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

Hui-Chun Huang[1,5], Sun-Sang Wang[2,5], Cho-Yu Chan[1,3,5], Yi-Chou Chen[1], Fa-Yauh Lee[1,4,5]*,
Full-Young Chang[1,5], Chi-Jen Chu[1,5], Han-Chieh Lin[1,5], Rei-Hwa Lu[1], Shou-Dong Lee[1,5]

Divisions of [1]Gastroenterology and [4]General Medicine, Department of Medicine and [3]Department of Medical Research and Education, Taipei Veterans General Hospital, [2]Taipei Municipal Gan-Dau Hospital, and [5]National Yang-Ming University School of Medicine, Taipei, Taiwan, R.O.C.

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

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 damage[6] 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

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^G-nitro-L-arginine methyl ester (L-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-α (TNF-α) after L-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-NAME and control groups.[12] Since NO acts in an autocrinic/paracrinic manner in that 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°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 Opto-Varimex 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 ($17 \times 17 \times 8$ 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 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 μL RT-PCR buffer containing Tris-HCl, KCl, $(NH_4)_2SO_4$ and 2.5 mmol/L $MgCl_2$; 2 μL deoxynucleotide mixture containing 400 μmol/L dATP, dCTP, dGTP and dTTP, respectively; 2 μL enzyme mixture containing Omniscript™ reverse transcriptase, Sensiscript™ reverse transcriptase, and HotStar Taq™ DNA polymerase; 1 μL RNase inhibitor (40 U); 3 μL of each random primer (10 pmol/μL), and 1 μg substrate RNA. RNase-free water was added in each reaction to the final volume of 50 μL. The sequences of the primers for eNOS were 5′-TACGGA-GCAGCAAATCCAC-3′ (sense) and 5′-CAGGCTG-CAGTCCTTTGATC-3′ (antisense), respectively. Those for iNOS were 5′-CCCTTCCGAAGTTTCTGGCA-GCAG-3′ (sense) and 5′-GGGCTCCTCCAAGGTG-TTGCCC-3′ (antisense), respectively. A constitutively expressed gene, β-actin, was analyzed as control, and the primers were 5′-TTGTAACCAACTGGGACGA-TATGG-3′ (sense) and 5′-GATCTTGATCTTCATG-GTGCTAGG-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°C for 30 minutes for reverse transcription, 95°C for 15 minutes for initial denaturation, followed by 35 cycles of the following sequential steps: 30 seconds at 94°C (denaturation), 45 seconds at 57.2°C for eNOS, 62°C for iNOS, 59.6°C for β-actin (annealing), and 45 seconds at 72°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.[15–17] 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 by the Bradford method.[18] An aliquot of 20–40 μg protein from 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% bomphenol 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).

