Altered mesenteric venous capacitance and volume pooling in cirrhotic rats are mediated by nitric oxide

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Li Y, Liu H, Gaskari SA, Tyberg JV, Lee SS. Altered mesenteric venous capacitance and volume pooling in cirrhotic rats are mediated by nitric oxide. Am J Physiol Gastrointest Liver Physiol 295: 252–259, 2008. First published June 12, 2008; doi:10.1152/ajpgi.00436.2007.—In cirrhosis, despite augmented blood volume, effective circulating volume is decreased. This implies abnormal regulation of blood volume, i.e., venous pooling. Because gut veins are the main blood reservoir, we studied mesenteric venous capacitance and compliance in a rat model of cirrhosis. Cirrhosis was induced by bile duct ligation (4 wk). Controls were sham operated. Changes in first-order mesenteric vein diameters induced by drugs, hemorrhage, and stepwise increases in portal pressure (inflatable cuff) were directly observed by intravital microscopy. Effects of nitric oxide on responses to acute graded hemorrhage were studied by use of selective NO synthase (NOS) isoform inhibitors. Pressures were related to diameters to assess capacitance and compliance. Compared with controls, cirrhotic rats demonstrated increased mesenteric venous capacitance and decreased compliance. Norepinephrine induced venoconstriction but did not affect compliance. Prazosin markedly diminished compliance in controls but not cirrhotics. Conversely, the nonselective NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) decreased compliance in cirrhotics, but not controls. Tetrodotoxin venodilated controls, venoconstricted cirrhotics, and markedly decreased compliance in both groups. When hemorrhaged, controls rapidly venoconstricted to compensate for initial hypotension, whereas cirrhotic rats remained hypotensive because venoconstriction was severely blunted. Pretreatment with L-NAME or the selective neuronal NOS inhibitors L-NAME and 7-nitroindazole normalized the homeostatic responses of cirrhotic rats, whereas the selective endothelial-constitutive NOS inhibitor N-iminoethyl-L-ornithine did not affect the response. In conclusion, mesenteric veins of cirrhotic rats showed enhanced capacitance, attenuated compensatory constrictive response to hemorrhage, and decreased compliance. The first two abnormalities were caused by neuronal NOS-derived nitric oxide.

METHODS

The protocol was approved by the institutional Animal Care Committee, according to guidelines of the Canadian Council on Animal Care.

Cirrhosis Model

In Sprague-Dawley rats initially weighing 175–200 g, cirrhosis was induced by chronic bile duct ligation (BDL), as previously described (15). Briefly, under halothane anesthesia, the common bile duct was doubly ligated with 4-0 silk and sectioned between the ligatures. Control rats (Sham) underwent identical procedures except that the bile duct was not ligated or sectioned. Animals were then returned to cages for 4 wk, by which time they weighed 300–400 g and cirrhosis had developed. Food but not water was withdrawn from the cages 12 h before study.

Experimental Protocols

Surgical procedure. Animals were anesthetized with pentobarbital sodium (50 mg/kg ip) and placed on a heating pad with rectal temperature maintained at 37°C. The right jugular vein was cannulated with a PE-10 polyethylene catheter for drug administration and the right carotid artery by a PE-90 catheter to measure mean arterial pressure (MAP). Care was taken not to damage the cervical vagus nerve during vessel cannulations. For hemorrhage experiments, a PE-50 catheter was inserted into the left femoral artery for blood withdrawal. All catheters were filled with heparinized saline. Through a midline incision, an inflatable silicone rubber cuff (DOCXS, Ukiah, CA) was placed around the prehepatic portal vein. Sequential 5-mmHg increments in portal pressure were achieved by inflating the cuff by a motorized syringe pump. To avoid ischemic damage, the cuff was inflated only long enough to obtain a stable pressure tracing.

