Role of Hepatic Nitric Oxide Synthases in Rats with Thioacetamide-induced Acute Liver Failure and Encephalopathy

Hui-Chun Huang1,5, Sun-Sang Wang2,5, Cho-Yu Chan1,3,5, Yi-Chou Chen1, Fa-Yauh Lee1,4,5*, Full-Young Chang1,5, Chi-Jen Chu1,3, Han-Chich Lin1,5, Rei-Hwa Lu1, Shou-Dong Lee1,5

Divisions of 1Gastroenterology and 4General Medicine, Department of Medicine and 5Department of Medical Research and Education, Taipei Veterans General Hospital, 2Taipei Municipal Gan-Dau Hospital, and 5National Yang-Ming University School of Medicine, Taipei, Taiwan, R.O.C.

Introduction

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome related to liver damage or portal-systemic anastomosis,1 but the exact mechanism remains obscure. The influences of nitric oxide (NO) on hepatic and brain injury are controversial, mainly derived from its diverse functions: NO may be protective or toxic at various concentrations, and its synthesis by NO synthase (NOS) is under delicate control.2 Regarding the influences on the brain, NO may be implicated in vascular and blood-brain barrier permeability regulation.3 It contributes to hemodynamic changes in the brain and also to the pathogenesis of acute hepatic failure-related cerebral edema.4 In the liver, intrinsic hepatic NO generation attenuates sinusoidal perfusion failure and improves liver tissue oxygenation, thus limiting hepatocyte injury during early reperfusion after hepatic low-flow ischemia.5 In addition, NO donors ameliorated apoptotic liver damage6 or ischemia/reperfusion (I/R) injury,7 and non-selective NOS inhibitors increased mortality and decreased tissue perfusion during endotoxemia.8 In contrast, excessive NO production can be detrimental since it may downregulate cytochrome

Background: Hepatic encephalopathy is neuropsychiatric derangement secondary to hepatic decompensation or portal-systemic shunting. Nitric oxide (NO) synthase inhibition aggravates encephalopathy and increases mortality in rats with thioacetamide (TAA)-induced acute liver failure, suggesting a protective role of NO. This study investigated the roles of endothelium-derived constitutive NO synthase (eNOS) and inducible NOS (iNOS) in the liver of rats with fulminant hepatic failure and encephalopathy.

Methods: Male Sprague-Dawley rats (300–350 g) were randomized to receive TAA 350 mg/kg/day, by intraperitoneal injection or normal saline for 3 days. Severity of encephalopathy was assessed with the Opto-Varimex animal activity meter. Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, and bilirubin were measured. Hepatic iNOS and eNOS RNA and protein expressions were assessed by reverse transcription-polymerase chain reaction and Western blot analyses, respectively.

Results: The TAA group showed lower motor activity counts than the normal saline group. Hepatic eNOS, but not iNOS, mRNA and protein expressions were enhanced in the TAA group. In addition, hepatic eNOS mRNA expression was negatively correlated with total movement but positively correlated with ALT and AST. Protein expression of hepatic eNOS was positively correlated with ALT, AST and bilirubin.

Conclusion: Upregulation of hepatic eNOS was observed in rats with TAA-induced fulminant hepatic failure and encephalopathy, which might play a regulatory role. [J Chin Med Assoc 2007;70(1):16–23]

Key Words: fulminant hepatic failure, hepatic encephalopathy, nitric oxide, nitric oxide synthase, thioacetamide

*Correspondence to: Dr Fa-Yauh Lee, Division of General Medicine, Department of Medicine, Taipei Veterans General Hospital, 201, Section 2, Shih-Pai Road, Taipei 112, Taiwan, R.O.C.
E-mail: fylee@vghtpe.gov.tw • Received: July 17, 2006 • Accepted: October 11, 2006
Nitric oxide and hepatic encephalopathy

P450, suppress liver protein and DNA synthesis, then induce apoptosis and necrosis.\(^9\) NO also reacts with superoxide radical to form peroxynitrite, a potent oxidant that inhibits cellular respiration and triggers apoptosis.\(^{10}\) Supplementation of the NO precursor L-arginine even exacerbated acetaminophen-induced hepatotoxicity in rats.\(^{11}\)