Prestained 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 interferon-γ and 1 μg/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 (Immun-Blot™ PVDF Membrane; Bio-Rad Laboratories) by a semidry electroblotting system (Trans-Blot® SD Semidry 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
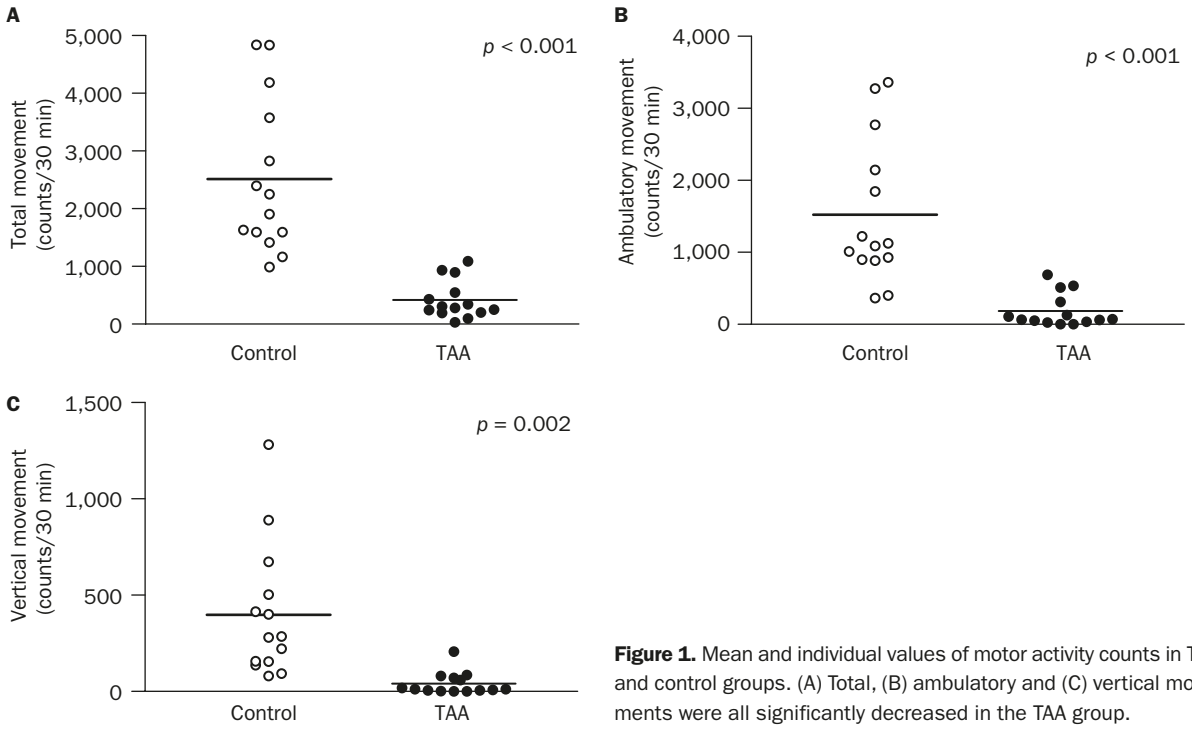
Figure 1. Mean and individual values of motor activity counts in TAA and control groups. (A) Total, (B) ambulatory and (C) vertical movements were all significantly decreased in the TAA group.
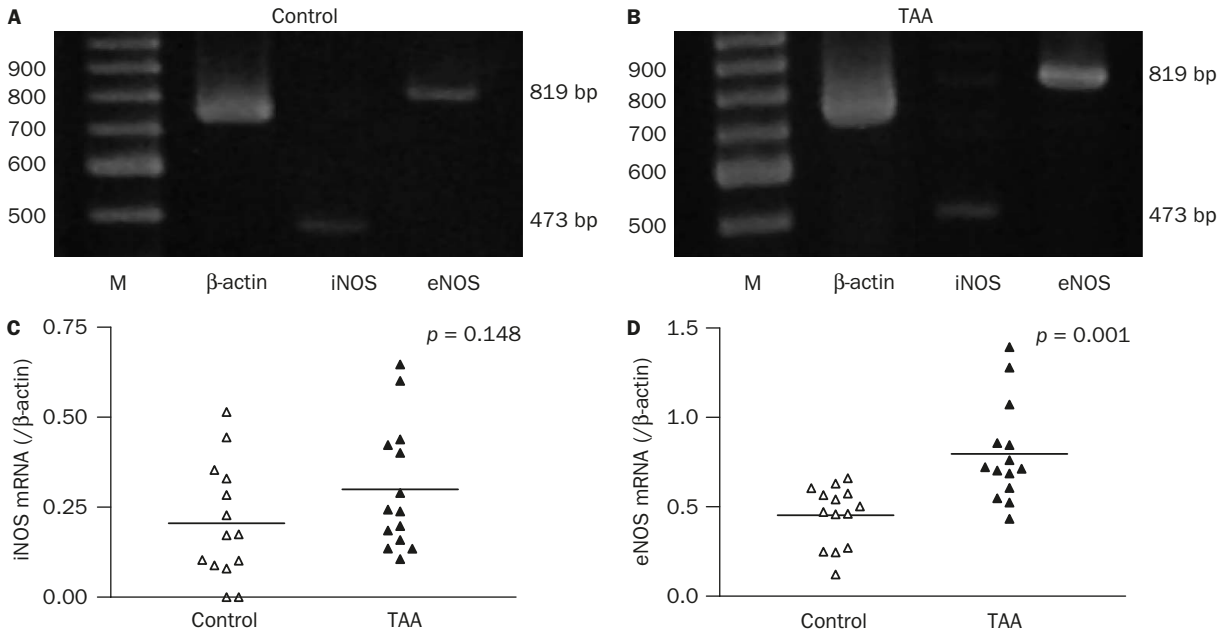

