Neuronal nitric oxide synthase and systemic vasodilation in rats with cirrhosis

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Received 1 March 2000; accepted in final form 3 August 2000

Xu, Lieming, Ethan P. Carter, Mamiko Ohara, Pierre-Yves Martin, Boris Rogachev, Kenneth Morris, Melissa Cadnapaphornchai, Mladen Knotek, and Robert W. Schrier. Neuronal nitric oxide synthase and systemic vasodilation in rats with cirrhosis. Am J Physiol Renal Physiol 279: F1110–F1115, 2000.—Cirrhosis is typically associated with a hyperdynamic circulation consisting of low blood pressure, low systemic vascular resistance (SVR), and high cardiac output. We have recently reported that nonspecific inhibition of nitric oxide synthase (NOS) with nitro-L-arginine methyl ester reverses the hyperdynamic circulation in rats with advanced liver cirrhosis induced by carbon tetrachloride (CCL4). Although an important role for endothelial NOS (eNOS) is documented in cirrhosis, the role of neuronal NOS (nNOS) has not been investigated. The present study was carried out to specifically investigate the role of nNOS during liver cirrhosis. Specifically, physiological, biochemical, and molecular approaches were employed to evaluate the contribution of nNOS to the cirrhosis-related hyperdynamic circulation in CCL4-induced cirrhotic rats with ascites. Cirrhotic animals had a significant increase in water and sodium retention. In the aorta from cirrhotic animals, both nNOS protein expression and cGMP concentration were significantly elevated compared with control. Treatment of cirrhotic rats for 7 days with the specific nNOS inhibitor 7-nitroindazole (7-NI) normalized the low SVR and mean arterial pressure, elevated cardiac index, and reversed the positive sodium balance. Increased plasma arginine vasopressin concentrations in the cirrhotic animals were also repressed with 7-NI in association with diminished water retention. The circulatory changes were associated with a reduction in aortic nNOS expression and cGMP. However, 7-NI treatment did not restore renal function in cirrhotic rats (creatinine clearance: 0.76 ± 0.03 ml/min·100 g body wt−1 in cirrhotic rats vs. 0.79 ± 0.05 ml/min·100 g body wt−1 in cirrhotic rats+7-NI; P NS.). Taken together, these results indicate that nNOS-derived NO contributes to the development of the hyperdynamic circulation and fluid retention in cirrhosis.

nitric oxide; liver disease; systemic hemodynamics

Experimental and Human Cirrhosis is characterized by a hyperdynamic circulation with low blood pressure, low systemic vascular resistance (SVR), and high cardiac output (CO) (13). Peripheral arterial vasodilation, mainly in the splanchnic circulation, plays a major role in the pathogenesis of this hyperdynamic circulation (29). Although all of the mechanisms have not been elucidated, nitric oxide (NO) has been implicated as a mediator of the arterial vasodilation (21, 37). In different models of portal hypertension, inhibition of NO production has a stronger vasopressor effect than in control animals, suggesting increased vascular NO production during portal hypertension (9, 19). In isolated blood vessels from cirrhotic rats, the hyporesponsiveness to vasoconstrictors such as epinephrine or ANG II is reversed by the administration of nitric oxide synthase (NOS) inhibitors (18, 32, 41). Finally, vascular production of NO in both experimental and human cirrhosis is increased (3, 7, 25, 28).

Arterial vasodilation and arterial underfilling have been proposed to activate the sympathetic nervous system and the renin-ANG-aldosterone axis and lead to the nonosmotic release of arginine vasopressin (AVP) (29). The activation of these vasoconstrictor and diuretic systems has been suggested to be central to the renal complications of cirrhosis (30, 31). Despite elevated systemic NO in cirrhosis, renal plasma flow (RPF) decreases (15), as does glomerular filtration rate (GFR) (14, 15, 21). The mediators of the cirrhotic-associated decrease in renal function have not been completely identified; however, several neurohormones and vasoactive molecules may be involved. ANG II (38) and endothelin-1 (34) are two of the most potent vasoconstrictors in the kidney, and these effects could be attenuated by NO.

We hypothesized that a 1-wk blockade of excessive NO production derived from neuronal NOS (nNOS) should be associated with an improvement in renal sodium and water excretion and normalization of systemic hemodynamics. The goals of this study were: 1) to investigate the effects of 1-wk treatment with low doses of the nNOS inhibitor 7-nitroindazole (7-NI) on...
renal sodium and water excretion in cirrhotic rats with ascites; 2) to investigate the cardiovascular effects of 1 wk of nNOS inhibition in cirrhotic rats with ascites; and 3) to investigate the effects of 1 wk of nNOS inhibition on plasma AVP levels and aortic cGMP expression in cirrhotic rats with ascites.