It has been shown that N\(^\text{\textcircled{G}}\)-nitro-L-arginine methyl ester (L-N\textsubscript{G}NAME), a non-selective NOS inhibitor aggravated HE and increased mortality in rats with thioacetamide (TAA)-induced acute hepatic failure.\(^{12}\) Based on elevated serum levels of bilirubin, alanine aminotransferase (ALT), endotoxin, and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) after L-N\textsubscript{G}NAME administration, the worsened HE was ascribed to aggravated liver damage by non-selective NO inhibition.\(^{12}\) However, the plasma nitrate/nitrite level, which represents the amount of NO generation, was similar between L-N\textsubscript{G}NAME and control groups.\(^{12}\) Since NO acts in an autocrinic/paracrinic manner in which the blood nitrate/nitrite level might not actually reflect the amount of hepatic NO synthesis and the expression of different NOS isoenzymes in liver during HE was not clear, we conducted this study to survey the molecular basis by which isoenzyme plays a major regulatory role in TAA-treated rats with fulminant hepatic failure and HE.

**Methods**

**Animal model**

Male Sprague-Dawley rats (300–350 g) were caged at 24\(^\degree\)C, with a 12-hour light-dark cycle and free access to food and water until the time of the experiments. Fulminant hepatic failure was induced by intraperitoneal injection of TAA (350 mg/kg in normal saline; Sigma Chemical Co., St Louis, MO, USA) every 24 hours for 3 consecutive days \((n=14)\). Rats treated with normal saline were categorized as controls \((n=14)\). To avoid hypoglycemia and electrolytes imbalance,\(^{13}\) 10% glucose water mixed with lactate ringer (25 mL/kg) was injected subcutaneously every 12 hours after the first injection of TAA. Motor activities were measured 3 days after the first administration of TAA. Heparinized blood samples were obtained afterwards from the inferior vena cava for ALT, aspartate aminotransferase (AST), alkaline phosphatase, and bilirubin measurements. The liver was dissected and removed for reverse transcription–polymerase chain reaction (RT-PCR) and Western blot analysis. The experiments were conducted according to the American Physiological Society guidelines for the care and use of laboratory animals.

**Measurement of motor activities**

Motor activities were determined using the OptoVarimex animal activity meter (Columbus Instruments Inc., Columbus, OH, USA).\(^{14}\) The sensors utilize high-intensity, modulated infrared light beams to detect motion. Animals were housed in transparent cages \((17 \times 17 \times 8 \text{ inches})\) through which 30 infrared beams pass in the horizontal plane, 15 on each axis. This device differentiates non-ambulatory movements (scratching, gnawing) from ambulation on the basis of consecutive interruption of the infrared monitoring beams. An additional row of infrared beams in the horizontal plane \((15 \text{ on each axis})\) about 10 cm above the floor was used to count the vertical movements. During the measurements, animals had no access to food or chow. All studies were performed under strictly standardized conditions in the dark room for 30 minutes. The count numbers of total, ambulatory, and vertical movements were recorded to reflect the motor activities.

**RNA isolation, RT and PCR**

Hepatic total RNA was extracted with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). A 1-step RT-PCR kit (Qiagen GmbH) was used with the following components: 10 \(\mu\)L RT-PCR buffer containing Tris-HCl, KCl, (NH\(_4\))\(_2\)SO\(_4\) and 2.5 mmol/L MgCl\(_2\); 2 \(\mu\)L deoxynucleotide mixture containing 400 \(\mu\)mol/L dATP, dCTP, dGTP and dTTP, respectively; 2 \(\mu\)L enzyme mixture containing Omniscript™ reverse transcriptase, Sensiscript™ reverse transcriptase, and HotStar Taq™ DNA polymerase; 1 \(\mu\)L RNase inhibitor \((40 \text{ U})\); 3 \(\mu\)L of each random primer \((10 \text{ pmol/}\mu\text{L})\), and 1 \(\mu\)g substrate RNA. RNase-free water was added in each reaction to the final volume of 50 \(\mu\)L. The sequences of the primers for cNOS were 5’-TACGGA-GCAGCAATCCAC-3’ (sense) and 5’-CAGGCTG-CAGTCTTTTGATC-3’ (antisense), respectively. Those for iNOS were 5’-CCCTTCCGAAGTTCTGGA-GCAG-3’ (sense) and 5’-GGGCTCCTCCAAG GTGGTTGCC-3’ (antisense), respectively. A constitutively expressed gene, \(\beta\)-actin, was analyzed as control, and the primers were 5’-TTGTAAACA CTGGGACGA-TATGG-3’ (sense) and 5’-GATCTTGATCTTCA GGTCTAGG-3’ (antisense), respectively. A negative control was included in each set of experiments.

