

Ischemia and Reperfusion of Liver Induces eNOS and iNOS Expression: Effects of a NO Donor and NOS Inhibitor

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Abstract

The aim of this study was to investigate the role of nitric oxide (NO) in hepatic ischemia-reperfusion (I/R) injury in rats. Immunohistochemistry was used to examine the protein expression of endothelial and inducible nitric oxide synthases (eNOS, iNOS) and nitrotyrosine after I/R challenges to the liver, and blood levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic dehydrogenase (LDH), hydroxyl radical and NO were measured before ischemia and after reperfusion. Ischemia was induced by occlusion of the common hepatic artery and portal vein for 40 min, followed by reperfusion for 90 min. Reperfusion of the liver induced a significant increase in the blood concentrations of AST, ALT, LDH ($n = 8$; $P < 0.001$), hydroxyl radical ($n = 8$; $P < 0.001$) and NO ($n = 8$; $P < 0.01$). The eNOS, iNOS, nitrotyrosine, SOD₁ and SOD₂ protein expression was also found to increase significantly after reperfusion ($n = 3$). Administration of the NOS inhibitor N^o-nitro-L-arginine methyl ester (L-NAME) ($n = 8$) had a protective effect on the I/R-related injury, but the NO donor L-arginine (L-Arg) ($n = 8$) potentiated the damage caused by I/R. These results suggest that reperfusion of the liver induces expression of NOS, which is related to the elevation of blood NO. The increase in hydroxyl radical concentration was accompanied by an increase in antioxidant enzyme expression (SOD₁ and SOD₂), and an increase in nitrotyrosine expression was also observed, reflecting the increased production of NO and oxygen radicals. We concluded from the protective effect of L-NAME and the potentiation by L-Arg that NOS expression and increases in NO and hydroxyl radical production have deleterious effects on the response to I/R in the liver.

Key Words: ischemia-reperfusion liver injury, NOS, NO donor, NOS inhibitor

Introduction

The liver is well recognized as a target during ischemia/ reperfusion (I/R) and inflammatory states

(20), and I/R-related injury in the liver remains an important clinical problem during shock, liver surgery, and liver transplantation. Mediators such as oxygen free radicals, nitric oxide and their reaction product

peroxynitrite have been found to be involved in I/R-mediated liver injury (3).

NO was first described in 1980 as endothelium-derived relaxing factor (EDRF) (7). It is produced by the reaction of L-Arg and nitric oxide synthase (NOS) in all mammalian cells, and the NOS inhibitor L-NAME, attenuates its production. Three forms of NOS have been identified: a constitutive form eNOS, which is mainly found in the endothelium; a neurally associated constitutive NOS nNOS, which is found in neurons; and an inducible enzyme iNOS, which causes large and continuous release of NO (18). NO is thought to play a central role in the physiology of and response to critical illness in the gastrointestinal tract (14). Endogenous NO, produced by an early and transient activation of constitutive NOS, protects both hepatocytes and endothelial cells against reperfusion injury in the liver (5). However, iNOS expression usually occurs after inflammatory responses (22).

Controversy exists regarding the effects of NO on the liver. It appears that NO plays a paradoxical role in liver physiology (11, 15). Small amounts of NO may have a cytoprotective effect (9, 11), while on the other hand, there is an increasing body of evidence indicating that overproduction of NO may damage liver function (16). Thus, NO may have both cytoprotective and cytotoxic properties, depending on the amount and isoform of NOS that causes NO production (1). This dual role for NO in hepatic I/R injury is, however, still controversial (12).

In this study we have investigated the relationship between reperfusion-related liver injury and the expression of eNOS and iNOS. We also examined the role of NO in I/R-related liver injury by elevating NO production with a NO donor and decreasing NO production with a NOS inhibitor.

