Gender differences in hepatic ischemic reperfusion injury in rats are associated with endothelial cell nitric oxide synthase-derived nitric oxide

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Abstract

AIM: This study was designed to examine the hypothesis that gender differences in I/R injury are associated with endothelial cell nitric oxide synthase (eNOS)-derived nitric oxide (NO).

METHODS: Wistar rats were randomized into seven experimental groups (12 animals per group). Except for the sham operated groups, all rats were subjected to total liver ischemia for 40 min followed by reperfusion. All experimental groups received different treatments 45 min before the laparotomy. For each group, half of the animals (six) were used to investigate the survival; blood samples and liver tissues were obtained in the remaining six animals after 3 h of reperfusion to assess serum NO, alanine aminotransferase (ALT) and TNF-α levels, liver tissue malondialdehyde (MDA) content, and severity of hepatic I/R injury.

RESULTS: Basal serum NO levels in female sham operated (FS) group were nearly 1.5-fold of male sham operated (MS) group (66.7±11.0 μmol/L vs 45.3±10.1 μmol/L, P<0.01). Although serum NO levels decreased significantly after hepatic I/R (P<0.01, vs sham operated groups), they were still significantly higher in female rat (F) group than in male rat (M) group (47.8±8.6 μmol/L vs 23.8±4.7 μmol/L, P<0.01). Serum ALT and TNF-α levels, and liver tissue MDA content were significantly lower in F group than in M group (370.5±46.4 U/L, 0.99±0.11 μg/L and 0.57±0.10 μmol/g vs 668.7±78.7 U/L, 1.71±0.18 μg/L and 0.86±0.11 μmol/g, respectively, P<0.01). I/R induced significant injury to the liver both in M and F groups (P<0.01 vs sham operated groups). But the degree of hepatocyte injury was significantly milder in F group than in M group (P<0.05 and P<0.01). The median survival time was six days in F group and one day in M group. The overall survival rate was significantly higher in F group than in M group (P<0.05). When compared with male rats pretreated with saline (M group), pretreatment of male rats with 17β-estradiol (E2) (M+E2 group) significantly increased serum NO levels and significantly decreased serum ALT and TNF-α levels, and liver tissue MDA content after I/R (P<0.01). The degree of hepatocyte injury was significantly decreased and the overall survival rate was significantly improved in M+E2 group than in M group (P<0.01 and P<0.05). The NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) treatment could completely abolish the protective effects of estrogen in both male and female rats.

CONCLUSION: The protective effects afforded to female rats subjected to hepatic I/R are associated with eNOS-derived NO.

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Key words: Gender identity; Liver; Reperfusion injury; Endothelial constitutive nitric oxide synthase

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INTRODUCTION

Hepatic ischemic reperfusion (I/R) injury is commonly seen in the field of hepatic surgery, such as in the course of hepatectomy, liver transplantation and resuscitation after shock. The injurious effect of I/R presents with a spectrum of clinical manifestations ranging from asymptomatic elevation of liver enzymes to acute liver failure and death. Although liver transplantation has become an accepted therapy for end-stage liver disease because of improved techniques, I/R injury resulting in primary liver graft dysfunction and failure continues to pose significant clinical problems[1].

An increasing body of evidence indicates that gender...
differences exist in cardiovascular and immunologic responses to various adverse circulatory conditions, including vascular occlusive disease, hypertension, stroke, shock, and atherosclerosis\[8,9\]. In addition, clinical studies have shown that female patients survived better than males after liver transplantation or hepatocellular carcinoma resection\[9,10\]. The underlying mechanism of these gender differences remains to be elucidated.

It has been suggested that the response of the hepatic endothelium to I/R plays a key role in the development of injury\[28,29\]. Estrogen has been postulated as a “survival factor” for endothelial cells\[30,31\]. Both women and men have functional α and β estrogen receptors expressed in endothelial cells\[31,32\]. Estrogen not only can rapidly induce the release of endothelial cell nitric oxide synthase (eNOS)-derived nitric oxide (NO) via a nongenomic manner\[31,32\], but also can transcriptionally activate the expression of eNOS in endothelial cells via the more classical genomic mechanisms\[33,34\]. Because we have previously demonstrated that constitutive isoform of NO synthase (eNOS, also known as eNOS)-derived NO plays an important role in limiting hepatic I/R injury in a model of full-size liver I/R\[10,19\], we hypothesized that the protective mechanism observed in females subjected to hepatic I/R may also depend on eNOS-derived NO.