METHODS

Cirrhosis induction. Male Sprague-Dawley rats (Sasco, Omaha, NE), weighing between 150–200 g, were housed in a controlled environment by using filter-top microisolators. The animals were allowed free access to food (Prolab 3000; Agway, Syracuse, NY; 22.5% protein and 0.44% sodium) and tap water. Cirrhosis was induced by weekly intragastric administration of carbon tetrachloride (CCl4; starting with 40 μl/wk and increasing progressively up to 300–400 μl/wk) along with phenobarbital in the drinking water (0.35 g/l), as previously described (27). The onset of ascites was heralded by a rapid weight gain associated with bulging flanks. When ascites developed, rats were given lower doses of CCl4 (80 μl/wk) for 1 wk, which were then discontinued. This method was chosen to avoid the spontaneous disappearance of ascites that occurs if CCl4 is stopped immediately after the onset of ascites (16). The experimental protocol started 7 days after the last dose of CCl4. Control rats were phenobarbital treated and age matched with cirrhotic rats.

Study design. Cirrhotic rats with ascites and control rats were divided into groups that received the nNOS inhibitor 7-NI or groups that received only vehicle. 7-NI (5 mg/kg body wt) were divided into groups that received the nNOS inhibitor and age matched with cirrhotic rats.

Measurement of cardiovascular and renal function. Mean arterial blood pressure (MAP) and CO were measured after 7 days of 7-NI treatment. Ketamine (40 mg/kg body wt) and xylazine (5 mg/kg body wt) were injected intraperitoneally for anesthesia. The right carotid artery and right jugular vein were catheterized with PE-50 tubing (26). The catheters were tunneled subcutaneously, exteriorized between the scapulae, and secured inside a plastic cup covered with a rubber cap. This prevented animal access to the catheters. Catheters were filled with a heparin-dextrose mixture (250 IU/ml in 50% dextrose) to avoid clotting and bacterial proliferation.

Catheters were flushed daily until MAP and CO were measured, generally 2–3 days after the surgery. On the day of the measurements, rats were placed unrestrained in a small cage and allowed to acclimate for 60 min. MAP and heart rate were monitored with a Statham transducer and an oscilloscope. CO was measured by injection of indocyanine green dye into one PE-50 right jugular vein catheter while blood was pumped at 3 ml/min from the carotid artery through a Waters cuvette into the other right jugular vein catheter. Three sequential cardiac outputs were calculated by using a CX3 analyzer (Beckman Instruments).

Statistics. Statistical analysis between groups was performed by using an ANOVA and F-Scheffé post hoc test. Results are expressed as means ± SE. P < 0.05 was considered significant.

RESULTS

The diagnosis of cirrhosis was confirmed by visual examination at laparotomy and by histological examination. Six rats died during the protocol from apparent complications of cirrhosis. Cirrhotic rats were typically studied between weeks 9 and 10 for the group treated with 7-NI and week 9 for the group treated with vehicle. Phenobarbital-treated control rats were studied after week 9 of phenobarbital administration.

Water and sodium balance. As is the hallmark of cirrhosis accompanied with ascites, cirrhotic rats had a significant increase in water and sodium balance (Fig. 1). Treatment with 7-NI reversed the positive sodium
and water balance. The cirrhotic rats with ascites demonstrated hyponatremia (134 ± 1 vs. 139 ± 1 mmol/l, \(P < 0.05\)) and mild hypoosmolality (297 ± 1 vs. 302 ± 2 mosmol/kg H₂O, \(P < 0.06\)) compared with control rats. Treatment of cirrhotic rats with 7-NI reversed hyponatremia (cirrhotic + 7-NI: 140 ± 1 vs. cirrhotic: 134 ± 1 mmol/l, \(P < 0.01\)) and hypoosmolality (cirrhotic + 7-NI: 305 ± 2 vs. cirrhotic: 297 ± 1 mosmol/kg H₂O, \(P < 0.05\)). Treatment of control rats with 7-NI did not affect serum sodium concentration (control + 7-NI: 139 ± 1 vs. control: 139 ± 1 mmol/l, \(P, 0.05\)) or plasma osmolality (control + 7-NI: 305 ± 2 vs. control: 302 ± 2 mosmol/kg H₂O, \(P, 0.05\)).