Figure 2. (A, B) Representative agarose gel electrophoresis of iNOS and eNOS cDNA in the liver of control and TAA-treated rats. Marker = 100-bp DNA ladder; iNOS = 473 bp; eNOS = 819 bp; bp = base pairs. (C, D) Semi-quantitative RT-PCR analysis of iNOS and eNOS expression.

group (Figure 2). 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
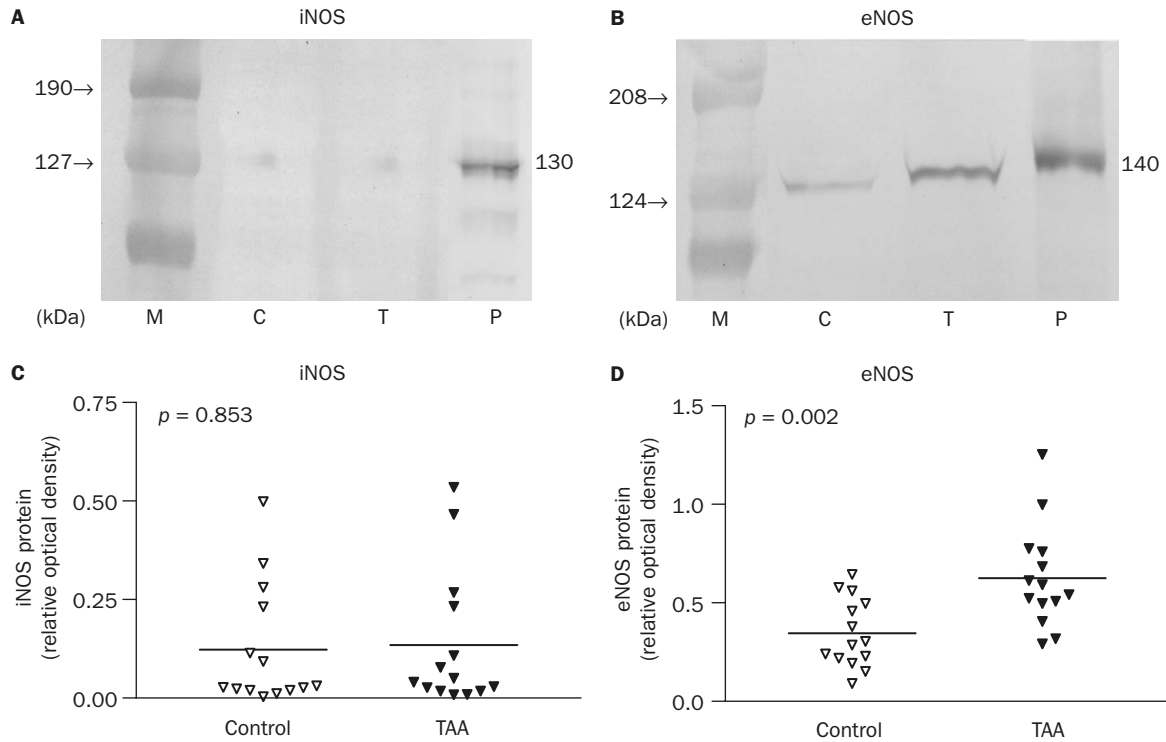
Figure 3. (A, B) Representative Western blots of iNOS (130 kDa) and eNOS (140 kDa) protein in the liver of control and TAA-treated rats. M = marker; C = control group; T = TAA group; P = positive control. (C, D) Semi-quantitative analysis of iNOS and eNOS protein expression in the liver using densitometric units.

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] N^{ω}-nitro-L-arginine (L-NNA), another non-selective NO inhibitor, also showed similar results in dimethylnitrosamine-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

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

	Total movements	ALT	AST	Bilirubin	eNOS protein
eNOS mRNA					
r	−0.543	0.543	0.514	0.321	0.056
p	0.003	0.003	0.005	0.096	0.777
eNOS protein					
r	−0.324	0.533	0.447	0.424	
p	0.093	0.004	0.017	0.025	
ALT					
r	−0.589				
p	0.001				
AST					
r	−0.614				
p	0.001				
Bilirubin					
r	−0.543				
p	0.015				