MATERIALS AND METHODS

Animals

Normal male and female Wistar rats weighing 290-350 g were purchased from the Center of Experimental Animal in Tongji Medical College, Huazhong University of Science and Technology. This project was approved by the Tongji Medical College, and the procedures were carried out according to the routine animal-care guidelines.

Reagents

N'-nitro-L-arginine methyl ester (L-NAME) and 17-β-estradiol (E\(_2\)) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Model of total hepatic I/R

A model of total hepatic I/R was used as described previously\[18,19\]. Briefly, rats were anesthetized with ether inhalation, and the midline laparotomy was performed to expose the liver. The total hepatic ischemia was achieved by occluding the hepatic arterial and portal venous blood by using a microaneurysm clip. Rats were then given an ip dose of heparin (200 U/kg) to prevent blood coagulation. After 40 min of ischemia, reperfusion was initiated by removal of the clip. Sham operated control rats were treated in an identical fashion but without vascular clamping. The abdominal cavity was closed, and the rats were allowed to recover with free access to food and water. Rats were then observed daily until d 7 post-surgery to assess survival or were killed after 3 h of reperfusion, and blood samples and liver tissues were obtained for analysis.

Blood and tissue analyses

Serum alanine aminotransferase (ALT) was measured by using standard techniques with a serum analyzer (HITACHI 7170A autoanalyzer, Japan). TNF-α concentration was determined with a commercial radioimmunoassay kit (East Asia Immunotechnology Institute, Beijing, China). Serum NO products nitrite/nitrate (NO\(_2\)/NO\(_3\)) were detected using a colorimetric NO detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The liver tissue malondialdehyde (MDA) concentration was determined using thiobarbituric acid test (assay kit was purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Morphometric assessment of I/R injury

Excised liver specimens were fixed in 40 g/L formaldehyde and embedded in paraffin. Hematoxylin-eosin-stained sections (5 μm) were evaluated at 200× magnification by a point-counting method for severity of hepatic injury by using an ordinal scale as follows\[20\]: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, loss of intercellular borders, and mild to moderate neutrophil infiltration; and grade 3, severe injury with disintegration of hepatic cords, hemorrhage, and severe polymorphonuclear cell infiltration. An average of 100 adjacent points on a 1-mm\(^2\) grid was graded for each specimen (n = 4).

Experimental design

Rats were randomized into seven experimental groups (12 per group). Apart from the sham operated groups, all rats were subjected to I/R injury. Each group received different treatments 45 min before the laparotomy. The seven groups consisted of group 1, male sham operated rats, 2 mL saline given intravenously (MS group); group 2, female sham operated rats, 2 mL saline given intravenously (FS group); group 3, male rats, 2 mL saline given intravenously (M group); group 4, female rats, 2 mL saline given intravenously (F group); group 5, female rats, L-NAME (10 mg/kg) dissolved in 2 mL saline, given intravenously (F + L-NAME group); group 6, male rats, E\(_2\) (4 000 μg/kg) dissolved in 2 mL saline, given intravenously (M+E\(_2\) group); and group 7, male rats, E\(_2\) (4 000 μg/kg)+L-NAME (10 mg/kg), dissolved in saline, given intravenously (M+E\(_2\)+L-NAME group). For each group, half of the rats (six) were used to investigate the survival, and blood samples and liver tissues were obtained in the remaining six rats for analysis after 3 h of reperfusion.

Statistical analysis

All values are expressed as mean±SD. Statistical significance between two groups of parametric data was evaluated by using an unpaired Student’s t test. Survival was analyzed by using the Kaplan-Meier method, and the difference in overall survival rate was evaluated using the log rank test. Statistical significance was accepted at P<0.05.

RESULTS

Differences in serum NO, ALT and TNF-α levels, and liver tissue MDA content

There were no significant differences between MS and FS groups with respect to serum ALT (Figure 1) and TNF-α
levels (Figure 2), and liver tissue MDA (Figure 3) content. But serum NO levels were significantly higher in FS group than those in MS group (66.7±11.0 μmol/L vs 45.3±10.1 μmol/L, P<0.01, Figure 4). Serum ALT and TNF-α levels, and liver tissue MDA content increased significantly, whereas serum NO levels decreased significantly after hepatic I/R both in M and F groups (P<0.01 vs sham operated groups). As compared with M group, serum NO levels were significantly higher (47.8±8.6 μmol/L vs 23.8±4.7 μmol/L, P<0.01), but serum ALT and TNF-α levels, and liver tissue MDA content were significantly lower in F group (370.5±46.4 μmol/L vs 668.7±78.7 μmol/L, P<0.01). Pretreatment with L-NAME in female rats or co-pretreatment with E₂ and L-NAME in male rats significantly increased serum NO levels vs those in F or M+E₂ group animals, respectively, after hepatic I/R (P<0.01 or P<0.05).

