Genetic overexpression of eNOS attenuates hepatic ischemia-reperfusion injury

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Although the role of NO in hepatic I-R injury has been controversial with a number of studies (3) that cite the protective and deleterious effects of NO therapy, it is now generally accepted that eNOS-derived NO is cytoprotective in I-R injury (4, 37). Studies (24, 37) have shown that eNOS significantly contributes to the cytoprotection of hepatic tissue against I-R injury and that injury is exacerbated in eNOS-deficient mice (16, 19). In correlation, eNOS overexpression has been reported (20) to reduce I-R injury.

The aim of the present study was to investigate the effects of chronic genetic overexpression of eNOS on the severity of hepatic I-R injury. We hypothesized that the enhanced production of NO would protect the ischemic liver via an NO-mediated pathway. In additional studies, we utilized pharmacological agents to investigate the cytoprotective mechanisms related to genetic overexpression of eNOS.

MATERIALS AND METHODS

Chemicals and Reagents

1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) was purchased from Alexis Biochemicals (San Diego, CA) and utilized as an inhibitor of soluble guanylyl cyclase (sGC) (14). It was administered intraperitoneally at a dose of 30 mg/kg at 22.5 min of ischemia, dissolved in a volume of 100 μl DMSO.

3-(5'-Hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), an sGC activator (13), was obtained from Alexis Biochemicals. It was dissolved in DMSO and diluted in normal saline, and 100 μl were injected intraperitoneally at a dose of 20 mg/kg at 22.5 min into ischemia.

Zinc (III) deuterooporphyrin IX-2,4-bisethyleneglycol (ZnDPBG) is an inhibitor of heme oxygenase-1 (HO-1) activity (6). It was purchased from Alexis Biochemicals (San Diego, CA) and utilized as an inhibitor of soluble guanylyl cyclase (sGC) (14). It was administered intraperitoneally at a dose of 30 mg/kg at 22.5 min of ischemia, dissolved in a volume of 100 μl DMSO.

Sildenafil citrate 1-({3-[6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl][4-ethoxyphe- nyl}sulfonyl)-4-methyl-piperazine citrate is a highly selective inhibitor of phosphodiesterase type 5 (PDE-5). It was dissolved in saline and administered intraperitoneally at a dose of 2 mg/kg at 22.5 min into ischemia.

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**Results**

**Characterization of eNOS-TG Mice**

Western blot analysis of hepatic protein lysate revealed a substantial increase in eNOS protein in both Kobe (bovine eNOS-TG) and RT (human eNOS-TG) mice (Fig. 1A). Calculation of relative optical density (Fig. 1B) demonstrated that the Kobe eNOS-TG mouse had an approximate sixfold increase in hepatic eNOS expression. Likewise, the RT eNOS-TG mouse was found to have an approximate sevenfold increase in hepatic eNOS protein expression.

**Liver Enzyme Determination**

Serum samples were analyzed for alanine aminotransferase (ALT) using Infinity ALT (GPT) reagent purchased from Thermo Electron. This transaminase is liver specific and is released during injury.

**Statistical Analyses**

Data were analyzed by Student’s t-test or one-way ANOVA with post-Tukey analysis where appropriate using Prism software (San Diego, CA). Data are reported as means ± SE. P values < 0.05 were considered statistically significant.

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Ion chromatographic analysis of plasma nitrate and nitrate in RT eNOS-TG mice revealed significant (P < 0.05) increases compared with that in WT littermates (Fig. 2). Plasma nitrite was increased by 44%, and in correlation plasma nitrate was increased by 47% in RT eNOS-TG mice. Hepatic nitrite levels
were also significantly increased from 0.685 ± 0.131 μM in WT mice to 2.058 ± 0.193 μM in RT eNOS-TG mice (Fig. 3A, *P < 0.001). Gas-phase chemiluminescence revealed that hepatic nitroso levels were 2.2-fold higher in RT eNOS-TG mice vs. WT littermates (Fig. 3B, **P < 0.01). Hepatic NO-Heme levels were also found to be increased, although not significantly, in eNOS-TG compared with those in nontransgenic control mice (Fig. 3C).

**Hepatic cGMP Levels in eNOS-TG Mice**

Quantitative ELISA analysis of hepatic lysate revealed a 60% increase in cGMP from 13.34 ± 1.30 pmol/mg protein in WT mice to 21.32 ± 4.17 pmol/mg protein in RT eNOS-TG mice.