Materials and Methods

Preparation of Animals

Male Sprague-Dawley rats (300 to 350 g, pathogen-free) were purchased from the National Animal Center. They were housed in a controlled environment at a temperature of $22\pm 1^\circ\text{C}$ under a 12 h/12 h light/dark cycle. Food and water were available *ad libitum*. Care and use of the animals were in accordance with the principles of the National Animal Center guidelines. Rats fasted overnight prior to the operation but were given free access to water 12 hr before the experiment. They were anesthetized with pentobarbital (50 mg/kg i.p.), and the right femoral artery was cannulated to permit administration of saline and blood sampling. The abdomen was shaved, and a mid line laparotomy was performed. Ligament attachments connecting the liver, diaphragm, ab-

dominal wall and neighboring organs were divided. The liver hilum was exposed to reveal the common hepatic artery and portal vein. Ischemia was induced by clamping the common hepatic artery and portal vein for 40 min (4). Thereafter, flow was restored and the liver was reperused for 90 min. Blood samples were obtained to determine changes in serum levels of AST, ALT, LDH, hydroxyl radical, and NO. All measurements were taken before ischemia and after reperfusion. During the operation, saline solution was given intravenously for volume replacement after each sampling. In the pilot experiments we did not find significant changes in the AST, ALT or LDH of blood samples 1 min after ischemia.

Quantification of Liver Injury by Measuring AST, ALT and LDH Activity in Serum

Blood samples were immediately centrifuged, and plasma was isolated. Plasma levels of AST, ALT and LDH were measured in an analyzer (Kodak Ektachem DT60, Rochester, NY, USA) and expressed in IU/L.

Measurement of Methyl Guanidine by Spectrofluorometry

Because the formation of methyl guanidine (MG) is an index of hydroxyl radical production in blood (20), we measured MG levels as a reflection of I/R-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. The assay was calibrated with authentic MG (Sigma M0377, St. Louis, MO, USA).

Measurement of NO

NO concentrations were measured with a chemiluminescence analyzer (Sievers 270 B NOA; Sievers Instrument, Denver, CO, USA). The quantitation of NO was based on the observation that ozone interacts with NO to generate chemiluminescent light, which can be measured by a sensitive photomultiplier tube. The chemiluminescence response is directly proportional to the NO level. The specimen was added to a gas purge chamber that had been previously evacuated to 3 mmHg to remove O_2 and stabilize NO. Signals from the detector were recorded and the peak response was used.

Immunohistochemistry

Liver tissues were dissected before ischemia and after I/R for immunochemical analysis of the

protein expression of eNOS, iNOS, SOD and nitrotyrosine.

- 1) Liver tissues from I/R-treated and sham-operated rats were fixed in tissue fix buffer and embedded in Super-Tek OCT compound (Gene Research Laboratory, Taiwan, catalog no: PS0001 and PS0002), then 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 I/R and the sham group 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, iNOS, SOD, or nitrotyrosine monoclonal antibody at a titer of 1:50; Chemicon MAb, 13421), and finally incubated with an anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody at a titer of 1:100. Sections were labeled and developed with HRP substrate solution, and counterstained with a hematoxylin and eosin 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, MD, USA) microimaging package. Data were collected and analyzed 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 GmbH, Germany) and stored as 8-bit color images. The immunostained tissue cells were automatically highlighted by Image-Pro Plus, and the area covered by immunohistochemically positive cells (with brown color) was scored as positive and divided by the total area.

Experimental Design

Animals were randomly divided into four groups. In the L-NAME group ($n = 8$) and L-Arg group ($n = 8$), rats received L-NAME (5 mg/kg; Sigma) and L-Arg (3 mg/kg; Sigma) by intravenous bolus injection 20 min prior to induction of liver I/R. In the control group ($n = 8$), rats were given no treatment except saline prior to clamping of the common hepatic artery and portal vein. Rats in the sham-operated group ($n = 7$) were prepared in the same manner as in the control group, but their vessels were not clamped. The dose used for L-NAME (5 mg/kg) was chosen because higher doses (> 5 mg/kg) have been found to raise the basal levels of liver injury indices (AST and ALT) by