**Cardiovascular and renal function during cirrhosis and 7-NI treatment.** The selective nNOS inhibitor 7-NI was used to evaluate the contribution of nNOS-derived NO to the hyperdynamic circulation during cirrhosis. CI, SVR, and MAP were measured in cirrhotic rats, with and without 7-NI administration. CI increased in the cirrhotic compared with control animals (Fig. 2A, \(P < 0.05\)), and this effect was reversed with 7-NI treatment. CI in the control rats was unaffected by 7-NI (control + 7-NI: 32.38 ± 3.0 vs. control: 32.97 ± 2.3 ml·min⁻¹·100 g body wt⁻¹; \(P, 0.05\)). SVR, computed from MAP and CI, decreased significantly in cirrhotic rats compared with control animals (Fig. 2B, \(P < 0.05\)). This effect was abolished by 7-NI (Fig. 2B). The 7-NI had no effect on SVR in the control rats (control + 7-NI: 3.99 ± 0.4 vs. control: 3.70 ± 0.3 mmHg·ml⁻¹·min⁻¹·100 g body wt⁻¹; \(P, 0.05\)). SVR, computed from MAP and CI, decreased significantly in cirrhotic rats compared with control animals (Fig. 2B, \(P < 0.05\)).

This effect was abolished by 7-NI (Fig. 2B). The 7-NI had no effect on SVR in the control rats (control + 7-NI: 3.99 ± 0.4 vs. control: 3.70 ± 0.3 mmHg·ml⁻¹·min⁻¹·100 g body wt⁻¹, \(P, 0.05\)). During cirrhosis, MAP significantly decreased (Fig. 2C, \(P < 0.05\) compared with control animals). The MAP increased with 7-NI administration (Fig. 2C); however, this change did not reach statistical significance. In the control rats, 7-NI did not have any effect on MAP (control + 7-NI: 121 ± 2 vs. control: 118 ± 2 mmHg, \(P, 0.05\)).

Creatinine clearance was used to estimate GFR. GFR was decreased in cirrhotic rats (0.76 ± 0.03 vs. 0.91 ± 0.02 ml·min⁻¹·100 g body wt⁻¹, \(P < 0.05\)) compared with control rats. Treatment of cirrhotic rats...
with 7-NI did not correct this fall in GFR (0.79 $\pm$ 0.05 ml·min$^{-1}$·100 g body wt$^{-1}$, P NS vs. control).

**Plasma AVP concentrations.** The plasma AVP concentrations were increased during cirrhosis, and this effect was reversed by 7-NI (Fig. 3). Plasma AVP in the control rats was unaffected by nNOS inhibition with 7-NI (1.42 $\pm$ 0.1 vs. 1.56 $\pm$ 0.2 pg/ml; P NS).

**nNOS protein expression.** Western blot analysis of nNOS protein expression was assessed in the aorta to determine its contribution to the systemic vascular changes associated with cirrhosis. The nNOS protein expression was significantly increased in cirrhotic rats compared with control rats, and the effect was reversed after 7 days of 7-NI treatment (Fig. 4). nNOS could not be consistently detected in the renal cortex of either control or cirrhotic rats.

**cGMP production in aorta.** cGMP is the second messenger of NO. cGMP production was measured in the aorta in control and cirrhotic rats both in the presence and absence of 7-NI. Levels of cGMP were significantly higher during cirrhosis compared with control (1.41 $\pm$ 0.2 vs. 1.05 $\pm$ 0.1 fmol/mg protein, P < 0.05). Treatment with 7-NI reduced cGMP levels somewhat; however, they were still significantly higher than control values (1.28 $\pm$ 0.06 fmol/mg protein, P < 0.05 vs. control). In the control aorta, 7-NI treatment did not alter cGMP expression (0.94 $\pm$ 0.1 fmol/mg protein, P NS vs. control).

**DISCUSSION**

Cirrhosis is characterized by a hyperdynamic circulation and renal sodium and water retention. It has been proposed that the peripheral arterial vasodilation, which occurs primarily in the splanchnic circulation, activates baroreceptor-mediated vasoconstrictor, antinatriuretic, and antidiuretic responses to counterregulate the relative underfilling of the arterial circulation (29). The basis of this hyperdynamic circulation is not completely understood; however, it appears to involve NO (5, 21). The resistance to the pressor responses of vasoconstrictors such as epinephrine (41), ANG II (8), endothelin (12), and vasopressin (24) in experimental cirrhosis is reversed by nonspecific NOS inhibitors. In addition, endothelial NOS (eNOS) protein expression is significantly upregulated in the aorta and mesenteric arteries from cirrhotic rats (20). On the basis of this background, it seems that alterations in the L-arginine-NO pathway are involved in the hyperdynamic circulation in cirrhosis.

