Estrogen improves the hyperdynamic circulation and hyporeactivity of mesenteric arteries by alleviating oxidative stress in partial portal vein ligated rats

Bin Zhang, Cheng-Gang Zhang, Quan-Bo Zhou, Wei Chen, Zhi-Yong Wu

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INTRODUCTION

Portal hypertension (PHT) is a major complication of liver cirrhosis and is associated with the development of hyperdynamic circulation, characterized by elevated portal pressure (PP), increased splanchnic (small intestine) blood flow, increased cardiac output, and development of an extensive network of portosystemic collaterals.[8] PP is determined by extrahepatic factors such as portal blood flow and collateral resistance in addition to intrahepatic resistance. Therefore, it would be triggered by persistent splanchnic vasodilation. Functional changes can be found in the portal hypertensive splanchnic vasculature, including splanchnic vasodilation and decreased responsiveness to vasoconstrictors as a result of endothelial dysfunction and impaired activation of vasoconstrictive mechanisms.[9] Although the levels of vasoconstrictors such as norepinephrine (NE) and angiotensin-II (Ang-II) are increased in the blood circulation and are accompanied by enhanced sympathetic excitability in PHT, the splanchnic vasculature remains dilated.[3] Hyporeactivity of the artery in response to vasoconstrictors plays a key role in blood vessel dilation and hyperdynamic circulation.[3,9] Vascular hyporeactivity has been observed in PHT animals in both the systemic circulation and in the mesenteric artery, and it is also affected by gender and estrogen[9]. In addition, experimental studies have shown that estradiol (E2) reduces PP and increases hepatic blood flow in castrated and cisthmotic model rats, but the effect is significantly inhibited by the estrogen antagonist ICI182, 780[7,9].

Both acute and chronic liver diseases are characterized by an increased formation of reactive oxygen species (ROS), as indicated by increased superoxide anion in the whole blood of cirrhotic patients with decompensated liver and increased urinary F2-isoprostanes and lipoperoxidation.[4,5] Increased oxidative stress may lead to impaired endothelial dysfunction and be involved in the pathogenesis of PHT. It has therefore been a subject of interest[5,9,10].

The aim of the present study was to investigate whether estrogen could attenuate the severity of hyperdynamic circulation and the underlying mechanism in PHT rats without cirrhosis, with a focus on oxidative stress. Thus, to exclude the hepatoprotective and antifibrogenic effects of estrogen, the PHT was induced by partial portal vein ligation (PPVL) but not by the injection of hepatotoxic drugs, such as CCl4 and dimethylnitrosamine, or by bile duct ligation.

MATERIALS AND METHODS

Animal model and treatment

This animal study was approved by the local animal ethics committee of Renji Hospital and performed according to the guidelines of the Laboratory Animal Care and Use Committee at School of Medicine, Shanghai Jiao Tong University (Shanghai, China). Fifty female Sprague-Dawley rats weighing 200-220 g underwent ovarietomy before the study. Three weeks after the initial surgery, the rats underwent PPVL or sham operation (SO) as previously described by Reiberger et al[1] and were divided into five groups: SO, PPVL + placebo (PLAC), PPVL + E2, PPVL + ICI and PPVL + E2 + ICI. Briefly, under ketamine anesthesia (intramuscular injection of 10 mg/100 g body weight), the portal vein was isolated, and stenosis was induced by a single ligature of 3-0 silk placed around both the portal vein and a 20-gauge blunt-tipped needle. The needle was then removed, leaving a calibrated stenosis of the portal vein. Portal hypertension was considered present at 14 d after surgery. In SO animals, the portal vein was isolated and similarly manipulated but not ligated. Starting on the day of surgery, the PPVL + E2, PPVL + ICI and PPVL + E2 + ICI groups were subcutaneously administered β-estradiol (2 μg/100 g body weight/d, R and D Systems, United States) or/and ICI182, 780 (2 μg/100 g body weight/d, R and D Systems, United States) for 14 d. The SO and PPVL groups were subcutaneously administered the same volume of the placebo (ethanol).