**Hemodynamic Analysis in eNOS-TG Mice**

WT, Kobe eNOS-TG, and RT eNOS-TG mice were implanted with radiotelemetry pressure transducers to assess arterial blood pressure and heart rate (Table 1). eNOS-TG (Kobe and RT) mice displayed no significant differences in heart rate, mean arterial blood pressure, systolic blood pressure, or diastolic blood pressure compared with those in WT control mice.

**eNOS Overexpression Attenuates Hepatic I-R Injury**

The systemic overexpression of bovine eNOS (Kobe eNOS-TG) significantly decreased hepatic tissue injury after 45 min of ischemia followed by 5 h of reperfusion (Fig. 4). Hepatic tissue injury was evaluated by serum ALT (Fig. 4A). WT control animals displayed a mean serum ALT level of 718.6 ± 54.37 U/l versus the Kobe eNOS-TG animals whose mean serum ALT level was 197.0 ± 48.38 U/l (*P < 0.001 vs. WT).

To further our understanding of genetic overexpression of eNOS, we looked at the effects of chronic overexpression of the human eNOS gene (RT eNOS-TG). We found that overexpression of human eNOS also protected against hepatic I-R injury, showing a 2.6-fold decrease in serum ALT (Fig. 4B).
The WT control group showed a mean serum ALT level of 732.3 ± 69.37 U/l, whereas the human eNOS-TG mouse showed a mean serum ALT level of 278.5 ± 48.38 U/l (P < 0.001 vs. WT). These data clearly demonstrate that increasing the availability of NO limits the extent of I-R injury.

**sGC-cGMP Pathway in Hepatic I-R Injury**

**sGC inhibition.** To examine the downstream signaling effects of NO, we evaluated the role of sGC in hepatic I-R injury (Fig. 5). First we looked at the inhibition of sGC in WT animals. Blocking sGC with the potent inhibitor ODQ (30 mg/kg) exacerbated hepatic I-R injury in WT mice (Fig. 5A), showing a mean serum ALT level of 1,278 ± 100.6 U/l versus 1,061 ± 68.88 U/l in mice receiving vehicle (P < 0.001 vs. vehicle).

**sGC activation.** In contrast, stimulation of sGC in WT animals with YC-1 (20 mg/kg) significantly attenuated hepatic I-R injury (Fig. 5B). The mean serum ALT value for vehicle was 1,061 ± 68.88 U/l compared with a mean value of 488.9 ± 93.38 U/l (P < 0.001 vs. vehicle) for the treated group. These results suggest that sGC plays a pertinent role in protecting against hepatic I-R injury.

**PDE-5 inhibition.** To further identify the role of the sGC-cGMP pathway in hepatic I-R injury, we utilized the potent and selective PDE-5A inhibitor sildenafil. Sildenafil was administered intraperitoneally at 22.5 min into ischemia to see if inhibiting the breakdown of cGMP would protect against hepatic I-R injury (Fig. 5C). Animals treated with sildenafil were significantly protected compared with animals receiving vehicle. The sildenafil group showed a mean serum ALT level of 210.6 ± 35.72 U/l versus 707.5 ± 61.42 U/l for the vehicle (P < 0.001). These results suggest the preservation of cGMP limits hepatic I-R injury.

**eNOS overexpression and sGC inhibition.** To further investigate the possible mechanism that sGC stimulation by NO leads to cytoprotection in hepatic I-R, we looked at the effect of sGC inhibition in the setting of eNOS overexpression (Fig. 5D). Inhibition of sGC in the eNOS-TG mouse increased tissue injury showing a mean serum ALT value of 511.6 ± 78.68 U/l versus vehicle (313.0 ± 28.87 U/l, P < 0.05). Although sGC inhibition abolished the protective effects of NO in the eNOS-TG mouse, these animals maintained significant protection compared with WT mice receiving ODQ (1,728 ± 100.6 U/l). Although these results suggest the involvement of sGC in NO-mediated protection (eNOS overexpression), other protective signaling pathways may also be involved.

**HO-1 Pathway in Hepatic I-R Injury**

**HO-1 inhibition.** We also investigated the possible participation of the HO-1 pathway in hepatic I-R (Fig. 6). First, we studied the effect of pharmacological inhibition with the enzyme ZnDPBG (Fig. 6A). In WT mice administered ZnDPBG, hepatic I-R injury was greatly exacerbated, displaying a mean serum ALT of 1,349 ± 1,349 U/l compared with the vehicle group with a mean ALT value of 926.3 ± 48.92 U/l (P < 0.05).