The tubes were placed in a thermocycler (Biometra® T Gradient thermocycler; Biometra GmbH, Göttingen, Germany) at 50\(^\degree\)C for 30 minutes for reverse transcription, 95\(^\degree\)C for 15 minutes for initial denaturation, followed by 35 cycles of the following sequential steps: 30 seconds at 94\(^\degree\)C (denaturation), 45 seconds at 57.2\(^\degree\)C for cNOS, 62\(^\degree\)C for iNOS, 59.6\(^\degree\)C for \(\beta\)-actin (annealing), and 45 seconds at 72\(^\degree\)C (extension). The
final extension was performed at 72°C for 10 minutes. The primers for eNOS, iNOS and β-actin were designed to allow amplification of 819, 473 and 764 base-pair fragments, respectively. Then, 10 μL of the PCR-amplified mixture was subjected to electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. Location of the predicted PCR products was confirmed by a 100-base pair ladder (GIBCO BRL, Gaithersburg, MD, USA). The gel was then photographed and the signal intensity quantitated by a digitalized software (Kodak Digital Science™ ID Image Analysis Software; Eastman Kodak Co., Rochester, NY, USA). The index of eNOS and iNOS signals were standardized against that of the β-actin signal from the same RNA sample and expressed as eNOS/β-actin and iNOS/β-actin ratios for comparison.

**Western blot analysis**

Livers were immediately frozen in liquid nitrogen and stored at −80°C until required. The protein extracts were made by pulverization in a grinder with liquid nitrogen, then using a ratio of 1 mL lysis buffer for each 100 mg powdered liver sample. The lysis buffer consisted of phosphate-buffered solution containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 0.05% protease inhibitor cocktail solution (Roche Diagnostics GmbH, Penzberg, Germany). Protein concentration was determined for each sample that dissolved in sample buffer (63 mmol/L Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.005% bromphenol blue) and 10 μg positive control were separated on denaturing SDS-10% polyacrylamide gels by electrophoresis (Mini-PROTEAN® 3 Cell; Bio-Rad Laboratories, Hercules, CA, USA).

Presaturated protein markers (SDS-PAGE Standards; Bio-Rad Laboratories) were used for molecular weight determinations. The positive controls included human aortic endothelial cell lysate (1 mg/mL) for eNOS, and mouse macrophages (RAW 264.7) stimulated with 10 ng/mL LPS for 12 hours (1 mg/mL) for iNOS (BD Transduction Laboratories, BD Biosciences Pharmingen, USA). Proteins were then transferred to a polyvinylidene difluoride membrane (Immuno-Blot™ PVDF Membrane; Bio-Rad Laboratories) by a semidyry electrophoretic system (Trans-Blot® SD Semidyry Electrophoretic Transfer Cell; Bio-Rad Laboratories) for 1.5 hours at 4°C. To block nonspecific binding, membranes were blocked for 30 minutes with 3% non-fat dry milk in TBS-T, pH 7.4 (25 mmol/L; Tris base, 137 mmol/L; NaCl 2.7 mmol/L; KCL, 1% Tween 20).

Blots were incubated with the first antibody, anti-eNOS, (1:2000) and anti-iNOS monoclonal antibodies (1:1000), diluted with 3% non-fat dry milk in TBS-T for 90 minutes at room temperature and washed. Then, the blots were incubated for 90 minutes with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG antibody, diluted with 3% non-fat dry milk in TBS-T; Sigma Chemical Co.) and washed. Subsequent detection of the specific proteins (140 kDa for eNOS, 130 kDa for iNOS) was performed by enhanced chemiluminescence (BCIP/NBT Solution; Amresco Co., Ohio, USA). With a computer-assisted video densitometer and digitalized software (Kodak Digital Science™ ID Image Analysis Software, Eastman Kodak Co.), the blots were scanned, photographed, and then the signal intensity (integral volume) of the appropriate bands was analyzed.

**Drugs**

TAA was purchased from Sigma Chemical Co. (St Louis, MO, USA).

**Statistical analysis**

All results are expressed as mean ± standard error of the mean. Statistical analyses were performed using independent t test and bivariate correlation with Pearson’s test, accordingly. Results were considered statistically significant at p < 0.05.

**Results**

**Motor activities**

The motor activities of the control and TAA groups are shown in Figure 1. The TAA group showed lower motor activity counts than the control group in total (TAA vs. control, 418 ± 88 vs. 2515 ± 356 counts/30 min, p < 0.001), ambulatory (186 ± 61 vs. 1523 ± 267 counts/30 min, p < 0.001), and vertical movements (40 ± 15 vs. 398 ± 92 counts/30 min, p = 0.002).