Hemodynamic measurements

Under anesthesia induced by an intramuscular injection of ketamine (10 mg/100 g body weight), the velocity and inside diameter of the portal vein (PV) proximal to the ligation and stroke volume (SV) were obtained using a Vevo770 High-Resolution Imaging System (Visual Sonics, Canada). A 22 G catheter filled with heparin saline was inserted into the femoral artery to obtain the mean arterial pressure (MAP) and heart rate (HR). Another 22 G catheter was introduced into the portal vein to measure PP, and one was inserted into the inferior vena cava to measure central venous pressure (CVP) after making an incision at the midline of the abdomen. All parameters were recorded using an SP840 pressure transducer and a multichannel recorder (Philips, United States).

Cardiac output (CO), cardiac index (CI), PV blood flow, portal venous inflow (PVI), systemic vascular resistance (SVR) and splanchnic arteriolar resistance (SAR) were calculated as follows: CO = SV × HR; CI = CO × 100/body weight (g); PVI = PV blood flow × 100/body weight (g); SVR = (MAP - CVP)/CI; SAR = (MAP - PP)/PVI. PV blood flow and CO were normalized by body weight and presented as CI and PVI. Resistances in the vascular systems were calculated from the ratio between the perfusion pressure (P) and blood flow (Q) of each vascular territory.

Determination of mesenteric arteriole reactivity to NE

Following the determination of hemodynamic measurements, the mesenteric arteries and the mesentry were removed. Using an SMZ-168 dissecting microscope (Motic, China), the third-order arterioles in the mesentry were carefully dissected and transferred to a vascular perfusion system containing a 3-(N-morpholino) propanesulfonic acid-buffered physiological salt solution (MOPS-PSS, 0-4 °C, pH 7.4, NaCl 145 mmol/L, KCl 5.0 mmol/L, CaCl2 2.0 mmol/L, MgSO4 1.0 mmol/L, NaH2PO4 1.0 mmol/L, NaHCO3 30 mmol/L) and oxygenated with 95% O2 and 5% CO2. After an equilibration period of 1 h, each vascular territory was subjected to a stepwise increase in vascular perfusion pressure (0-90 mmHg) at 10-mmHg intervals. The responses of the mesenteric arterioles to NE were determined. Two areas were measured: the arteriolar lumen diameter and the cross-sectional area of the mesenteric arteriole. The arteriolar lumen diameter and cross-sectional area of the mesenteric arteriole were measured using a computerized image analysis system (Fujifilm, Japan). Each arteriolar lumen diameter and cross-sectional area were measured three times and the average value was calculated.
Table 1 Hemodynamic data of the five animal groups and maximum contraction and EC50 in the five groups

|                   | SO          | PPVL + PLAC | PPVL + E2 | PPVL + ICI | PPVL + E2 + ICI |
|-------------------|-------------|-------------|-----------|------------|-----------------|
| Body weight (g)   | 248 ± 15    | 261 ± 22    | 245 ± 18  | 231 ± 23^a | 244 ± 13        |
| HR (bpm)          | 440 ± 32    | 412 ± 25^a  | 415 ± 35^a| 419 ± 21^a | 406 ± 28^a      |
| MAP (mmHg)        | 95 ± 6      | 85 ± 11^a   | 88 ± 9^a  | 89 ± 5^a   | 84 ± 8^a        |
| PP (mmHg)         | 4.6 ± 1.2   | 14.9 ± 0.9^b| 12.2 ± 1.6^b| 14.2 ± 1.5^b| 13.9 ± 1.3^b    |
| PV flow (mL/min)  | 8.52 ± 2.3  | 12.71 ± 3.1 | 9.76 ± 2.8| 11.87 ± 3.5| 13.04 ± 3.8     |
| PVI (mL/min/100 g)| 3.43 ± 1.2  | 4.87 ± 1.6^a| 3.98 ± 1.4^a| 5.14 ± 1.8^a| 5.34 ± 1.9^a    |
| CO (mL·min−1)     | 54.68 ± 4.9 | 61.79 ± 5.9 | 52.07 ± 5.1| 56.82 ± 6.6| 57.91 ± 6.0     |
| CI (mL/min/100 g) | 22.05 ± 1.9 | 23.67 ± 2.1 | 21.25 ± 2.0| 24.60 ± 2.2| 23.79 ± 2.5     |
| SVR (mmHg·min100 g/mL) | 4.08 ± 0.48 | 13.49 ± 2.2 | 19.29 ± 1.8^a| 14.94 ± 1.7 | 13.12 ± 1.5^b |
| SAR (mmHg·min100 g/mL) | 26.33 ± 0.27 | 50.47% ± 3.48^c | 70.65% ± 2.42^d| 51.37% ± 4.12^d| 54.33% ± 4.71% |
| Emax              | 75.18% ± 4.52% | 50.47% ± 3.48% | 70.65% ± 2.42% | 51.37% ± 4.12% | 54.33% ± 4.71% |
| EC50 (10−6 mol/L) | 2.77 ± 0.74 | 5.27 ± 0.88^b | 3.77 ± 0.69^a | 4.89 ± 0.76c | 3.85 ± 0.52^d   |