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and never more than 1 min each time. The cuff was fully deflated and portal pressure returned to baseline between each pressure measurement. The portal vein was punctured by a 20-gauge needle caudad to portal pressure returned to baseline between each pressure measurement. Twenty minutes after onset of hemorrhage, the withdrawn blood was reinfused over 3 min by reversing pump direction. The effect of NOS was investigated using different NOS inhibitors including L-NAME, L-thiocitrulline (SMTC; 1 mg/kg ip) and 7-nitroindazole (7-NI; 5 mg/kg ip), or the selective endothelial NOS (eNOS) inhibitor N-iminoethyl-L-ornithine (1-NIO; 20 mg/kg ip). These chemicals were purchased from Sigma (Pittsburgh, PA) except L-NAME (Cayman Chemicals, Ann Arbor, MI). The acute hemorrhage was started 1 h after the NOS inhibitors were administered, MAP and mesenteric venous diameters were recorded.

Table 1. Mesenteric venous diameters and diameter-pressure slopes

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>BDL</th>
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<tbody>
<tr>
<td>Basal diameter, μm</td>
<td>125.0 ± 2.9</td>
<td>156.7 ± 4.4*</td>
</tr>
<tr>
<td>Basal portal vein pressure, mmHg</td>
<td>6.1 ± 0.3</td>
<td>14.1 ± 0.7**</td>
</tr>
<tr>
<td>Basal D/P slope, mmHg/mmHg</td>
<td>2.15 ± 0.07</td>
<td>1.63 ± 0.03*</td>
</tr>
<tr>
<td>Basal unstressed diameter, μm</td>
<td>112.0 ± 1.3</td>
<td>134.0 ± 0.7*</td>
</tr>
<tr>
<td>NE change in diameter</td>
<td>−17.3 ± 2.1%†</td>
<td>−13.4 ± 5.3%†</td>
</tr>
<tr>
<td>NE D/P slope, μm/mmHg</td>
<td>0.08 ± 0.03</td>
<td>0.89 ± 0.06†</td>
</tr>
<tr>
<td>NE unstressed diameter, μm</td>
<td>89.1 ± 0.6†</td>
<td>124.3 ± 2.8†</td>
</tr>
<tr>
<td>Prazosin change in diameter</td>
<td>21.4 ± 9.9%‡</td>
<td>15.7 ± 4.0%*</td>
</tr>
<tr>
<td>Prazosin D/P slope, μm/mmHg</td>
<td>0.87 ± 0.04†</td>
<td>1.35 ± 0.08*‡</td>
</tr>
<tr>
<td>Prazosin unstressed diameter, μm</td>
<td>141.0 ± 0.9†</td>
<td>147.6 ± 1.9†‡</td>
</tr>
<tr>
<td>L-NAME change in diameter</td>
<td>−15.4 ± 2.1%†</td>
<td>−27.5 ± 6.7%‡</td>
</tr>
<tr>
<td>L-NAME D/P slope, μm/mmHg</td>
<td>2.11 ± 0.08</td>
<td>1.16 ± 0.10*</td>
</tr>
<tr>
<td>L-NAME unstressed diameter, μm</td>
<td>94.3 ± 1.6†</td>
<td>120.2 ± 1.9†‡</td>
</tr>
<tr>
<td>TTX change in diameter</td>
<td>16.4 ± 2.5%‡</td>
<td>−18.0 ± 3.1%†</td>
</tr>
<tr>
<td>TTX D/P slope, μm/mmHg</td>
<td>1.12 ± 0.04†</td>
<td>1.01 ± 0.09†</td>
</tr>
<tr>
<td>TTX unstressed diameter, μm</td>
<td>113.7 ± 2.4</td>
<td>149.8 ± 0.7†‡</td>
</tr>
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</table>

Values are mean ± SE. D/P, diameter-to-pressure ratio; NE, norepinephrine; L-NAME, N-nitro-l-arginine methyl ester; TTX, tetrodotoxin. *Significantly different from sham control group, P < 0.05. †Significantly different from basal value, P < 0.05.