**Differences in degree of hepatocyte injury**

No significant differences in degree of hepatocyte injury between the MS and FS groups were noted (Table 1). Liver architecture was well conserved and liver cells were morphologically normal with a typical plate appearance. I/R induced significant injury to the liver both in M and F groups (P<0.01 vs sham operated groups). But the degree of hepatocyte injury was significantly milder in F group vs M group determined by a point-counting method with an ordinal scale (P<0.05 and P<0.01).

**Differences in survival**

A survival advantage was seen in F group vs M group. The median survival time was six days in F group vs one day in M group. The overall survival rate was significantly higher in F group vs M group (P<0.05).

**Worsening of hepatic I/R injury of female rats by NOS inhibitor L-NAME**

Significantly lower serum NO levels and significantly higher serum ALT and TNF-α levels, and liver tissue MDA content were documented after I/R in female rats pretreated with L-NAME (F+L-NAME group) compared with female rats pretreated with saline (F group) (P<0.01, Figures 1-4). The degree of hepatic injury was exacerbated (Table 1) and the overall survival rate was significantly decreased after hepatic I/R in F+L-NAME group rats compared with F group rats (P<0.01 and P<0.05). The median survival time decreased from six days in F group to one day in F+L-NAME group.
Suppression of the protective effects of 17β-estradiol (E2) on hepatic I/R injury by L-NAME in male rats

When compared with male rats pretreated with saline (M group), pretreatment of male rats with E2 (M+E2 group) significantly increased serum NO levels and significantly decreased serum ALT and TNF-α levels, and liver tissue MDA content after I/R (P<0.01, Figures 1-4). The degree of hepatocyte injury was significantly decreased (Table 1) and the overall survival rate was significantly improved in M+E2 group vs M group (P<0.01 and P<0.05). The median survival time increased from one day in M group to less than seven days in M+E2 group. Significantly lower serum NO levels and significantly higher serum ALT and TNF-α levels, and liver tissue MDA content were shown after hepatic I/R in male rats pretreated with E2 and L-NAME (M+E2+L-NAME group) compared with male rats given E2 alone (M+E2 group) (P<0.01, Figures 1-4). The protection of E2 on I/R injury of the liver was completely reverted by the co-administration of L-NAME in male rats. The degree of hepatocyte injury was exacerbated (Table 1) and the overall survival rate was significantly decreased after hepatic I/R in M+E2+L-NAME group vs M+E2 group rats (P<0.01 and P<0.05). The median survival time decreased from less than seven days in M+E2 group to 1.5 d in M+E2+L-NAME group.

**DISCUSSION**

In the present study we found that female rats were protected to a much greater extent from the injurious effects of hepatic I/R than were male rats, and E2 pretreatment could induce protection to male rats on hepatic I/R injury. We also found that the NOS inhibitor L-NAME could abolish these gender differences and revert protection induced by estrogen pretreatment on male rats. As we have previously proved that hepatic I/R does not induce the expression of inducible NOS (iNOS) in our model[14,22], the results of the current study clearly demonstrated that the protective effect afforded by estrogen was associated with eNOS-derived NO.