**Plasma ALT, AST, alkaline phosphatase and total bilirubin**

Plasma concentrations of ALT (TAA vs. control, 196 ± 24 vs. 33 ± 2 U/L, p < 0.001), AST (542 ± 53 vs. 93 ± 6 U/L, p < 0.001), alkaline phosphatase (241 ± 12 vs. 160 ± 11 U/L, p < 0.001), and bilirubin (1.1 ± 0.3 vs. 0.1 ± 0.0 mg/dL, p = 0.003) were all higher in the TAA group than in the control group.

**Analysis of NOS expression**

Semi-quantitative RT-PCR of hepatic eNOS expression was higher in the TAA group than in the control
Nitric oxide and hepatic encephalopathy

The mean intensity of eNOS signal standardized against that of β-actin signal (eNOS/β-actin) in the TAA group vs. the control group was 0.79 ± 0.07 vs. 0.45 ± 0.04 (p = 0.001). However, iNOS expression (iNOS/β-actin, 0.30 ± 0.05 vs. 0.21 ± 0.04, p = 0.148) was similar between the 2 groups.

Western blot analysis
In the control group, Western blot of liver homogenates revealed a single band at 140 kDa stained with anti-eNOS antibody and a very faint band at 130 kDa stained with anti-iNOS antibody (Figure 3). In the TAA group, eNOS protein expression was 1.8-folds
higher than in the control group (TAA vs. control, 0.63 ± 0.07 vs. 0.35 ± 0.05 [relative optical density], \(p=0.002\)), whereas iNOS protein expression was not significantly different between TAA and control rats (0.13 ± 0.05 vs. 0.12 ± 0.04, \(p=0.853\)).

**Correlations between severity of hepatic injury, HE and hepatic eNOS expression**

Plasma concentrations of ALT, AST and bilirubin showed negative correlations with total movements, indicating that more severe liver damage was associated with aggravated HE. Hepatic eNOS mRNA expression was negatively correlated with total movements and positively correlated with ALT, AST and bilirubin. In addition, liver eNOS protein showed positive correlations with ALT, AST and bilirubin, suggesting that hepatic injury and HE are accompanied by eNOS mRNA and protein upregulation (Table 1, Figure 4).

**Discussion**

NO plays a distinct role in the pathogenesis of HE. Excessive NO formation in portal hypertensive states leads to vasodilatation,\(^{19}\) which may participate in the development of HE.\(^{20}\) Cerebral vasculature dilatation increases capillary surface area and facilitates the diffusion of ammonia.\(^{21}\) In addition, cerebral blood flow autoregulation defect was found in fulminant hepatic failure.\(^{22}\) These conditions can be responsible for brain swelling and intracranial hypertension in hepatic coma. A retrospective study also revealed a strong relationship among cerebral blood flow, brain swelling, outcome and depth of coma.\(^{23}\)

According to our previous study, chronic L-NAME administration aggravates liver injury, with detrimental effects on survival and HE in TAA-treated rats.\(^{12}\) \(\text{N}^\text{ω}-\text{nitro-L-arginine (L-NNA)},\) another non-selective NO inhibitor, also showed similar results in dimethyl-nitrosamine-treated rats.\(^{24}\) NO has been known for its effects on vasodilatation and platelet aggregation inhibition: L-NNA aggravated I/R injury partly by vasoconstriction with compromised hepatic blood flow\(^{25}\) and NO prevented hepatic microvascular shutdown during endotoxemia.\(^{26}\) In a mouse model of immunologic liver damage, NO also protects the liver through vasodilatation and/or platelet aggregation inhibition.\(^{27}\) Among the NOS isoenzymes, iNOS inhibition resulted in similar changes in sinusoidal perfusion and hepatocyte injury in rats that had hepatic reperfusion injury.\(^{5}\) These findings suggest the role of eNOS in protecting the liver against various insults. In fact, constitutive
Nitric oxide and hepatic encephalopathy

Table 1. Correlation coefficients ($r$) and $p$ values between total movements, liver biochemistry parameters, and mRNA and protein expression of hepatic eNOS

<table>
<thead>
<tr>
<th></th>
<th>Total movements</th>
<th>ALT</th>
<th>AST</th>
<th>Bilirubin</th>
<th>eNOS protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>-0.543</td>
<td>0.543</td>
<td>0.514</td>
<td>0.321</td>
<td>0.056</td>
</tr>
<tr>
<td>$p$</td>
<td>0.003</td>
<td>0.003</td>
<td>0.005</td>
<td>0.096</td>
<td>0.777</td>
</tr>
<tr>
<td>eNOS protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>-0.324</td>
<td>0.533</td>
<td>0.447</td>
<td>0.424</td>
<td></td>
</tr>
<tr>
<td>$p$</td>
<td>0.093</td>
<td>0.004</td>
<td>0.017</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>$r$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>-0.589</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p$</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>$r$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>-0.614</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p$</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>$r$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>-0.543</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p$</td>
<td>0.015</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ALT = alanine aminotransferase; AST = aspartate aminotransferase.