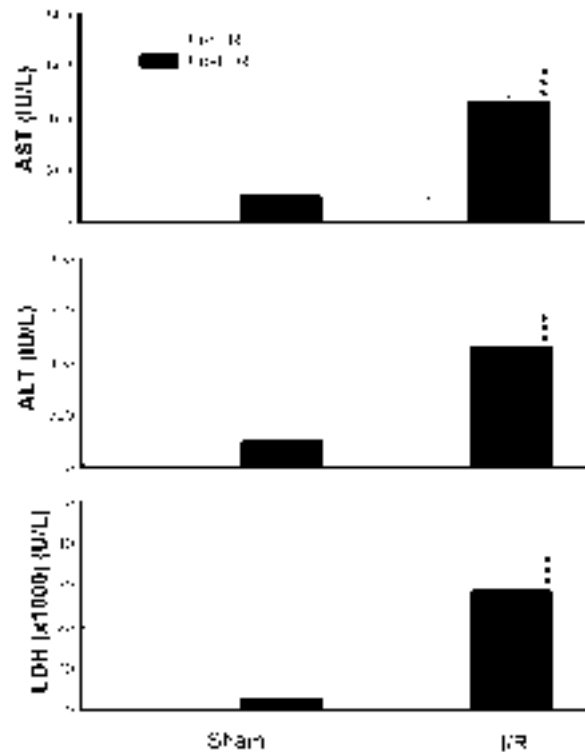


Fig. 1. AST, ALT and LDH change in the sham-operated and ischemia/reperfusion groups. ***, $P < 0.001$ significantly different between sham and post I/R group.

30%. L-Arg was used at 3 mg/kg because higher doses (4 or 5 mg/kg) are associated with very high mortality, and the basal levels of AST and ALT are also increased.

Data Analysis

Data were expressed as means \pm SEM. Comparisons among groups were made with one-way ANOVA and Scheffe's comparison. Comparisons within each group for a given parameter were made using paired Student's t tests. Values of $P < 0.05$ were considered statistically significant.

Results

Figure 1 shows that in the control group receiving saline before I/R, there was a significant increase in serum AST, ALT and LDH after exposure to I/R ($P < 0.001$). In contrast, AST, ALT, and LDH were not significantly increased in the sham-operated group.

MG, the product of hydroxyl radicals, increased significantly in the control group after I/R ($P < 0.001$, significantly different between pre I/R and post I/R); MG levels did not change in the sham-operated group before or after I/R. The results for NO changes mirrored those for MG ($P < 0.01$, significantly

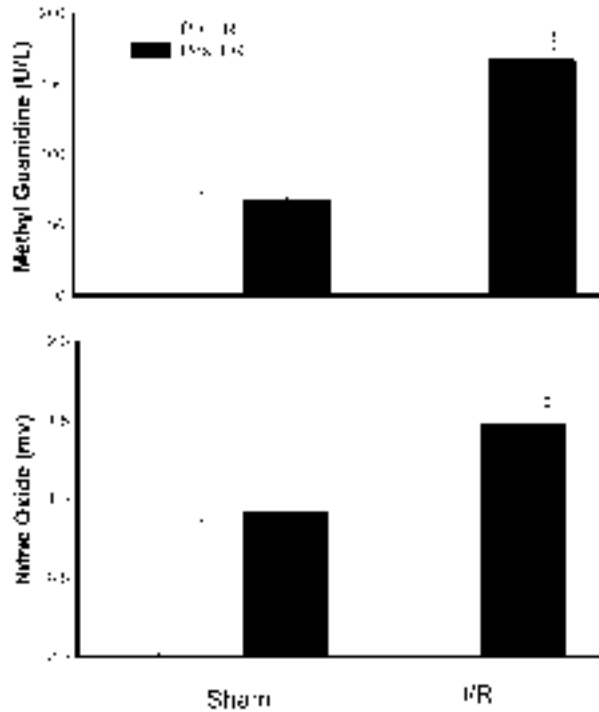


Fig. 2. Changes in methyl guanidine (MG) which represent formation of hydroxyl radicals in the sham and I/R groups. $***P < 0.001$ significantly different between pre -I/R and post -I/R; changes in nitric oxide (NO) content in the sham and I/R groups. $**P < 0.01$ significantly different between pre -I/R and post -I/R.

different between pre I/R and post I/R) (Fig. 2).

When we examined the immunohistochemical expression of eNOS and iNOS in liver tissue, we observed a marked increase in the expression of both eNOS (5.0 ± 0.6 -fold increase) in Kupffer cells and of iNOS (21.2 ± 1.6 -fold increase) in Kupffer cells and endothelial cells in the I/R group. However, hepatocytes were negative for both eNOS and iNOS. Immunostaining of eNOS and iNOS was negligible in the sham group (Fig. 3). Similarly, liver tissue showed a marked increase in cytosolic Cu/Zn-SOD (SOD_1) (4.2 ± 1.3 -fold increase) and mitochondrial Mn-SOD (SOD_2) expression (20.0 ± 1.2 -fold increase) in Kupffer cells of the I/R group (Fig. 4), but a negligible amount of immunostaining in liver tissues of the sham group. When the expression of SOD_1 and SOD_2 was compared, the I/R-induced antioxidant enzymes expression was found to occur mainly in the mitochondria.