Fig. 1. Basal levels of mesenteric vein diameters, portal vein pressures, and slopes of diameter-pressure relationships in both sham and bile duct ligation (BDL) rats. Data are expressed as means ± SE for 5 animals each group. *P < 0.05, slope compared with corresponding sham rats.
nNOS and MeSenteric VEnous Function in Cirrhosis

(Applied Biosystems), 0.5 µl 20× mix of oligonucleotide primers and probes (Hs00694747_m1, Applied Biosystems), and 2 µl of diluted cDNA solution. The total reaction volume was 10 µl. The expression of nNOS was calculated as the ratio of threshold cycle value of the target gene and the housekeeping gene (nNOS/GAPDH).

nNOS protein content in mesenteric veins was examined by Western blotting. Veins were homogenized in PBS with protein inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) and centrifuged. The equivalent denatured protein was loaded and separated on SDS-PAGE 8%. Proteins were transferred to nitrocellulose by electroblotting and blocked at 4°C with 10% skim milk overnight. After wash, membranes were subsequently incubated with anti-nNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000) and horseradish peroxidase-linked goat anti-mouse immunoglobulin (Santa Cruz, 1:1,000). The blots were detected by chemiluminescence (ECL Western blot kit, Amersham), using -actin as an internal control. The relative expression of nNOS and -actin was quantified by Quantity One software.

Statistical Analysis

Data are presented as means ± SE. Diameter-pressure slopes were calculated by linear regression analysis of the mean values. Paired or unpaired Student’s t-tests were used to compare two observations in the same animal or two groups, respectively. For more than two groups, ANOVA followed by a Bonferroni correction for multiple comparisons was used. Statistical significance was set at P < 0.05.

RESULTS

Basal Parameters

Compared with sham controls, at baseline, BDL rats had increased mesenteric vein diameter, but decreased diameter-pressure slope, i.e., decreased compliance (Table 1 and Fig. 1). The unstressed volume, defined as the zero intercept of the diameter-pressure slope (27), or the calculated vessel diameter at zero distending pressure, was significantly higher in the cirrhotic rats compared with controls (Table 1). Capacitance is defined as the volume that the vein contains at a given pressure.

Fig. 2. Effects of norepinephrine (NE) on diameter-pressure relationship in both sham and BDL rats. Data are expressed as means ± SE (n = 5 per group). Squares represent shams; circles represent BDL-cirrhotic rats. Solid symbols denote NE results; open symbols are the basal results from Fig. 1 (without error bars for ease of viewing).

Fig. 4. Effects of N-nitro-L-arginine methyl ester (L-NAME) on diameter-pressure relationship in both sham and BDL rats. Data are expressed as means ± SE (n = 5 per group). Squares represent shams; circles represent BDL-cirrhotic rats. Solid symbols denote L-NAME results; open symbols are the basal results from Fig. 1 (without error bars).

Fig. 3. Effects of prazosin on diameter-pressure relationship in both sham and BDL rats. Data are expressed as means ± SE (n = 5 per group). Square symbols represent shams; circles represent BDL-cirrhotic rats. Solid symbols denote prazosin results; open symbols are the basal results from Fig. 1 (without error bars).

Fig. 5. Effects of tetrodotoxin (TTX) on diameter-pressure relationship in both sham and BDL rats. Data are expressed as means ± SE (n = 5 per group). Square values represent shams; circles represent BDL-cirrhotic rats. Solid symbols denote TTX results; open symbols are the basal results from Fig. 1 (without error bars).
Thus the two D/P lines reveal that at any given pressure value the diameter of the BDL vein is higher. For example, at a portal pressure of 20 mmHg, the average BDL diameter would be ~165 μm and the Sham-control diameter 152 μm (Fig. 1). Therefore, capacitance is higher in the cirrhotic mesenteric vein.

