Deficit in nitric oxide production in cirrhotic rat livers is located in the sinusoidal and postsinusoidal areas

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Deficit in nitric oxide production in cirrhotic rat livers is located in the sinusoidal and postsinusoidal areas. Am J Physiol Gastrointest Liver Physiol 284: G567–G574, 2003. First published December 18, 2002; 10.1152/ajpgi.00452.2002.—Intrahepatic nitric oxide (NO) production is decreased in cirrhotic livers. Our objective was to identify, in cirrhotic rat livers, intrahepatic vascular segments where the deficit of NO facilitates the effect of vasoconstrictors. By using a modified rat liver perfusion system with measurement of both the perfusion and sinusoidal (wedged hepatic vein) pressures, we studied the effect of the NO synthase blocker Nω-nitro-l-arginine (l-NNA) on the response to methoxamine (α1-adrenoreceptor agonist) in different segments of the intrahepatic circulation of normal and cirrhotic rat livers. l-NNA enhanced the presinusoidal, sinusoidal, and postsinusoidal responses to methoxamine in normal livers as well as the presinusoidal response in cirrhotic livers. However, l-NNA did not change the already enhanced sinusoidal/postsinusoidal response to methoxamine in cirrhotic livers. The postsinusoidal response to methoxamine was higher in cirrhotic rats with ascites than in those without ascites. We concluded that NO modulates the presinusoidal, sinusoidal, and postsinusoidal vascular tone in normal livers. NO production in cirrhotic rat livers is severely impaired in the sinusoidal and postsinusoidal areas but is preserved in the presinusoidal area, as evidenced by its normal response to l-NNA. We speculate that an increased postsinusoidal response to catecholamines may participate in the genesis of ascites in cirrhosis.

The intrahepatic circulation in cirrhosis. We recently described a fourth mechanism, which is the inability of the diseased vasculature to fully respond to NO (7).

On the other hand, an increased NO production determines an abnormal splanchnic and systemic vasodilatory state in cirrhotic patients (23, 37). This vasodilatory state is opposed by different compensatory mechanisms, including an increased sympathetic nervous activity that is characterized by an increased plasma concentration of catecholamines (35) and may contribute to enhancement of the intrahepatic vascular tone. In fact, the use of α1-adrenergic blockers was shown to reduce the hepatic vascular resistance and the hepatic venous pressure gradient in cirrhotic patients (1).

The objective of this study was to identify the location within the vasculature of cirrhotic livers where the deficit of NO facilitates the effect of vasoconstrictors. Using a modified rat liver perfusion system, we studied the effect of Nω-nitro-l-arginine (l-NNA), a NO synthase (NOS) inhibitor, on the vascular response to the α1-adrenoreceptor agonist methoxamine in different segments of the intrahepatic vasculature in normal and cirrhotic rats.

MATERIALS AND METHODS

Seventy-seven normal and cirrhotic adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) underwent in situ liver perfusion. We adhered to the American Physiological Society guiding principles for the care and use of animals.

Induction of cirrhosis by CCl4. Rats weighing 100–125 g (beginning weight) underwent inhalation exposure to CCl4 three times a week. Phenobarbital (0.35 g/l) was added to the drinking water as described previously (25). This technique produces a high yield of micronodular cirrhosis in ∼10–12 wk of treatment. Perforusions were performed 6–12 days after the animal received the last dose of CCl4 or phenobarbital.