ALT = alanine aminotransferase; AST = aspartate aminotransferase.
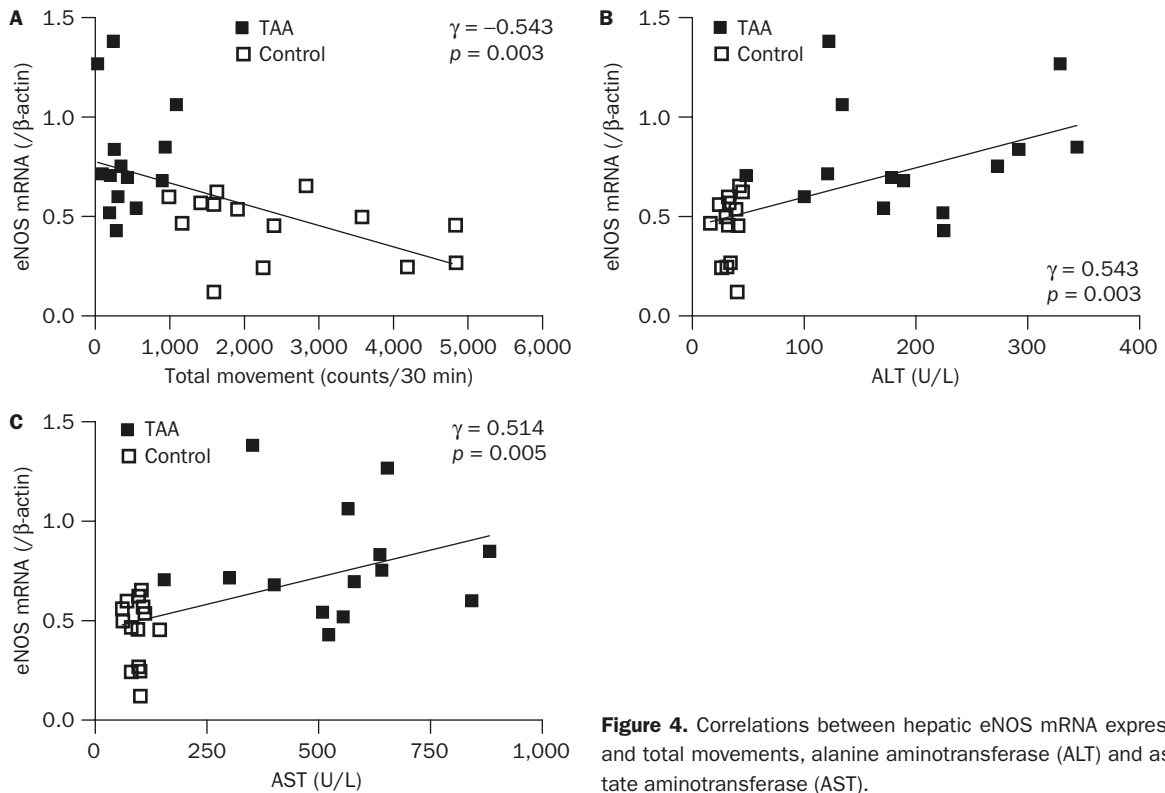
Figure 4. Correlations between hepatic eNOS mRNA expression and total movements, alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

NOS (cNOS) inhibition aggravated I/R- or endotoxin-related liver injury.[5,28] An increase of I/R-induced liver injury was also demonstrated in mice genetically deficient in eNOS.[29]

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,[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 upregulation 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.[30] In mice with genetic iNOS deficiency, they also suffered from similar LPS-induced liver damage in comparison with wild-type mice.[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.[32] Furthermore, iNOS even protected the liver against LPS-induced apoptotic damage.[33] The paradoxical and dual effects may be attributed to a great variety of inhibitors, dosages and experimental designs. Considering the similar studies on TAA-induced liver injury, it has been found that aminoguanidine pretreatment markedly diminished injury severity by decreasing oxidative stress and lipoperoxidation.[34] Aminoguanidine pretreatment also significantly reduced the severity of TAA (500 mg/kg, 2 intraperitoneal injections, 8 hours apart)-induced hepatic damage and improved mortality.[35] However, the beneficial effect might not be attributed to iNOS inhibition by aminoguanidine, since iNOS mRNA is only detected in the post-necrotic proliferative hepatocytes[34] and guanidines can even scavenge peroxynitrite.[36]

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.[35] In fact, the same group reported a mortality of 75%,[37] which is much higher than the 18% mortality in our previous study.[16] Therefore, the degree and onset of hepatic injury do vary and may have different influences on iNOS expression.