**Effects of Vasoactive Drugs on Compliance and Capacitance**

NE vasoconstricted both sham and BDL animals (Table 1). NE shifted the diameter-pressure lines downward in both groups; this effect was more pronounced in the controls (Fig. 2). The D/P slopes were not changed by NE in either group (Fig. 2). The unstressed diameter and capacitance remained significantly higher in the BDL group (Table 1 and Fig. 2).

Prazosin venodilated both groups, but much more so in the controls (Table 1). Prazosin shifted the D/P line upward in both groups, in a parallel fashion in the cirrhotics, i.e., D/P slope was unchanged in this group (Fig. 3). In contrast, the D/P slope was markedly reduced by prazosin in the control group. Moreover, the unstressed diameters after prazosin became similar in both groups (Table 1). Capacitance remained higher in the BDL group (Fig. 3).

L-NAME significantly vasoconstricted both groups, more so in cirrhotic rats (Table 1 and Fig. 4). It significantly shifted the D/P lines downward in both groups, in a parallel fashion in the sham controls, and with a significant reduction in the D/P slope in the BDL rats. Unstressed diameters were reduced in both groups but the significant difference between the two groups

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**Fig. 6.** Effects of hemorrhage (H) and reinfusion (R) on mesenteric vein diameter and arterial pressure. Basal values with equivolumic normal saline (A and B), after L-NAME (C and D) and S-methyl-l-thiocitrulline (SMTC; E and F). Data are expressed as means ± SE for 5 animals per group.
remained after L-NAME administration. Capacitance remained higher in the BDL group at all portal pressure values until the D/P lines converged at \(25–30\) mmHg (Fig. 4).

Topical application of TTX resulted in totally opposite changes in sham and BDL rats. TTX significantly venodilated sham controls but constricted cirrhotic veins (Table 1 and Fig. 5). Thus TTX shifted the D/P line upward in controls but downward in cirrhotic rats. It also significantly decreased the D/P slopes in both groups, rendering this parameter virtually identical in the two groups. The calculated unstressed diameter remained unchanged in the sham controls but increased in the cirrhotic group, accentuating the difference between the two groups. However, capacitance was reversed by TTX: the directionally opposite shifts in the D/P lines resulted in the sham control capacitance being significantly higher than the BDL (Fig. 5).

**Effects of Acute Hemorrhage**

Baseline MAP of BDL rats was lower than sham controls (Fig. 6). Small volumes of normal saline (200–300 \(\mu\)l), equivalent to those used for NOS inhibitor injections, did not affect any parameter in either group. In the sham controls, hemorrhage induced mesenteric venoconstriction, which was maintained during the period of hemorrhage (Fig. 6A). Reflecting homeostatic adjustments to defend circulating arterial volume including a mobilization of mesenteric venous blood, MAP initially decreased but quickly returned to baseline values. These responses in BDL rats were dramatically blunted. Mesenteric venoconstriction was delayed and minimal (Fig. 6A), whereas MAP remained low during the entire period of hemorrhage (Fig. 6B).

Pretreatment with L-NAME did not affect basal mesenteric vein diameter or MAP in the sham controls. However, it decreased basal mesenteric vein diameter and increased basal MAP in BDL rats during the 1-h stabilization period. When these rats were then bled, the blunted response of the BDL rats was normalized (Fig. 6, C and D). In other words, the mesenteric venoconstrictive response to defend arterial pressure became similar to the controls. In sham controls, L-NAME did not affect the normal homeostatic response. The selective nNOS inhibitor SMTC showed the same effect as L-NAME in the BDL rats and did not affect the sham controls (Fig. 6, E and F). Results using a different selective nNOS inhibitor 7-NI also showed a similar pattern to the L-NAME and SMTC responses (Fig. 7, A and B). In contrast, the selective eNOS inhibitor...
l-­NIO (Fig. 7, C and D) exerted a markedly different pattern of response compared with the l-­NAME or the two selective nNOS inhibitors. Although l-­NIO, as expected, modestly increased MAP in both control and BDL rats and decreased mesenteric venous diameter in the controls, all within the first 20 min after administration, it did not affect the responses of the vein or MAP to hemorrhage. In other words, both cirrhotic and control rats showed the identical patterns of response to hemorrhage as those in the control experiment with normal saline infusion.