Antegrade in situ rat liver perfusion. Animals were anesthetized with ketamine hydrochloride (Ketaset, 100 mg/kg body wt; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (Rompum, 40 mg/animal; Bayer, Shawne Mission, KS) and then submitted to liver perfusion in situ via the portal vein with the use of a recirculating system. Briefly, the abdomen was opened and examined for evidence of portal ascites; liver microcirculation; rat liver perfusion; nitric oxide synthase; wedged hepatic venous pressure

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hypertension and cirrhosis, i.e., splenomegaly, collateral circulation, ascites, and liver nodularity. All cirrhotic rats used for antegrade liver perfusion had ascites. The bile duct was cannulated with polyethylene tubing (PE-10). Loose ligatures were placed around the portal vein, common hepatic artery, and posterior vena cava (PVC) just cranially to the confluence of the right renal vein. A 500-unit dose of heparin was administered into the PVC. The portal vein was cannulated with a 14-gauge Teflon catheter, and liver exsanguination was initiated by infusion (40 ml/min) of oxygenated (carbogen gas, 95% O2-5% CO2) Krebs-Henseleit solution containing heparin (2 U/ml) and dextrose (11 mM) in a nonrecirculating mode. The PVC was immediately cut caudally to the loose ligature, thus allowing both the perfusate to escape and the vein catheterization with a second 14-gauge Silastic tubing lung (19) interposed between the perfusate and to the wedged catheter, respectively. A three-way stopcock connected to the wedged catheter allowed either the flow of the perfusate toward the reservoir during the incubation period or the sinusoidal pressure measurement during the vascular response study. The interruption of flow through the catheter, with connection to the transducer, created a zone of stasis in hepatic vein tributaries of a small segment of the left lobe, and the pressure measured was that in vessels immediately proximal to the zone of stasis, that is, the sinusoidal pressure. During all experiments, the outflow catheter allowed the drainage of the liver in both antegrade and retrograde perfusions. Before each experiment, both pressure-measurement systems were calibrated with the zero point at the level of the hepatic hilum. Perfusion and sinusoidal pressures were continuously recorded (except for sinusoidal pressure during incubation) by using Chart 3.6 software on MacLab/4e hardware (AD Instruments).

The perfusion pressure increase during either antegrade or retrograde perfusions, the difference between the perfusion (total) and the sinusoidal pressure increase is determined by the sum of the increases in presinusoidal, sinusoidal, and postsinusoidal vascular resistances. In antegrade perfusions, the sinusoidal pressure increase is determined by the sum of the sinusoidal and postsinusoidal vascular resistance increases. Consequently, in antegrade and retrograde perfusions, the difference between the perfusion (total) and the sinusoidal pressure increases is determined by the presinusoidal and postsinusoidal vascular resistance increases, respectively. A schematic representation of pressure measurements and their interpretation in both antegrade and retrograde experiments is shown in Fig. 1.

According to Ohm’s concept, since the perfusion flow is kept constant, this technique also provides continuous recording of changes in vascular resistance. Pressure or vascular resistance increases or decreases in response to vasoactive substances are due exclusively to the hepatic vascular structures. Although the tubing does not interfere with these parameters, we used identical tubing in all experiments. Therefore, we could compare the basal perfusion pressure values obtained from experiments with normal and cirrhotic livers.

**Experiment design.** Normal and cirrhotic rat livers were perfused for 20 min in the absence or the presence of the NOS inhibitor l-NNA (10−4 M). During this period of incubation, as well as during the preceding exsanguination, the flow through the wedged catheter was maintained to ensure an equal distribution of l-NNA through the vasculature. The outflow through both the PVC catheter (or the portal vein catheter in retrograde experiments) and the wedged catheter drained into the perfusate reservoir. After incubation, the wedged catheter outflow was interrupted, allowing the measurement of the sinusoidal pressure. Then, a concentration-effect curve was performed by using three cumulative doses of methoxamine (3 × 10−9, 10−8, and 3 × 10−7 M) with a 2-min interval between them. The bile production was measured during the period of incubation. Twenty normal livers (10 in the absence and 10 in the presence of l-NNA) and twenty-five cirrhotic livers (12 in the absence and 13 in the presence of l-NNA) were perfused by using the antegrade perfusion model. Eighteen normal livers (11 in the absence and 7 in the presence of l-NNA) and 14 cirrhotic livers (7 from ascitic rats and 7 from nonascitic rats, all 14 in the absence of l-NNA) were perfused in the retrograde direction.
Samples of liver tissue were fixed in 10% formalin solution, and their sections were analyzed by light microscopy after hematoxylin and eosin staining.