From 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.

Acknowledgments

References

1. Mousseau DD, Butterworth RF. Current theories on the pathogenesis of hepatic encephalopathy. *Proc Soc Exp Biol Med* 1994;206:329–44.
2. Hortelano S, Genaro AM, Bosca L. Phorbol esters induce nitric oxide synthase activity in rat hepatocytes. *J Biol Chem* 1992;267:24937–40.
3. Janigro D, West GA, Nguyen TS, Winn HR. Regulation of blood-brain barrier endothelial cells by nitric oxide. *Circ Res* 1994;75:528–38.
4. Master S, Gottstein J, Blei AT. Cerebral blood flow and the development of ammonia-induced brain edema in rats after portacaval anastomosis. *Hepatology* 1999;30:876–80.
5. Pannen BH, Al-Adili F, Bauer M, Clemens MG, Geiger KK. Role of endothelins and nitric oxide in hepatic reperfusion injury in the rat. *Hepatology* 1998;27:755–64.
6. Mannick JB, Asano K, Izumi K, Kieff E, Stamler JS. Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. *Cell* 1994;79:1137–46.
7. Ferraresso M, Burra P, Cadrobbi R, Calabrese F, Pettenazzo E, Sarzo G, Parnigotto A, et al. Protective effect of L-arginine on liver ischemia-reperfusion injury. *Transplant Proc* 1997;29:393–4.
8. Minnard EA, Shou J, Naama H, Cech A, Gallagher H, Daly JM. Inhibition of nitric oxide synthesis is detrimental during endotoxemia. *Arch Surg* 1994;129:142–8.
9. Milosevic N, Schawalder H, Maier P. Kupffer cell-mediated differential down-regulation of cytochrome P450 metabolism in rat hepatocytes. *Eur J Pharmacol* 1999;368:75–87.

10. Wahl SM, McCartney-Francis N, Chan J, Dionne R, Ta L, Orenstein JM. Nitric oxide in experimental joint inflammation. Benefit or detriment? *Cells Tissues Organs* 2003;174:26–33.

11. Gardner CR, Heck DE, Yang CS, Thomas PE, Zhang XJ, DeGeorge GL, Laskin JD, et al. Role of nitric oxide in acetaminophen-induced hepatotoxicity in the rat. *Hepatology* 1998;27:748–54.

12. Chu CJ, Wang SS, Lee FY, Chang FY, Lin HC, Hou MC, Chan CC. Detrimental effects of nitric oxide inhibition on hepatic encephalopathy in rats with thioacetamide-induced fulminant hepatic failure. *Eur J Clin Invest* 2001;31:156–63.

13. Gammal SH, Basile AS, Geller D, Skolnick P, Jones EA. Reversal of the behavioral and electrophysiological abnormalities of an animal model of hepatic encephalopathy by benzodiazepine receptor ligands. *Hepatology* 1990;11:371–8.

14. Ribeiro J, Nordlinger B, Ballet F, Cynober L, Coudray-Lucas C, Baudrimont M, Legendre C, et al. Intrasplenic hepatocellular transplantation corrects hepatic encephalopathy in portacaval-shunted rats. *Hepatology* 1992;15:12–8.

15. Tanoue K, Ohta M, Tarnawski AS, Wahlstrom KJ, Sugimachi K, Sarfeh IJ. Portal hypertension activates the nitric oxide synthase genes in the esophageal mucosa of rats. *Gastroenterology* 1996;110:549–57.

16. Ohta M, Tarnawski AS, Itani R, Pai R, Tomikawa M, Sugimachi K, Sarfeh IJ. Tumor necrosis factor α regulates nitric oxide synthase expression in portal hypertensive gastric mucosa of rats. *Hepatology* 1998;27:906–13.

17. Morales-Ruiz M, Jimenez W, Perez-Sala D, Ros J, Leivas A, Lamas S, Rivera F, et al. Increased nitric oxide synthase expression in arterial vessels of cirrhotic rats with ascites. *Hepatology* 1996;24:1481–6.

18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.