We next examined the expression of nitrotyrosine by immunochemical staining in liver tissues of the sham and I/R groups (Fig. 5). Liver tissue in the I/R group showed a marked increase in nitrotyrosine (27.2 ± 3.3 -fold increase). In contrast, nitrotyrosine immunostaining was almost absent in the sham-operated group.

When we assayed for AST, ALT and LDH in the

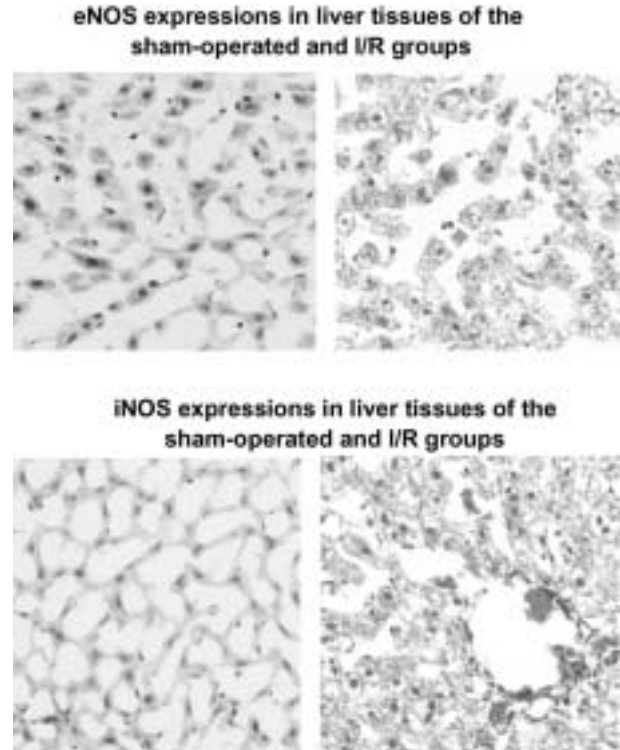


Fig. 3. Immunohistochemical staining of eNOS and iNOS activity in liver tissues of sham-operated (right panel) and I/R groups (left panel). In the sham group eNOS and iNOS immunostaining was negligible; however, in the I/R group liver tissue showed a marked increase in the eNOS (5.0 ± 0.6 -fold increase) and iNOS expression (21.2 ± 1.6 -fold increase) in the sinusoidal areas.

sham and I/R group in the presence of the NO donor L-Arg and NOS inhibitor L-NAME, the results showed that L-Arg potentiated the liver injury while L-NAME attenuated it (Fig. 6). ($P < 0.001$, significantly different between pre I/R and post I/R group $P < 0.05$; $P < 0.01$; and $P < 0.001$, significantly different between post-I/R and interventions with L-Arg and L-NAME).

Discussion

In this study we have demonstrated that L-NAME, a non-specific NOS inhibitor, was able to attenuate I/R-related liver injury and that the NO donor, L-Arg, aggravated I/R-mediated liver injury. Our results provided evidence that reperfusion liver injury induced increased in eNOS and iNOS expression in liver tissue and elevated NO in blood, and they strongly suggested that overproduction of NO was deleterious in reperfusion liver injury.

Endogenous NO produced by eNOS has been shown to protect both hepatocytes and endothelial cells against reperfusion injury (5, 13). Plasma transaminase levels were also significantly increased in eNOS knockout animals when compared to wild-type