**Drugs.** Methoxamine, l-NNA, bovine serum albumin, and methylene blue were purchased from Sigma (St. Louis, MO). Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ). l-NNA and methoxamine solutions were prepared fresh daily with Krebs solution. Methoxamine was kept on ice and foil wrapped. A similar volume of Krebs solution was added to the perfusate when l-NNA solution was not used.

**Statistical analysis.** All data are expressed as means ± SE. Weights, bile production, and basal pressures (pressure after the 20-min incubation in the absence of l-NNA) were compared by using the unpaired **t**-test. The vascular responses to methoxamine in different groups were compared by using the **t**-test or ANOVA for each concentration of vasoconstrictor. Since we used three different methoxamine concentrations (multiple comparison), only **P** values <0.017 (0.05 divided by 3) were considered significant (12). StatView software (Abacus Concepts, Berkeley, CA) was used for statistical analyses.

**RESULTS**

**Vascular response to methoxamine in antegrade experiments.** In antegrade experiments, body weight and liver weight were similar between compared groups. Cirrhotic rats showed higher spleen weight (1.22 ± 0.04 vs. 0.94 ± 0.02 g; **P** < 0.01) and lower bile production (0.38 ± 0.03 vs. 1.40 ± 0.08 μl·g⁻¹·min⁻¹; **P** < 0.01) than normal rats. All cirrhotic animals that were used in antegrade perfusions had ascites. After incubation in the absence of l-NNA, the basal perfusion pressure in cirrhotic livers was higher than in normal livers (12.4 ± 0.4 vs. 10.4 ± 0.4 cmH₂O; **P** < 0.05). The basal sinusoidal pressure was also higher in cirrhotic livers (8.0 ± 0.8 vs. 4.8 ± 0.4 cmH₂O; **P** < 0.01). The difference between perfusion and sinusoidal basal pressures in cirrhotic livers was lower than in normal livers (4.0 ± 0.4 vs. 5.6 ± 0.4 cmH₂O; **P** < 0.01), suggesting the presence of a lower basal presinusoidal vascular resistance in cirrhosis.

In the absence of l-NNA, the total vascular resistance increase induced by methoxamine in cirrhotic livers was significantly higher than in normal livers. However, the presence of the NOS inhibitor enhanced the total vascular response to methoxamine more in normal than in cirrhotic livers, bringing the vascular resistance increases to the same level. This indicated that the higher response to the vasoconstrictor in cir-
rhotic livers was caused by a deficient NO production (Fig. 2).

In normal livers, the estimated presinusoidal response to methoxamine was higher than the sinusoidal/postsinusoidal response. Both the presinusoidal and the sinusoidal/postsinusoidal responses were enhanced by the presence of L-NNA (Fig. 3), indicating that NO modulates both the presinusoidal and the sinusoidal/postsinusoidal vascular tone under normal conditions. On the other hand, in cirrhotic livers, the sinusoidal/postsinusoidal response to methoxamine was higher than the presinusoidal response. The presinusoidal response in cirrhotic livers was enhanced by the presence of L-NNA. However, this NOS inhibitor did not change the already enhanced sinusoidal/postsinusoidal response (Fig. 4). Together, these results indicate that, although NO modulates both the presinusoidal and sinusoidal/postsinusoidal vascular tone in cirrhotic livers as well, the sinusoidal/postsinusoidal NO production is severely impaired in cirrhosis.

Vascular response to methoxamine in retrograde experiments. In retrograde experiments using normal rats, body weight, liver weight, and spleen weight were similar between the compared groups. The inversion of flow direction produced a slight liver swelling and transudation of perfusate through the liver capsule. Both swelling and transudation were enhanced with the perfusion pressure increase induced by methoxamine. The swelling reversed completely with the interruption of flow at the end of the experiment. Therefore, in normal livers, the basal vascular resistance is higher in the presinusoidal than in the sinusoidal or the postsinusoidal segments. Although the postsinusoidal resistance increase in the presence of L-NNA was very small, it was significantly higher than the resistance increase in the absence of the inhibitor (Fig. 5), indicating that NO modulates the postsinusoidal vascular tone under normal conditions.