**nNOS mRNA and Protein Expression**

nNOS mRNA transcription was significantly increased in BDL mesenteric arteries compared with controls (Fig. 8A). In contrast, BDL mesenteric veins showed no significant differences compared with sham controls in both mRNA (Fig. 8B and C) and protein expression (Fig. 8D) of nNOS (n = 6 for each group).

**DISCUSSION**

In mesenteric veins of our cirrhotic rats, capacitance was increased and compliance decreased. Although we did not measure total blood volume in this study, we previously demonstrated in this BDL rat model a 16% increase in this variable (19). The most striking abnormality was the inability of cirrhotic rats to mobilize their mesenteric volume reservoir to defend circulating arterial pressure. Administration of l-­NAME or the nNOS inhibitors SMTC and 7-­NI (but not the specific eNOS inhibitor l-­NIO) corrected this blunted homeostatic response, indicating that NO derived from nNOS is responsible for this defect.

Our method of measuring venous compliance was modified from the method of Shoukas and Bohlen (31), who directly observed in situ mesenteric venules and altered venular pressure through micropipette cannulation. The validity of our method is suggested by the sham control results, which are reassuringly similar to previous studies using a variety of different methods. In particular, the linearity of the diameter-­pressure relationship through the fairly wide range of portal pressure values is now firmly established in the literature. Additionally, the parallel shifts of the D/P line toward the pressure axis with NE also agree with previous studies using an entirely different method of assessing mesenteric venous compliance (nuclear scintigraphy) in dogs (29) and humans (24).

Normally, venoconstrictor tone is predominantly due to sympathetic activity. In that respect, hemorrhage, norepinephrine, and prazosin, all acting by sympathetic adrenergic mechanisms, showed blunted responses in our cirrhotic rats. These results agree with numerous previous studies demonstrating arterial hyporesponsiveness to sympathetic stimulation in cirrhosis. Proposed mechanisms include desensitization and adrenergic receptor downregulation (16, 21), or overactivity of the vasodilator NO, which impairs arterial vasoconstriction (4, 9, 13, 32).

TTX depolarizes nerve cell membranes by blocking a population of Na+ channels but does not directly affect vascular smooth muscle. To our knowledge, TTX has not previously been studied in cirrhotic veins. The venodilation in our sham controls agrees with previous work demonstrating that TTX dilated normal rat mesenteric venules in situ by a neural mechanism but lacked any direct effect on vascular smooth muscle (23). The diametrically opposite effects on cirrhotic vs. control veins suggest that net neural tone is normally constricive but dilatory in cirrhotic mesenteric veins.

We believe this is the first study to directly measure mesenteric venous compliance in cirrhosis. Because true compliance measurement requires a stepwise manipulation of either pressure or volume, it is likely to be ethically and methodologically unfeasible in patients with cirrhosis. However, two previous studies have quantified compliance of the entire systemic vasculature in cirrhotic patients (2, 5). Both studies measured right atrial pressure increment after a rapid volume expansion (300 ml). By this method, total systemic vascular compliance was found to be increased. Besides these human studies, two rat studies have also examined the total vascular
capacitance in a similar manner, with rapid volume infusions in the CCl4-cirrhotic rat (7) or infusions and withdrawal in the prehepatic portal-hypertensive rat (1). The Ingles et al. study (7) found increased capacitance but no change in compliance in the cirrhotic rat, whereas the Andreu et al. study (1) reported increased compliance. Thus all four previous studies are consistent in showing an enhanced capacitance or compliance of the overall systemic veins in cirrhosis and portal hypertension. Although we also showed enhanced mesenteric capacitance, compliance was decreased. This pattern in the mesenteric veins may not necessarily conflict with the four previous studies, since it is entirely possible that regional venous beds behave differently from the total systemic venous bed.