Significant differences are marked with superscripts showing the P values. ^aP < 0.05, ^bP < 0.01 vs sham operation (SO); ^cP < 0.05, ^dP < 0.01 vs partial portal vein ligation (PPVL) + placebo (PLAC); ^E2: Estrogen; HR: Heart rate; MAP: Mean arterial pressure; PP: Portal pressure; PV: Portal vein; CO: Cardiac output; CI: Cardiac index; PVI: Portal venous inflow; SVR: Systemic vascular resistance; SAR: Splanchnic arteriolar resistance.

mmol/L, glucose 5.0 mmol/L, pyruvate 2.0 mmol/L, EDTA 0.02 mmol/L and MOPS 3.0 mmol/L). A glass micropipette containing MOPS-PSS (top diameter, 50 μm) was inserted into one end of the arteriole and fixed with 11-0 single strands. Blood was flushed out at a perfusion pressure of 8 mmHg. Another glass micropipette was then inserted into the other end of the arteriole and fixed. The two glass micropipettes were suspended in organ baths containing 60 mL of MOPS-PSS (37 °C, pH 7.4). The arteriole was equilibrated under a pressure of 80 mmHg for 30 min prior to the experiments. After the equilibration period, cumulative NE concentration response curves (10^−6 mol/L-10^−4 mol/L) were obtained by increasing the concentration in quarter-log increments. The level of hydrogen peroxide (H₂O₂) was measured by a hydrogen peroxide assay kit (Abcam, United States). In the presence of horseradish peroxidase (HRP), the OxiRed Probe reacts with H₂O₂ to produce product with color. The superior mesenteric artery was cleaned of connective tissue, precipitated with RIPA (Beyotime, China; 200 μL RIPA/20 mg tissue) for 15 min and then centrifuged for 15 min at 1000 g. A total of 5 μL of the supernatant was diluted with 46 μL of assay buffer, mixed with 50 μL of the Reaction Mix (assay buffer 46 μL, OxiRed Probe 2 μL, HRP 2 μL) and then incubated at room temperature for 10 min. The OD570 nm was read with a Synergy 4 Multi-Mode Microplate Reader (BioTek, United States), and the H₂O₂ concentration was calculated according to a standard concentration curve.

Statistical analysis
The change in the reactivity of the mesenteric arteriole in response to NE was presented as a dose-response curve, which was fitted by a nonlinear regression analysis (GraphPad Software Inc., San Diego, CA, United States). Maximal responses (Emax) and effective concentrations causing half maximum responses (EC50, calculated by regression analysis) were obtained from concentration response curves. Values are expressed as the means ± SD. Statistical comparisons were performed using one-way ANOVA. P values < 0.05 were considered significant. All statistical analyses were performed using the Statistical Package for the Social Sciences 13.0 (SPSS Inc., United States).