Differently from antegrade experiments, in which all cirrhotic rats had ascites, half of the cirrhotic rats that were used in retrograde experiments had no ascites, although they had been previously treated with CCl4 and phenobarbital for a longer period of time (22.3 ± 0.3 vs. 19.4 ± 0.8 wk; P < 0.01). Nonascitic cirrhotic rats showed a greater liver weight than the ascitic cirrhotic animals (15.8 ± 1.0 vs. 11.7 ± 0.8 g; P < 0.01). Although the spleen weight in nonascitic rats was smaller than in ascitic rats (1.45 ± 0.06 vs. 1.94 ± 0.08 g; P < 0.01), it was higher (P = 0.01) than in normal rats (1.00 ± 0.04 g). The light microscopy confirmed the presence of cirrhosis in all CCl4-treated livers that underwent retrograde perfusion.

In retrograde experiments, the basal perfusion pressure in livers from ascitic and nonascitic cirrhotic livers (16.8 ± 0.4 and 16.4 ± 0.8 cmH2O, respectively) was similarly increased (P = 0.0416) compared with normal livers (14.4 ± 0.8 cmH2O). The sinusoidal/presinusoidal vascular resistance increase induced by methoxamine in livers from ascitic and nonascitic cirrhotic rats was similarly elevated compared with normal livers (Fig. 6). However, the total and, consequently, the postsinusoidal vascular resistance increases induced by methoxamine in livers from ascitic rats were higher than in livers from nonascitic rats. Interestingly, the postsinusoidal response to methoxamine in livers from nonascitic cirrhotic rats was similar to the response in normal livers (Fig. 6).
DISCUSSION

In 1951, Myers and Taylor (30) described the hepatic vein wedge catheterization for estimation of portal pressure in patients. The determination of the hepatic vein pressure gradient, the difference between wedged and free hepatic vein pressures, is a technique of unquestionable value in both the differential diagnosis of portal hypertension (15) and the prognosis on variceal bleeding in cirrhosis (29). The wedged hepatic venous pressure (WHVP) corresponds to the sinusoidal pressure. In cirrhotic livers, the blockage of intersinusoidal communications impairs the effective decompression of the static column at the sinusoidal level. In this situation, WHVP gives an excellent approximation of actual portal pressure (16).

The hepatic vein catheterization in rats was initially described for in vivo collection of hepatic venous blood, which allowed the study of both hepatic hemodynamics and liver function (32, 24). Jenkins and collaborators (21) used the in vivo hepatic vein wedge catheterization to study the effect of propranolol on hepatic hemodynamics in cirrhotic and noncirrhotic rats. Now we describe an in situ rat liver perfusion model in which not only the perfusion pressure but also the sinusoidal pressure is measured by using the hepatic vein wedge catheterization.

Similarly to the WHVP measurement in patients, blockage of intersinusoidal communications in cirrhotic rat livers could extend the zone of stasis to portal branches, resulting in misinterpretation of the sinusoidal pressure measurement. However, compared with the measurement in patients, the measurement of sinusoidal pressure during rat liver perfusion has fundamental differences: although hepatic nerves have not been cut, it is a denervated system; there is no arterial flow; the perfusion flow is constant and higher than the physiological flow; although the perfusate contains 2% albumin, it is still much less viscous than blood; and finally, humoral vasoactive factors are absent in the experimental model. Although all these factors seem to be enough to cause effective decompression of the static column at the sinusoidal level, it is possible that terminal portal vessels have been functionally added to the sinusoidal area in cirrhotic livers. However, if this were the case, the system would not be able to detect an increased sinusoidal/pressinusoidal vascular response in cirrhotic livers during retrograde perfusions as it was already detected.