19. Lee FY, Colombato LA, Albillos A, Groszmann RJ. N$^\omega$–nitro-L-arginine administration corrects peripheral vasodilatation and systemic capillary hypotension and ameliorates plasma volume expansion and sodium retention in portal hypertensive rats. *Hepatology* 1993;17:84–90.

20. Groszmann RJ. Hyperdynamic circulation of liver disease 40 years later: pathophysiology and clinical consequences. *Hepatology* 1994;20:1359–63.

21. Lockwood AH, Yap EW, Wong WH. Cerebral ammonia metabolism in patients with severe liver disease and minimal hepatic encephalopathy. *J Cerebr Blood F Met* 1991;11:337–41.

22. Larsen FS, Knudsen GM, Paulson OB, Vilstrup H. Cerebral blood flow autoregulation is absent in rats with fulminant hepatic failure. *J Hepatol* 1994;21:491–5.

23. Agarwal S, Kramer D, Yonas H, Obrist W, Kang Y, Martin M, Policare R. Cerebral hemodynamic changes in fulminant hepatic failure: a retrospective study. *Hepatology* 1994;19:80–7.

24. Lukivskaya O, Lis R, Zwierz K, Buko V. Effect of the nitric oxide donor and the nitric oxide synthase inhibitor on the liver of rats with chronic hepatitis induced by dimethylnitrosamine. *Pol J Pharmacol* 2004;56:599–604.

25. Cottart CH, Do L, Blanc MC, Vaubourdolle M, Descamps G, Durand D, Galen FX, et al. Hepatoprotective effect of endogenous nitric oxide during ischemia-reperfusion in the rat. *Hepatology* 1999;29:809–13.

26. Nishida J, McCuskey RS, McDonnell D, Fox ES. Protective role of NO in hepatic microcirculatory dysfunction during endotoxemia. *Am J Physiol* 1994;267:1135–41.

27. Wang GS, Liu GT. Role of nitric oxide in immunological liver damage in mice. *Biochem Pharmacol* 1995;49:1277–81.

28. Vos TA, Gouw AS, Klok PA, Havinga R, van Goor H, Huitema S, Roelofsen H. Differential effects of nitric oxide synthase inhibitors on endotoxin-induced liver damage in rats. *Gastroenterology* 1997;113:1323–33.

29. Kawachi S, Hines IN, Laroux FS, Hoffman J, Bharwani S, Gray L, Leffer D, et al. Nitric oxide synthase and post-ischemic liver injury. *Biochem Biophy Res Co* 2000;276:851–4.

30. Hines IN, Harada H, Bharwani S, Pavlick KP, Hoffman JM, Grisham MB. Enhanced post-ischemic liver injury in iNOS-deficient mice: a cautionary note. *Biochem Biophy Res Co* 2001;284:972–6.

31. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, et al. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 1995;81:641–50.

32. Isobe M, Katsuramaki T, Hirata K, Kimura H, Nagayama M, Matsuno T. Beneficial effects of inducible nitric oxide synthase inhibitor on reperfusion injury in the pig liver. *Transplantation* 1999;68:803–13.

33. Akahori M, Yamada S, Takeyama N, Tanaka T. Nitric oxide ameliorates actinomycin D/endotoxin-induced apoptotic liver failure in mice. *J Surg Res* 1999;85:286–93.

34. Diez-Fernandez C, Sanz N, Alvarez AM, Zaragoza A, Cascales M. Influence of aminoguanidine on parameters of liver injury and regeneration induced in rats by a necrogenic dose of thioacetamide. *Br J Pharmacol* 1998;125:102–8.

35. Rahman TM, Hodgson HJ. The effects of early and late administration of inhibitors of inducible nitric oxide synthase in a thioacetamide-induced model of acute hepatic failure in the rat. *J Hepatol* 2003;38:583–90.

36. Szabo C, Ferrer-Sueta G, Zingarelli B, Southan G, Salzman AL, Radi R. Mercaptoethylguanine and guanidine inhibitors of nitric-oxide synthase react with peroxynitrite and protect against peroxynitrite-induced oxidative damage. *J Biol Chem* 1997;272:9030–6.

37. Rahman TM, Hodgson HJ. L-NAME and aminoguanidine (AMG)-selectivity and temporal differences in iNOS inhibition in a rat model of acute hepatic failure (AHF). *J Hepatol* 2000;32:395.