RESULTS

Hemodynamics
Compared to SO, PPVL resulted in a lowered HR (P < 0.05) and MBP (P < 0.05) and significantly increased PP (P < 0.01) in the four groups of prehepatic PHT. However, measurement of MAP and HR revealed no significant differences between the four PPVL groups (P > 0.05). PP significantly decreased by 18.5% in PPVL + E2 rats compared to PPVL + PLAC rats (3.98 ± 1.4 mL/min per 100 g vs 4.06 ± 2.8 mL/min per 100 g, P < 0.01) (Table 1).

PVI was significantly increased in the four groups of rats that underwent PPVL compared to the corresponding SO group (P < 0.01). Treatment with E2 resulted in a reduction in PVI in PPVL + E2 rats compared to PPVL + PLAC rats (3.98 ± 1.4 mL/min per 100 g vs 4.87 ± 1.6 mL/min per 100 g, P < 0.05) (Table 1).

Although CI was slightly increased in the four groups...
of PPVL rats compared to the SO group, there were no significant differences among the five different groups (P > 0.05). SVR was lower in the four groups of PPVL rats compared to SO rats due to the decreased MBP (P < 0.05), which was increased 16.9% by E2 treatment in PPVL + E2 rats compared to PPVL + PLAC rats (3.95 ± 0.41 mmHg/min-100 g/mL vs 3.38 ± 0.35 mmHg/min-100 g/mL, P < 0.05) (Table 1).

SAR was significantly lower in the four groups of PPVL rats compared to SO rats (P < 0.01). Rats treated with E2 showed significantly higher SAR, with a 34.1% increase compared to PPVL + PLAC rats (19.29 ± 1.8 mmHg/min-100 g/mL vs 14.39 ± 2.2 mmHg/min-100 g/mL, P < 0.01) (Table 1).

ICI182, 780 treatment alone did not influence PP, PVI, CI, SVR or SAR compared to PPVL + PLAC rats, except that the body weight was slightly lower (P < 0.05). However, ICI182, 780 reversed the beneficial effects of E2 on PVI, CI, SVR and SAR. Thereby, the systemic circulation and splanchnic hyperdynamic circulation were more deteriorated (Table 1).

**Contractility of isolated rat mesenteric arterioles in response to NE**

Compared with the SO group, the dose-response curve of the mesenteric arteriole in response to NE was lower and shifted right, with decreased Emax (P < 0.01) and increased EC50 (P < 0.01, Figure 1; Table 1) in the four PPVL groups. In the mesenteric arterioles of PPVL rats treated with E2, the dose-response curve was shifted left, and the EC50 was decreased (P < 0.01), compared with the PPVL + PLAC rats. ICI182, 780 alone did not influence the dose-response curve or EC50 compared to PPVL rats treated with placebo (P > 0.05). However, ICI182, 780 decreased the response to NE of arterioles treated with E2, thereby the dose-response curve was shifted right.

**Superoxide anion production**

Low-intensity fluorescence was observed throughout SO vessels, confirming that all layers of the normal vessel produced low amounts of O$_2^-$. Fourteen d after the surgery, O$_2^•$ production was increased in the four groups of PPVL rats (Figure 2). However, the staining was particularly weak in PPVL + E2 rats, and the administration of ICI182, 780 blocked the beneficial effect that E2 provided.

**Hydrogen peroxide production**

In the superior mesenteric arteries, H$_2$O$_2$ generation was nearly 2.5-fold higher in PPVL + PLAC rats than in SO rats (8.2 ± 1.5 μmol/L vs 3.3 ± 0.9 μmol/L, P < 0.01; Figure 3). Exogenous E2 reduced H$_2$O$_2$ levels in PPVL + E2 rats when compared with those in PPVL + PLAC rats (4.9 ± 1.0 μmol/L vs 8.2 ± 1.5 μmol/L, P < 0.01). The H$_2$O$_2$ levels in mesenteric arterioles treated with ICI182, 780 alone were similar to those found in PPVL + PLAC rats (7.9 ± 1.8 μmol/L vs 3.3 ± 0.9 μmol/L, P < 0.01). However, the administration of ICI182, 780 to PPVL + E2 rats suppressed the antioxidant effect of E2 (6.6 ± 1.3 μmol/L vs 4.9 ± 1.0 μmol/L, P < 0.05).