Direct microscopy observation of hepatic vascular structures has brought valuable information about isolated sinusoids in hemodynamic studies (5). However, differently from intravital microscopy, in which a few peripheral sinusoids are accessed, the WHVP measurement during liver perfusion allows the hemodynamic study of the sinusoids as a functional network. Considering the sinusoidal recruitment, a fundamental characteristic of the intrahepatic circulation, this advantage is particularly important.

When flow/pressure curves are obtained from organ perfusion data, a positive inlet pressure is usually detected at flow zero (critical closing pressure; \( P_Q=0 \)) (2). This phenomenon occurs in consequence of vessel collapse caused by a positive perivascular-intravascular pressure gradient (2). Collapsible tubes and blood vessels have particular hydrodynamic (hemodynamic) characteristics (6). Among them, the flow/pressure slope has been shown to reflect the vascular resistance upstream from the site regulating \( P_Q=0 \) and not the entire vessel vascular resistance (6, 28). However, this aphorism is valid only for partially collapsed vessels. Experiments using Penrose tubes showed that both

Fig. 4. In antegrade perfusions of cirrhotic livers, the presinusoidal response (A) but not the sinusoidal/postsinusoidal response (B) to methoxamine was enhanced by L-NNA (unpaired \( t \)-test).

Fig. 5. In retrograde perfusions of normal livers, the postsinusoidal response to methoxamine in the presence of L-NNA was higher than in the absence of the inhibitor (unpaired \( t \)-test).
completely collapsed and completely distended tubes behave like a Poiseuille resistance whose value in the former state is much higher than in the subsequent state (6).

Although some authors have attributed changes in $P_Q/I$ and flow/pressure slope to hemodynamic changes in the sinusoidal and presinusoidal areas, respectively (33), these correlations may not be applicable for any experimental model of liver perfusion. Without the correct localization of collapsing vessels into the intrahepatic circulation, the flow/pressure slope is useless for identification of the vascular segment involved in this function. Similarly to the main point of vascular resistance (28), the critical closing point in the intrahepatic circulation probably varies among different species and different conditions such as cirrhosis.

We have previously shown that NO modulates the global hepatic vascular tone in normal rats (26, 27) and that the production of this vasodilator is decreased in cirrhotic livers (17, 36). In the present paper, the L-NNA-induced enhancement of the total vascular response to methoxamine in normal and cirrhotic livers to the same level indicates that the deficit in NO production plays an important role on the increased intrahepatic vascular tone observed in cirrhosis.

By measuring both the perfusion and the sinusoidal pressures in antegrade and retrograde experiments, we could directly demonstrate that NO modulates the vascular tone in both the presinusoidal and postsinusoidal areas in normal livers. Although we could not obtain a direct measurement of the sinusoidal response to methoxamine (a limitation of our experimental mod-

Fig. 6. In retrograde perfusions of normal and cirrhotic livers, the sinusoidal/presinusoidal response to methoxamine (A) in cirrhotic livers from ascitic and nonascitic rats was similarly increased compared with the response in normal livers; an increased postsinusoidal response (B) in livers from ascitic rats but not in livers from nonascitic rats determined the stepping up of the total response (C) (ANOVA).
el), indirect evidence indicated that NO also modulates the sinusoidal vascular tone in normal livers. At the methoxamine concentration of $3 \times 10^{-5}$ M, the effect of L-NNA on the sinusoidal pressure increase in antegrade experiments (caused by both the sinusoidal and postsinusoidal vascular resistance increases) was maximum (Fig. 3B), whereas it was negligible on the postsinusoidal resistance increase in retrograde experiments (Fig. 5). This observation suggests that the L-NNA-induced enhancement of the sinusoidal pressure increase induced by methoxamine in antegrade experiments was determined by the sinusoidal component. In addition to our previous study showing that sinusoidal endothelial cells produce NO (39), the reports of relaxation of the stellate cell in response to sodium nitroprusside (22) and enhancement of the endothelin type B receptor agonist-induced sinusoidal constriction (colocalized with stellate cells) by $\text{N}^\circ\text{G}$-nitro-L-arginine methyl ester (5) support our conclusion that NO modulates the sinusoidal vascular tone.