Estrogen may increase NO production from eNOS by genomic and/or nongenomic responses[13-17]. It was confirmed that estrogen might up-regulate eNOS gene expression to increase NO production in vascular endothelial cells as well as sinusoidal endothelial cells from liver[16,21]. Additionally, engagement of estrogen receptor-α for estrogen can mediate Ca²⁺ uptake and “activate” existing eNOS. It was demonstrated that this rapid nongenomic production of eNOS-derived NO involved a rapid, PI3-kinase-dependent activation of Akt and consequent serine phosphorylation of eNOS[14,22]. Consistent with these observations, we found that basal serum NO levels in female sham operated rats were nearly 1.5-fold of those in males. Although serum NO levels decreased significantly after hepatic I/R (P<0.01, sham operated groups), which was due to endothelial cell injury or dysfunction in our full size hepatic I/R model, they were still significantly higher in F group than those in M group rats (P<0.01). The precise mechanisms by which eNOS-derived NO protects female rats from the injurious effects of I/R remain to be identified. One possible mechanism may involve the antioxidant properties of eNOS-derived NO[23]. NO is known to inhibit reactive oxygen species (ROS)-mediated reactions and it has been suggested that the protective effects in a variety of conditions are due to the ability of NO to detoxify ROS such as O₂⁻, OH⁻; and/or ferryl hemoprotein[24]. This might be one of the main causes why liver tissue MDA content was significantly lower in F group than that in M group. Another mechanism by which eNOS-derived NO may exert protection in our model of I/R is vasodilation and enhanced perfusion of the posts ischemic tissue[14,25]. We have previously demonstrated that eNOS-derived NO may counteract the physiological effect of endothelin-1 (ET-1), a potent and long-lasting vasoconstrictive peptide[26,27]. Other mechanisms by which eNOS-derived NO may protect the liver from hepatic I/R-induced injury may be related to its inhibition of platelet aggregation and adhesion as well as attenuation of endothelium-leukocyte interactions, all of which may be beneficial to reduce hepatic I/R injury[26]. TNF-α has been shown to play a critical role in inflammatory responses during hepatic I/R[27]. We found in this study that serum TNF-α levels were significantly lower in F group than those in M group. It was postulated that the anti-inflammatory properties of estrogen might be due to its inhibition of transcription factor nuclear factor κB[28] and this might be via endogenous signal molecule NO or other signal molecules. It was also found that eNOS-derived NO may protect animals after hepatic I/R by protecting the liver from the injurious effects of TNF-α[29].

We also studied the protective effect of estrogen on I/R injury of the liver in male rats. In previous studies, administration of 4 000 μg/kg E2 was found to increase plasma E2 in mice comparable to levels seen in female mice in the proestrous state[30]. We found that male rats pretreated with 4 000 μg/kg E2 were protected from the injurious

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**Table 1** Degree of hepatocyte injury (determined by a point-counting method with an ordinal scale) (n = 4, mean±SD)

| Group       | Grade 0 (%) | Grade 1 (%) | Grade 2 (%) | Grade 3 (%) |
|-------------|-------------|-------------|-------------|-------------|
| MS          | 99.4±0.1    | 0.6±0.1     | 0           | 0           |
| FS          | 99.4±0.2    | 0.6±0.2     | 0           | 0           |
| M           | 0.2±0.1     | 8.6±1.7     | 76.9±2.4    | 14.4±7.2    |
| F           | 2.7±1.0     | 37.2±3.7    | 52.2±3.2    | 7.9±1.4     |
| F+L-NAME    | 1.0±0.3     | 16.0±4.6    | 75.1±4.1    | 8.0±1.0     |
| M+E2        | 4.0±1.2     | 57.7±3.9    | 32.9±5.3    | 5.5±0.5     |
| M+E2+L-NAME | 1.6±0.6    | 28.5±1.9    | 62.3±3.1    | 7.6±1.3     |

There were no significant differences between MS and FS groups with respect to degree of hepatocyte injury. *P*<0.01 vs MS group, *P*<0.01 vs FS group, *P*<0.05 vs M group, *P*<0.01 vs M group, *P*<0.05 vs F group, *P*<0.01 vs F group. Significant lower serum NO levels, and liver tissue MDA content were shown after hepatic I/R in male rats pretreated with E2 (M+E2 group) compared with male rats given E2 alone (M+E2 group) (P<0.01, Figures 1-4).
effects of hepatic I/R to an extent not less than that of female rats. The role of eNOS-derived NO as a crucial component for the protective effects afforded by estrogen was confirmed in studies using L-NAME-treated female and male rats. Since some of the protective effects of estrogen are NO-independent, it was puzzling that L-NAME treatment could completely abolish the protective effects of estrogen in both male and female rats in this study. We postulated that L-NAME treatment would aggravate endothelial cell injury or dysfunction in our full size hepatic I/R model, which might conceal the NO-independent protective effects of estrogen.

In conclusion, the results of this study demonstrate that the protective effects afforded to female rats subjected to hepatic I/R are associated with eNOS-derived NO. These results may have important implications for the design of novel strategies against ischemic reperfusion injury in the future.

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