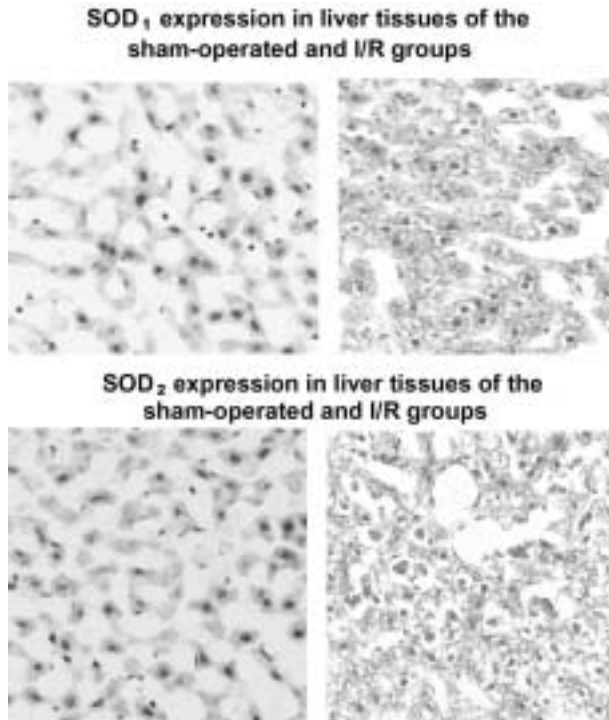


Fig. 4. Immunohistochemical staining of cytosolic Cu/Zn-SOD (SOD₁) and mitochondrial Mn-SOD (SOD₂) in liver tissues of the sham-operated and I/R groups. In the sham group (right panel) SOD₁ and SOD₂ immunostaining was negligible, whereas in the I/R group (left panel) liver tissue showed a marked increase in SOD₁ (4.2±1.3-fold increase) and SOD₂ expression (20.0±2.2-fold increase) in the sinusoidal areas.

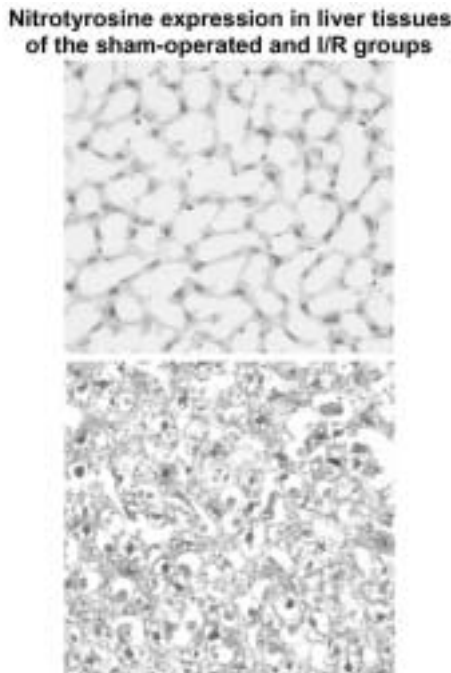


Fig. 5. Immunohistochemical staining of nitrotyrosine in liver tissues of the sham-operated (upper panel) and I/R group (lower panel). In the sham group nitrotyrosine immunostaining was almost absent. In the I/R group the liver tissue showed a marked increase in nitrotyrosine (27.2±3.2-fold increase).

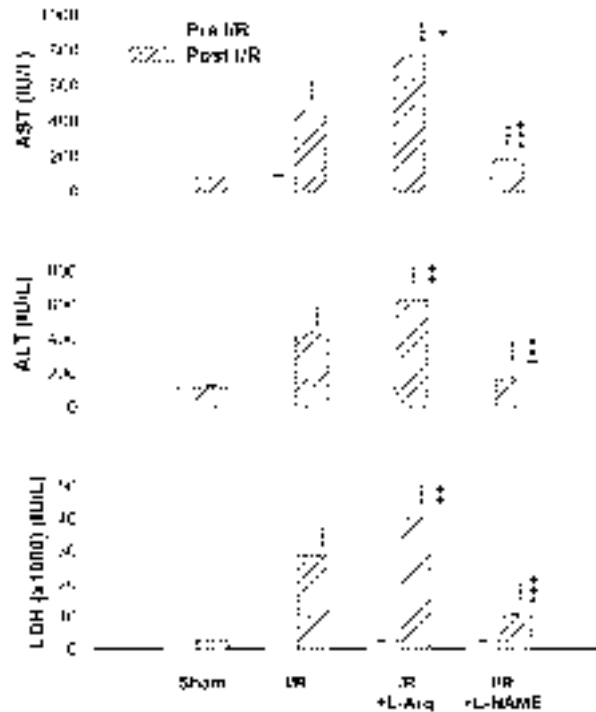


Fig. 6. AST, ALT and LDH levels in the sham-operated and I/R groups and the effect of L-arginine (L-Arg) and NOS inhibitor L-NAME. ***, $P < 0.001$ significantly different between pre -I/R and post -I/R group. +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$, significantly different between post I/R and interventions with L-Arg and L-NAME.

controls (15). Kawachi and coworkers (11) have also established that eNOS-derived NO plays an important role in limiting I/R-induced liver injury *in vivo* following 5 hr of reperfusion.