NOS (cNOS) inhibition aggravated I/R- or endotoxin-related liver injury. An increase of I/R-induced liver injury was also demonstrated in mice genetically deficient in eNOS.

In the current study, enhanced hepatic eNOS mRNA expression and protein synthesis in rats with TAA-induced fulminant hepatic failure and HE were noted. Of interest, they showed negative correlations with total movements and positive correlations with plasma ALT, AST and bilirubin levels. In addition, negative correlations between total movements and ALT, AST, and bilirubin were found, compatible with...
the hypothesis that more severe liver injury is associated with more severe HE. One may debate the actual role of hepatic eNOS: if enhanced hepatic eNOS activity is harmful, it would be accompanied by higher ALT, AST and bilirubin concentrations and lower movement counts. However, the assumption is contradictory to recent studies on NOS inhibitors,\textsuperscript{5,12,24,28} which showed that eNOS inhibition led to detrimental effects on hepatic injury or, additionally, HE. Therefore, the inverse might be inferred: after TAA administration, hepatic damage and HE are accompanied by eNOS up-regulation in the liver. In addition, the more severe the liver damage and HE, the higher the hepatic eNOS expression, suggesting molecular evidence of NO modulation by eNOS during fulminant hepatic failure and HE. Though there was no significant correlation between eNOS mRNA and protein levels, the condition might be attributed to the following factors: first, the involvement of post-transcriptional and post-translational regulators; second, the liver was harvested at the same time for both mRNA and protein assay. Therefore, eNOS mRNA or even protein might have undergone various degrees of degradation at the time of analysis (however, as compared with the control group, eNOS mRNA and protein in TAA-administered rats did show significantly enhanced expressions).

No significant changes in hepatic iNOS mRNA and protein expression were identified in this study. Similar to our findings, iNOS mRNA upregulation was not detected in the post-ischemic liver of mice and iNOS inhibition did not exacerbate post-ischemic liver injury.\textsuperscript{30} In mice with genetic iNOS deficiency, they also suffered from similar LPS-induced liver damage in comparison with wild-type mice.\textsuperscript{31} These observations suggest that iNOS-derived NO does not play a significant role in hepatic damage. However, contradictory results have been reported: iNOS inhibition exerted beneficial effects on reperfusion liver injury in pigs.\textsuperscript{32} Furthermore, iNOS even protected the liver against LPS-induced apoptotic damage.\textsuperscript{33} The paradoxical and dual effects on hepatic injury or, additionally, HE. Therefore, the degree and onset of hepatic injury do vary and may have different influences on NO expression.

From previous studies on NOS inhibitors and the current study on hepatic NOS isoforms in rats that have undergone TAA-induced hepatic damage and HE, eNOS may be a remarkable modulator of acute hepatic injury, although NO may at the same time aggravate HE. Further tests on the optimal dose and timing of eNOS inhibition to ameliorate HE without compromising the hepatic NO level required to maintain liver homeostasis may have potential in the management of acute liver failure and HE.

Acknowledgments

The authors gratefully acknowledge Yun-Ni Hsieh and Jia-Yi Liao for their excellent technical assistance. This work was supported by grants from the National Science Council (NSC 93-2314-B-075-060) and Taipei Veterans General Hospital (VGH-93-224), Taiwan, R.O.C.

References


Furthermore, TAA 350 mg/kg injections once per day for 3 consecutive days were used in the current study, with a lower dose, a longer dosing interval and different evaluation time as compared with the previous study.\textsuperscript{35} In fact, the same group reported a mortality of 75%,\textsuperscript{37} which is much higher than the 18% mortality in our previous study.\textsuperscript{16} Therefore, the degree and onset of hepatic injury do vary and may have different influences on iNOS expression.

In previous studies of NOS inhibitors and the current study on hepatic NOS isoforms in rats that have undergone TAA-induced hepatic damage and HE, eNOS may be a remarkable modulator of acute hepatic injury, although NO may at the same time aggravate HE. Further tests on the optimal dose and timing of eNOS inhibition to ameliorate HE without compromising the hepatic NO level required to maintain liver homeostasis may have potential in the management of acute liver failure and HE.


