Neuronal nitric oxide synthase and systemic vasodilation in rats with cirrhosis

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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 tetra-chloride (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⁻¹·100 g body wt⁻¹ in cirrhotic rats vs. 0.79 ± 0.05 ml·min⁻¹·100 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.

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 vasodilatation, 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 vasodilatation (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 vasodilatation 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 antiadrenergic 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 cirrhotically-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 aged 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) 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 tunnelled 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 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 tunnelled 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 the computer and averaged. CO, expressed as cardiac index (Cl, ml·min⁻¹·100 g body wt⁻¹), and MAP were used to calculate SVR, mmHg·ml⁻¹·min⁻¹·100 g body wt⁻¹. GFR was estimated from 24-h creatinine clearance. Animals were placed in metabolic cages subsequent to 7 days of 7-NI treatment and measurement of systemic hemodynamics. Urine was collected for a 24-h period. Serum and urine creatinines were measured by using a Beckman creatinine analyzer (Beckman Instruments, Fullerton, CA).

Determination of plasma vasopressin, sodium, and osmolality. After acetone-ether extraction, plasma AVP concentrations were measured by RIA as previously described (17). The rat antibody (2849) for AVP RIA was generously provided by Dr. Jacques Durr (Bay Pines Veterans Affairs Medical Center, Bay Pines, FL). AVP concentration was expressed as picograms per milliliter. Plasma sodium concentrations were measured by using a CX3 analyzer (Beckman Instruments).

Western blot analysis. Western blots were performed, as previously described, on aorta (18). After pulverization, frozen tissue was glass homogenized in a lysis buffer in 50 mM β-glycerophosphate, 100 μM Na₅VO₄, 2 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, and 1 mM DTT containing protease inhibitors (20 μM pepstatin, 20 μM leupeptin, 1,000 U/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The protein concentration was determined for each sample by using the Bradford method (Bio-Rad Laboratories, Richmond, CA). Fifty micrograms of total aortic protein were loaded per lane. The proteins were separated on denaturing SDS/7.5% polyacrylamide gels by electrophoresis. Fractionated proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Blots were blocked overnight at 4°C with 5% nonfat dry milk in TBS-T, pH 7.5 (20 mM Tris base, 137 mM NaCl, 0.1% Tween 20). Western blot analysis was performed by using an antinNOS monoclonal antibody (Transduction Laboratories, Lexington, KY). Blots were incubated with the primary antibody (1:1,500 in TBS-T) for 70 min at room temperature and then washed with TBS-T. Blots were then incubated with the secondary antibody conjugated to horseradish peroxidase (1:1,500 in TBS-T) for 70 min. After washing, antibodies were detected by using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) per manufacturer’s instructions. Molecular weights were confirmed by using prestained protein markers (Sigma, St. Louis, MO). Rat pituitary lysate (Transduction Laboratories) served as the positive control for nNOS. Intensity of antigenic signals on the ECL film was videomagnified and quantitated by using a software package (NIH Image version 1.61). Densitometric integrated values (area × density of the band) were determined, and differences were expressed as percent change from the mean of the cirrhotic samples within a given blot because those values were always the highest.

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 balance.
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 NS) or plasma osmolality (control + 7-NI: 305 ± 2 vs. control: 302 ± 2 mosmol/kg H2O, P NS).

**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 NS). 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 NS). 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 NS).

Creatinine clearance was used to estimate GFR. 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 NS). 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 NS).

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 ± 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 ± 0.1 vs. 1.56 ± 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 ± 0.2 vs. 1.05 ± 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 ± 0.06 fmol/mg protein, P < 0.05 vs. control). In the control aorta, 7-NI treatment did not alter cGMP expression (0.94 ± 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.

**Fig. 3.** Plasma arginine vasopressin (AVP) concentration during cirrhosis and with nNOS inhibition. Levels of plasma AVP were measured in control rats and in cirrhotic rats in the absence and presence of nNOS inhibition with 7-NI, as described in METHODS. *Significantly greater than the other 2 groups.

**Fig. 4.** Western blot analysis of aortic nNOS expression during cirrhosis and with 7-NI treatment. Homogenates of aorta (50 μg protein/lane) were resolved by SDS-PAGE, blotted, and probed with nNOS monoclonal antibody (top, representative blot). From a series of blots, relative expression was determined by using densitometry (bottom). *Significantly increased compound with control, P < 0.05.
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.

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