Inducible NOS expression usually occurs after the inflammatory response and can aggravate the liver injury (22). Hierholzer and coworkers (8) have shown that upregulation of iNOS plays an essential role in the initiation of the inflammatory response in the liver following hemorrhagic shock, and iNOS inhibition by N^G-(iminoethyl)-L-lysine during hemorrhagic shock likewise reduces liver injury in mice. In the swine model, reperfusion liver injury is triggered by centrilobular iNOS expression and is attenuated by inhibition of iNOS (10). Furthermore, Lee and coworkers (15) have shown that iNOS knockout mice develop significantly less hepatic injury than do wild-type mice subjected to I/R.

In the present study we used a nonspecific NOS inhibitor to attenuate I/R-related liver injury; thus, it appeared that both eNOS- and iNOS-induced overproduction of NO could mediate liver injury. Furthermore, administration of a NO donor further aggravated the liver injury. The results of this study seemed to be somewhat contradictory to other previous reports.

Serraciuo-Inglott and coworkers (21) found that eNOS was downregulated, while iNOS was expressed after hepatic lobe ischemia and reperfusion in Wistar rats. Cottart and coworkers (5) also reported that endogenous NO exerted a protective effect on I/R-related liver injury, and Liu and coworkers (16) found that L-NAME (10 mg/kg) enhanced I/R-related liver injury. These discrepancies could be attributed to differences in experimental procedure: In particular, these studies utilized hepatic lobe clamping to produce partial hepatic ischemia, whereas in the present and one previous study (4), whole-liver ischemia was produced by clamping the common hepatic artery and portal vein, thereby excluding all the blood supply to the liver. This model of whole-liver ischemia might cause endotoxin and bacteria translocation during reperfusion. Also, the dose of L-NAME or L-NNA (N^o-nitro-L-arginine) in these previously cited studies was 10 mg/kg (5, 16), which was higher than the dose (5 mg/kg) we used in our study. Different results might also be obtained in different strains or species of experimental animals. The above studies mentioned used Wistar rats (21) or rabbits (25), whereas in this study we used Sprague-Dawley rats. Finally, the reperfusion time might also have affected the results, since the reperfusion time after release of the liver lobe clamping was as long as 4 to 6 hr in the previous studies (5, 16, 21) whereas we subjected the liver to reperfusion for only 90 min.

In addition to NO, reactive oxygen radicals are also involved in the I/R-related liver injury. Oxidative species are thought to play a pivotal role in the pathogenesis of I/R-related injury (24). Bilzer and coworkers (2) have also shown that the generation of reactive oxygen species by activated Kupffer cells contributes to liver injury following I/R, shock, or endotoxemia. In the present study we demonstrated that I/R induced an elevation in the blood concentration of hydroxyl radicals which was accompanied by an increase in antioxidant enzymes expression in liver tissue, and in particular of mitochondrial Mn SOD (SOD₂). The proposed mechanisms of liver injury during I/R included massive oxygen radical and NO production. When NO was elevated, its most thermodynamically favorable reaction was with the superoxide radical to form peroxynitrite, a potent oxidant (6). Substantial evidence has indicated that peroxynitrite could act on the mitochondria to inhibit cellular respiration, triggering apoptosis. In this process peroxynitrite could act to nitrate proteins and such nitrated proteins have been identified in the liver, as shown in this study and others (20, 23).

The liver injury generated in the present study was attenuated by the administration of the NOS inhibitor L-NAME, perhaps as the result of a reduction in peroxynitrite, hydroxyl radical and nitro compound

production. Similar results have been observed by Meguro *et al.* (19) and Kimura *et al.* (12). In both studies they demonstrated that NOS inhibition by ONO-1714 and aminoguanidine could attenuate reperfusion liver injury. On the other hand, administration of a NO donor, L-Arg, exacerbated the liver damage. One possible explanation for this result is that the NO donor provided more nitric oxide to react with the oxygen radicals to produce peroxynitrite, hydroxyl radical and nitrosative compounds during I/R challenge to the liver.

In summary, reperfusion of ischemic liver tissue induced protein expression of eNOS and iNOS, through whose action large amounts of NO were released to induce nitrosative stress. At the same time, oxidative stress induced an increase in hydroxyl radical production, and the interaction of oxidative stress and nitrosative stress potentiated the damage to liver tissue. The NOS inhibitor attenuated the posts ischemic and reperfused liver injury, and a NO donor aggravated the injury, demonstrating that upregulated NOS expression after reperfusion was involved in the reperfusion-related liver injury.

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