The presence of similar effect of L-NNA on the presinusoidal response to methoxamine in normal and cirrhotic livers (increase of 111% at the methoxamine concentration of $3 \times 10^{-4}$ M) indicates that the presinusoidal NO production is preserved in cirrhotic livers. Actually, we observed that the presinusoidal vascular resistance in cirrhotic livers was lower than in normal livers and that the presinusoidal response to methoxamine in cirrhotic livers was significantly lower than in normal livers either in the absence or the presence of NNA. This finding is in accordance with an in vitro study from Heller and collaborators (20) showing that endothelium-denuded portal vein (equivalent to NOS blocker-treated vessel) obtained from cirrhotic patients has a decreased response to methoxamine. Although factors such as the presence of portal venule compression and the development of periportal fibrosis should cause an increase in the presinusoidal vascular resistance in cirrhotic livers, this increase could be limited to small intrahepatic portal vessels while the portal vein and its main branches would behave as part of the splanchnic circulation, a vasodilated system. Therefore, the presinusoidal vascular resistance as a whole could be decreased in cirrhosis. It is important to emphasize that the presence of a decreased presinusoidal vascular resistance (estimated in vitro) in cirrhotic livers does not mean that the venous pressure into this vascular system (measured in vivo) is not increased, since it actually is because of the increased sinusoidal and postsinusoidal vascular resistances.

In antegrade experiments, the absence of effect of NNA on the sinusoidal/postsinusoidal response to methoxamine in cirrhotic livers, which was already increased compared with normal livers, indicated that the NO production in both the sinusoidal and postsinusoidal areas is severely impaired in cirrhotic livers.

Some of the cirrhotic rats that were used in retrograde experiments developed no ascites and showed a smaller increase in spleen weight, indicating that they developed less severe portal hypertension than ascitic rats. The presence of similar basal perfusion pressures in livers from ascitic and nonascitic cirrhotic rats emphasizes the importance of functional factors on the development of this complication in cirrhosis. Actually, we observed that an increased response to methoxamine in livers from ascitic rats was determined by an increased postsinusoidal response to the $\alpha_1$-agonist. An increased sensitivity of the postsinusoidal bed to catecholamines and perhaps to other vasoconstrictors may play an important role in the development of ascites.

Nitrates, NO precursor drugs, have been shown to reduce portal pressure in cirrhotic patients (9, 18) probably by acting via different mechanisms, including both a decrease in the intrahepatic vascular resistance (16) and by causing arterial hypotension (11). The ideal vasodilator for treatment of portal hypertension should have a relatively high selectivity for specific hepatic vascular beds depleted in NO, avoiding an unwanted vasodilatory effect on systemic and splanchnic circulations (3). Chemical manipulation of NO donor molecules could produce liver-selective drugs (8, 38) that would deliver NO exclusively to a desired segment of the hepatic vasculature. On the basis of the results of the present study, drugs designed to deliver NO into the intrahepatic circulation should aim mainly at the sinusoidal and postsinusoidal segments.

In summary, using a modified rat liver perfusion system, we observed that NO modulates the vascular tone in the presinusoidal, sinusoidal, and postsinusoidal segments of the intrahepatic circulation. The NO production in cirrhotic livers is preserved in the presinusoidal area but severely impaired in both the sinusoidal and the postsinusoidal areas. An increased postsinusoidal response to catecholamines may participate in the genesis of ascites in cirrhotic rats. Finally, NO donors and $\alpha_1$-adrenoreceptor blockers specifically designed to act on the sinusoidal and postsinusoidal vascular segments would be useful in the management of portal hypertension and its complications in cirrhosis.

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