophen was enhanced [28]. Furthermore, induction of heat shock proteins, inhibition of NF-κB, and proinflammatory cytokines with reduction in liver injury and mortality in rat following endotoxaemia [27–31]. In the present study we used quercetin, an inhibitor of Hsp70. The findings that inhibition of HSP enhanced the WBH liver damage support the contention that HSP exerts a protective role in this model of hyperthermia. These studies have provided evidence for the protective role of heat shock proteins in organ injury induced by different challenges. In a previous study using alteration in the perfusion, we found that the major site of NO production through the iNOS system was from the lung [32]. Whether WBH-hepatic injury is induced by NO generation in the liver remains a subject to be determined.

Conclusions

In summary, the present study has demonstrated that WBH increases NO production, iNOS, eNOS and Hsp70 expression. It elevates the plasma nitrate/nitrite, methyl guanidine, AST and ALT. The WBH-hepatic injury is associated with white cell...
These biochemical and pathological changes are exacerbated by NOS inhibitors with L-NAME and AG, while attenuated by L-arginine. The results suggest that NO production through the iNOS isoform is protective to this type of organ injury. A Hsp70 inhibitor, quercetin aggravated the WBH liver injury, indicating the beneficial role of HSP.

**Acknowledgements**

The authors appreciate the kind supply of quercetin and technical assistance in the real-time PCR analysis from Y.L. Yang at Chiayi University, Taiwan. We are also grateful to A. Huang and W.H. Wang for the editing of this manuscript.

**Declaration of interest:** This study was supported in part by a grant from the National Science Council (NSC 99-2320-B-320-010-MY3). The authors alone are responsible for the content and writing of the paper.

**References**