**HO-1 activation.** HO-1 activation was also examined pharmacologically using the compound CoPP. Tissue injury was significantly decreased (2.8-fold) in animals receiving CoPP (Fig. 6B). Serum ALT levels measured 195.4 ± 45.56 U/l in the treated group and 554.3 ± 88.05 U/l in the vehicle group (P < 0.01 vs. vehicle). These results suggest the HO-1 pathway is involved in limiting the extent of I-R injury.

**eNOS overexpression and HO-1 inhibition.** Inhibition of HO-1 in the eNOS-TG mouse was investigated to determine whether NO-mediated protection was dependent on HO-1.
signaling (Fig. 6). Our findings did not show a significant difference between the ZnDPBG group and the vehicle group. The mean serum ALT concentrations for the treated versus nontreated groups were 441.4 ± 94.6 U/l and 336.6 ± 59.86 U/l (P not significant), respectively. These data suggest that the cytoprotective effects of NO in this experimental model system are independent of HO-1 signaling.

DISCUSSION

In the present study we found that genetic overexpression of eNOS protects against hepatic I-R injury. We investigated two distinct strains of eNOS-overexpressing mice. One mouse (Kobe eNOS-TG) featured the bovine eNOS gene and a second mouse featured the human eNOS gene (RT eNOS-TG). In both mouse models, we observed a 2.5- to 3.5-fold decrease in serum ALT levels after 45 min of ischemia and 5 h of reperfusion. These results, which support our initial hypothesis, were expected because Lefer and colleagues (20) had previously observed a reduction of myocardial infarct size in both mouse models after subjecting them to myocardial I-R. Whereas eNOS overexpression was found to be associated with elevated levels of NO-related metabolites in blood and liver and higher concentrations of hepatic cGMP, enhanced systemic NO formation was not accompanied by significant differences in heart rate and blood pressure, rendering hemodynamic changes an unlikely contributor to eNOS-mediated protection against hepatic I-R injury.

The soluble isoform of guanylyl cyclase is an important cellular target of NO. Thus, to further investigate the protective role of eNOS overexpression in hepatic I-R, a series of pharmacological approaches aimed at modulating cGMP availability was used to assess the suspected involvement of the NO-cGMP pathway. First, we looked at the downstream effects of direct pharmacological inhibition of sGC by ODQ. NO binds to the heme group of sGC, resulting in enzyme activation and accumulation of the second messenger cGMP. ODQ oxidizes the heme group of sGC from the ferrous to the ferric form, to which NO has a poorer binding affinity (32), preventing enzyme stimulation. Direct sGC inhibition increased serum ALT levels in WT animals after hepatic I-R, suggesting that sGC function plays a pivotal role in attenuating I-R injury.

We next examined the effects of sGC activation with YC-1 on the severity of hepatic I-R injury in WT animals. This compound allosterically binds to sGC and instigates a conformational change, resulting in an enhancement of stimulation by NO (13). In addition, YC-1 can slow down sGC deactivation by maintaining the association of NO with sGC for a longer time period (2). YC-1 stimulation of sGC attenuated hepatic injury in WT mice subjected to 45 min of ischemia followed by 5 h of reperfusion.

In an attempt to further define the role of the sGC-cGMP pathway, we looked at the direct effects of preventing cGMP breakdown using the potent PDE-5A inhibitor sildenafil. Mice treated with sildenafil were significantly protected against hepatic I-R injury. Taken together, these data suggest that the NO-sGC-cGMP axis plays a critical role in limiting the extent of hepatic I-R injury.

The pharmacological inhibition of sGC was next utilized to investigate the downstream pathways responsible for hepato-cellular protection in eNOS-TG mice. ODQ partially reduced
In the bars represent the number of animals used in each group.

P* mice (Kobe eNOS-TG) receiving DMSO vehicle or ZnDPBG (10 mg/kg).

administered saline vehicle or the HO-1 activator cobalt (III) protoporphyrin IX chloride (CoPP, 5 mg/kg).

or the HO-1 inhibitor HO-1 zinc (III) deuteroporphyrin IX-2,4-bisethyleneglycol (ZnDPBG, 10 mg/kg).

support of the notion that the sGC-cGMP pathway is protective

protection was dependent on cGMP production. In further

shown to inhibit apoptosis, but the protective effects of the NO

failure in mice, two NO donors and two cGMP analogs were

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was cited the protective effects of the sGC-cGMP pathway in

hepatoprotection in the eNOS-TG mouse. This would suggest that HO-1 is not featured in

mediated signaling.