The goal of the present study was to investigate the role of nNOS in contributing to the cirrhosis-associated hyperdynamic circulation and renal abnormalities. The investigation of nNOS during cirrhosis was undertaken for several reasons. First, although the contributions of eNOS and iNOS to the cirrhosis-mediated hyperdynamic circulation have been studied (22), the role of nNOS has not been investigated. A recent study has suggested that nNOS plays a significant role in blood pressure in chronic renal failure (43), and another study suggests a role of nNOS in the hyposensitivity of cirrhotic rats to nerve stimulation (33). Second, the locations of nNOS protein expression [aorta, macula densa, and collecting duct (1, 2, 23, 40)] make it a candidate to potentially contribute to the cirrhosis-associated peripheral vasodilation and changes in renal function leading to sodium and water retention. On the basis of this background, we hypothesized that nNOS function is involved in the peripheral arterial vasodilation and the renal perturbations in cirrhosis. The major findings of the present study were 1) nNOS protein expression in aorta was upregulated in cirrhotic rats; 2) specific inhibition of nNOS with 7-NI partially reversed not only the increased nNOS protein expression in aorta but also the decreased SVR in the cirrhotic rats; and 3) water and sodium retention during cirrhosis were reversed by 7-NI treatment.
Compared with eNOS, 7-NI has been shown to preferentially inhibit nNOS by a severalfold margin (4). Interestingly, similar to the effect of the nonspecific NOS inhibitor, nitro-L-arginine methyl ester to decrease eNOS protein expression, 7-NI decreased the protein expression of nNOS in the aorta. The mechanism whereby these NOS inhibitors downregulate the NOS proteins is not clear. Because the second messenger of NO is cGMP, the aortic cGMP would be expected to be elevated in cirrhosis and at least partially reversed with the 7-NI treatment. This was indeed the case. However, the failure of the aortic cGMP to return totally to normal during the 7-NI treatment likely indicates the involvement of the other NOS isoforms, namely, eNOS and iNOS. Another possible explanation is the previously described elevation in atrial natriuretic peptide in cirrhosis (10, 11, 21), because cGMP is also the second messenger for that hormone (39).

The present study also provided information about the water retention and hyponatremia associated with cirrhosis. According to the peripheral vasodilation hypothesis, the relative arterial underfilling that accompanies the diminution in SVR should cause a nonosmotic stimulation of the antidiuretic hormone AVP and contribute to water retention and hyponatremia. In the present study, plasma AVP concentrations were elevated in the cirrhotic animals, thus confirming previous observations (21, 25). The administration of 7-NI, which reversed the diminished SVR and thus the arterial underfilling, also reversed the elevation in plasma AVP in the cirrhotic animals. Although this supports a baroreceptor-mediated effect on the nonosmotic release of AVP, a direct central effect cannot be excluded because NOS and AVP are colocalized in the supraoptic and paraventricular nuclei (6).

Despite the reversal in systemic hemodynamics, treatment with 7-NI failed to correct the decreased GFR seen in cirrhotic rats. The reversal of systemic vasodilation with 7-NI would be expected to improve renal perfusion pressure. However, it is possible that the intrarenal inhibition of nNOS may have obscured this potentially beneficial effect by enhancing the response to renal vasoconstrictors (e.g., renal nerves, ANG II, endothelin, etc.). The reversal of positive sodium and water balance is consistent with a decrease in tubular reabsorption during 7-NI treatment.

In summary, nNOS appears to contribute to the peripheral arterial vasodilation and hyperdynamic circulation in experimental cirrhosis, in addition to any effects of eNOS and iNOS. The chronic (7-day) inhibition of nNOS with 7-NI reversed the systemic arterial vasodilation as well as the sodium and water retention.

This work was supported by a grant from the National Institutes of Health (NIH; DK-19928). Ethan P. Carter was supported by HL-07171P32 and the American Heart Association (Scientist Development Award). Melissa Cadnapaphornchai was supported by a National Research Service Award from the NIH (HD-08566–01).

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