The ensemble of the D/P lines and their shifts in position and slope with the pharmacological stimuli generally agree with what is known about normal mesenteric venomotor tone. In the normal gut, venomotor tone is predominantly due to α-adrenergic influence (27). The D/P slopes after prazosin in the controls, l-NNAME in the cirrhotics, and TTX in both control and cirrhotic veins were remarkably similar, ~1 µM/mmHg. Presumably this D/P slope value reflects the intrinsic myogenic elasticity of the venous smooth muscle, devoid of neural vasoactive influences. This again suggests that cirrhotic mesenteric veins are under a net tonic dilatory influence, likely mediated by a NO-dependent mechanism. However, although NO is likely involved in the increased capacity of the mesenteric vein in cirrhosis, it is not responsible for the decreased mesenteric compliance, given that L-NNAME further decreased the D/P slope rather than normalizing it. The mechanism underlying decreased compliance in cirrhotic mesenteric veins remains unclear.

The hemorrhage experiments conclusively demonstrate, for the first time, the oft-speculated “sump” effect in cirrhosis whereby the gut veins pool blood and are unable to mobilize this reservoir when needed. Our data argue strongly for a dominant role of NO derived from nNOS in the pathogenesis of this sump effect. The ability to mobilize the gut venous reservoir to defend the circulation when needed is mediated through baroreflexes (26, 27). Baroreflex function is known to be impaired in cirrhosis (6, 14). But the normalization of this homeostatic response in the cirrhotic rat by a selective nNOS inhibitor indicates that NO plays a role in some aspect of baroreflex regulation of gut venomotor responses. In that respect, nonadrenergic, noncholinergic (NANC) nerves are abundant in the gut vasculature (25), and the majority of such NANC nerves likely use nitricergic transmission (17, 22). Indeed, it has been reported that NANC nerves contain nNOS (33). However, it remains unclear exactly how NO-NANC nerves interact with baroreflexes to mediate the effects seen in our study.

Our results in the cirrhotic mesenteric vein agree with recent studies demonstrating an important role of nNOS in the pathogenesis of arterial hyperdynamic circulation in cirrhosis. Xu and colleagues (35) showed that 7-NI blocked the development of hyperdynamic circulation in cirrhotic rats. Furthermore, increased nNOS mRNA and/or protein levels have been documented in aortae of BDL mice (3), mesenteric arteries of portal hypertensive rats (10), and hepatic arteries of cirrhotic patients (3). Finally, a preeminent role of nNOS would explain the persistence of hyperdynamic circulation in portal hypertensive double-knockout mice lacking the genes for both eNOS and inducible NOS (8) and cirrhotic knockout mice lacking the eNOS gene (12).

Although the increased nNOS mRNA transcription in our cirrhotic mesenteric arteries agrees with these previous studies, we could not detect any difference in mRNA nor protein content of nNOS between the cirrhotic and control mesenteric veins. The reasons for this remain unclear. nNOS function may not correlate closely with levels of the nNOS mRNA or protein in the vessel, owing at least in part to posttranslational modification or regulation. It is also possible that the changes in venous diameters observed with hemorrhage were due to changes in the upstream mesenteric arteries mediated by nNOS. In other words, since veins are the drainage vessels of arteries, vasoactive events in the artery will influence downstream venous flow and thus diameter. In summary, mesenteric veins of cirrhotic rats show decreased compliance, increased capacitance, and a markedly blunted ability to mobilize its reserve volume to defend the circulation. Nitric oxide derived from nNOS mediates the last two abnormalities. These changes in venous function contribute to a sump effect and thus to the decreased effective circulating volume of cirrhosis.

GRANTS
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