Extensive evidence (8, 23, 35) exists showing that HO-1 is

protective in I-R injury, although the mechanism of this still

remains unclear. Our data are congruent with existing findings

regarding the actions of this enzyme during I-R. It has been

reported that HO-1 activity within the liver decreases leukocyte

interaction with the endothelium (38), possibly by suppressing the

induction of adhesion molecules (36). HO-1 has also been re-

ported to suppress inflammatory cytokine pathways involved in

hepatic I-R injury (34). These findings suggest that HO-1 is

central in limiting the inflammatory response in hepatocytes.

Inhibition of this enzyme has also been shown to increase the

magnitude and duration of apoptotic cell death (28), whereas

overexpression has been shown (23) to limit apoptosis after I-R

injury. However, although we found HO-1 to be protective in WT

animals, it seemed to play no role in the protection seen in

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utilized pharmacological stimulation of

HO-1 with the compound CoPP (22) significantly decreased by

2.8-fold serum ALT levels in the liver after 45 min of ischemia

and heme oxygenase is the rate-limiting step in the oxidative degra-

dation of heme. The heme breakdown products carbon monoxide (21)

and biliverdin (12) have been shown to be protective in I-R injury.

HO-1 belongs to the class of heat shock proteins, and its

expression is quickly induced in many cell types in response to

oxidative insults, such as in I-R injury (33).

We first examined the effects of pharmacologically inhibiting

the HO-1 enzyme in WT mice utilizing ZnDPBG. ZnDPBG is a metalloporphyrin that has previously been shown to

selectively inhibit liver heme oxygenase at very low concentrations (6). We found that inhibition of HO-1 significantly

increased hepatocellular injury by 3.8-fold after hepatic I-R. In agreement with these findings, pharmacological stimulation of

HO-1 with the compound CoPP (22) significantly decreased by

2.8-fold serum ALT levels in the liver after 45 min of ischemia

and 5 h of reperfusion. These results suggest that HO-1 does

indeed play a role in limiting the extent of hepatic I-R injury.

We next sought to determine whether the hepatoprotection

observed after I-R injury in eNOS-TG mice also involved the

HO-1 pathway. We subjected the eNOS-TG mouse to 45 min of ischemia and 5 h of reperfusion after administration of the

HO-1 inhibitor ZnDPBG and found no significant difference in

serum ALT levels compared with those in the vehicle group.

These data demonstrate that NO-mediated hepatoprotection

seen in the eNOS-TG mouse is likely independent of HO-1-

mediated signaling.

In conclusion, we have shown that eNOS overexpression sig-

nificantly attenuates hepatic I-R injury. Utilizing pharmacological

modulation, we investigated the role of sGC-cGMP and HO-1

pathways in I-R injury. We found that both pathways were

prominent in limiting the extent of I-R injury in WT mice, but

only the sGC-cGMP pathway was found to play a role in the

protection resulting from eNOS overexpression. These results

suggest that NO-mediated hepatoprotection in I-R injury involves

the protective effects of eNOS overexpression. These data suggest that hepatic cytoprotection in the eNOS-TG mouse

may involve the sGC-cGMP pathway. However, NO-mediated

hepatoprotection was not completely reduced with ODQ, sug-

gesting that additional pathways contribute to the protection provided by eNOS overexpression.

In agreement with our results, many recent reports have

cited the protective effects of the sGC-cGMP pathway in

hepatic I-R injury. In a study (1) of endotoxin-induced hepatic

failure in mice, two NO donors and two cGMP analogs were

shown to inhibit apoptosis, but the protective effects of the NO

donors were abolished by a sGC inhibitor, suggesting that the

protection was dependent on cGMP production. In further

support of the notion that the sGC-cGMP pathway is protective

in hepatic I-R, a study utilizing a rat model (7) found the cGMP

analog (8-bromo-cGMP) to protect the ischemic liver. Furthermore, preserving endogenous cGMP levels with sildenafil has

been shown to protect against myocardial I-R injury in num-

erous animal models (26). These aforementioned studies along

with our current findings point to the sGC-cGMP pathway as being critical in NO-mediated cytoprotection.

To address the nature of additional protective pathways, we

next investigated the potential role of HO-1 in hepatic I-R injury. Heme oxygenase is the rate-limiting step in the oxidative degra-
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