**DISCUSSION**

Recent studies have already provided evidence of the importance of vascular hyporeactivity in the development and maintenance of PHT. Aortic ring hypococontractility has been investigated both in bile duct ligation and CCl4-induced cirrhotic models[13-15]. Ferlitsch et al[16] have shown that forearm artery responses to NE and Ang-II are decreased in patients with cirrhosis. Clear sex differences have also been observed in the vasoconstrictor responsiveness of aortic rings from rats with and without PHT[17]. In contrast to male rats, PHT does not induce vascular hyporesponsiveness in female rats. Estrogen has been shown to be effective in animal models of established PHT with cirrhosis by suppressing hepatic fibrosis and relaxation of the hepatic sinusoid[18]. However, the efficacy of estrogen in the setting of de novo PHT and the underlying mechanism had not been characterized in detail. To exclude the effect of cirrhosis on oxidative status and inflammatory cytokines, we established this animal model of prehepatic PHT to further describe both the hemodynamic and anti-oxidant effects of estrogen treatment in the PPVL rat model. The hypothesis of our study was that estrogen could reverse the severity of hyperdynamic circulation and the vascular hyporeactivity of the mesenteric arteries by alleviating oxidative stress in portal hypertensive rats without cirrhosis in which liver function was normal.

The results of our study suggested that mesenteric arteriole sensitivity and contractility in response to NE were decreased in PPVL rats, indicating the hyporesponsiveness of the splanchnic vessels to vasoconstrictors, in accordance with deteriorative splanchnic hemodynamics. Treatment with E2 significantly ameliorated the hyperdynamic splanchnic circulation in PPVL rats. The reduction
in splanchnic blood inflow represented by decreased PVI could be explained by an improved contractile reaction of the splanchnic vessels to the vasoconstrictor and a subsequently increased SAR. Additionally, the production of ROS was decreased in PPVL-E2 rats compared to PPVL-SA rats, indicating a profound amelioration of oxidative stress and corresponding to the improvement of systemic and splanchnic hyperdynamic circulation.

The fact that PPVL resulted in oxidant injury was first demonstrated by Fernando et al. [18], who concluded that the formation of ROS may be important in the pathogenesis of hemodynamic changes and that anti-oxidants can ameliorate oxidant injury and prevent the development of hyperdynamic circulation [19]. Estrogen played a protective role by acting as an antioxidant, which granted it action as a scavenger of free radicals, decreasing the formation of ROS. In castrated rats, significant increases in the activity of antioxidant enzymes were observed, which may have occurred to compensate for the absence of circulating E2 promoted by castration; even then, it was not effective in reducing lipid peroxidase levels [8,20,21]. Estrogen replacement can reverse this effect, reducing lipid peroxidase to the values of control animals [21]. In addition, estrogen stimulates eNOS expression in SECs and increases NO production, contributing to a reduction in portal pressure in a model of intrahepatic PHT [7].

In summary, we conclude that improvements in oxidative stress after estrogen administration manifest as a functional improvement in the contractile response to vasoconstrictors. Indeed, we observed improvements in both the systemic and splanchnic hyperdynamic circulation. Further studies on the clinical administration of estrogen should be performed.

**COMMENTS**

**Background**

Portal hypertension, a major complication of liver cirrhosis, is associated with the development of hyperdynamic circulation and would be triggered by persistent splanchnic vasodilation. Functional changes can be found in the portal hypertensive splanchnic vasculature, including splanchnic vasodilation and decreased responsiveness to vasoconstrictors as a result of endothelial dysfunction and impaired activation of vasoconstrictive mechanisms.
The manuscript is an interesting manuscript with novel observations. The authors proposed that treatment with estrogen could improve the systemic and splanchic hyperdynamic circulation in partial portal vein ligation (PPVL) rats. The authors found that improvements in oxidative stress after estrogen administration manifest as a functional improvement in the contractile response of mesenteric arteries to vasoconstrictors in partial portal vein ligation (PPVL) rats. The authors proposed that treatment with estrogen could improve the systemic and splanchic hyperdynamic circulation in partial portal hypertensive rats. The findings are straightforward and manuscript is well written and nicely discussed.

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