

# Nitric Oxide Synthase (NOS) Inhibition for One Week Improves Renal Sodium and Water Excretion in Cirrhotic Rats with Ascites

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## Abstract

Normalization of the increased vascular nitric oxide (NO) generation with low doses of  $N^G$ -nitro-L-arginine methyl ester (L-NAME) corrects the hemodynamic abnormalities of cirrhotic rats with ascites. We have undertaken this study to investigate the effect of the normalization of vascular NO production, as estimated by aortic cyclic guanosine monophosphate (cGMP) concentration and endothelial nitric oxide synthase (eNOS) protein expression in the aorta and mesenteric artery, on sodium and water excretion. Rats with carbon tetrachloride-induced cirrhosis and ascites were investigated using balance studies. The cirrhotic rats were separated into two groups, one receiving 0.5 mg/kg per day of L-NAME (CIR-NAME) during 7 d, whereas the other group (CIR) was administered the same volume of vehicle. Two other groups of rats were used as controls, one group treated with L-NAME and another group receiving the same volume of vehicle. Sodium and water excretion was measured on days 0 and 7. On day 8, blood samples were collected for electrolyte and hormone measurements, and aorta and mesenteric arteries were harvested for cGMP determination and nitric oxide synthase (NOS) immunoblotting. Aortic cGMP and eNOS protein expression in the aorta and mesenteric artery were increased in CIR as compared with CIR-NAME. Both cirrhotic groups had a similar decrease in sodium excretion on day 0 (0.7 versus 0.6 mmol per day, NS) and a positive sodium balance (+0.9 versus +1.2 mmol per day, NS). On day 7, CIR-NAME rats had an increase in sodium excretion as compared with the CIR rats (sodium excretion: 2.4 versus 0.7 mmol per day,  $P < 0.001$ ) and a negative sodium balance (-0.5 versus +0.8 mmol per day,  $P < 0.001$ ). The excretion of a water load was also increased after L-NAME administration (from  $28 \pm 5\%$  to  $65 \pm 7$ ,  $P < 0.05$ ). Plasma renin activity, aldosterone and

arginine vasopressin were also significantly decreased in the CIR-NAME, as compared with the CIR rats. The results thus indicate that normalization of aortic cGMP and eNOS protein expression in vascular tissue is associated with increased sodium and water excretion in cirrhotic rats with ascites. (*J. Clin. Invest.* 1998. 101:235–242.) Key words: cirrhosis • ascites • nitric oxide • vasodilation • edema • eNOS

## Introduction

A hyperdynamic circulation with low blood pressure, low systemic vascular resistance, and high cardiac output is characteristic of experimental and human cirrhosis (1). Peripheral arterial vasodilation, mainly in the splanchnic circulation, plays a major role in the pathogenesis of this hyperdynamic circulation (2). The cause of the arterial vasodilation in cirrhosis is not completely elucidated, but a strong body of evidence supports a role for an increased vascular production of nitric oxide (NO),<sup>1</sup> a potent vasodilator (3). Indeed, since this hypothesis was raised by Vallance and Moncada (4), several studies have addressed this issue. In different animal models of portal hypertension, inhibition of NO production has a stronger vasopressor effect than in the control animals suggesting an increased NO production (5, 6). The characteristic in vitro hyporesponsiveness of vessels from cirrhotic rats to vasoconstrictors such as epinephrine or angiotensin II is reversed by the administration of nitric oxide synthase (NOS) inhibitors (7–9). Estimation of vascular NO production either in vitro or in vivo has demonstrated an increased NO production in experimental cirrhosis (10, 11). In addition, there is evidence that NO production is increased in human cirrhosis (12, 13).

Previously, we have demonstrated that aortic cyclic guanosine monophosphate (cGMP), the intracellular second messenger that is mainly responsible for the vasodilating effect of NO in vascular smooth muscle cell, is a good index of vascular NO production (10). Aortic cGMP was increased in the carbon tetrachloride (CCl<sub>4</sub>)-phenobarbital rat model of cirrhosis, more in cirrhotic rats with than without ascites, and correlated inversely with blood pressure and systemic vascular resistance (10). By titrating different doses of  $N^G$ -nitro-L-arginine methyl ester (L-NAME), a nonspecific inhibitor of NOS that was administered orally for 7 d, a dose (0.5 mg/kg per day) was found to normalize aortic cGMP content in cirrhotic rats with ascites to the same level as control rats (14). This treatment was ac-

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1. *Abbreviations used in this paper:* ANP, atrial natriuretic peptide; AVP, arginine vasopressin; CCl<sub>4</sub>, carbon tetrachloride; cGMP, cyclic guanosine monophosphate; CIR, cirrhotic rats; CIR-NAME, cirrhotic rats treated with  $N^G$ -nitro-L-arginine methyl ester; CTL, control rats; CTL-NAME, control rats treated with  $N^G$ -nitro-L-arginine methyl ester; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; L-NAME,  $N^G$ -nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; PRA, plasma renin activity; PVL, partial portal vein ligation.

accompanied by a normalization of the systemic hemodynamics in cirrhotic rats with ascites, as blood pressure and systemic vascular resistance increased and cardiac index decreased (14).

Previous studies have reported that peripheral arterial vasodilation is associated with sodium and water retention of cirrhosis and there is evidence that administration of vasoconstrictors such as 8-ornithine vasopressin and norepinephrine improves sodium and water excretion in human cirrhosis (15, 16). However, use of these vasoconstrictors alone usually does not result in a significant improvement of renal excretion if not associated with other treatment, such as head-out water immersion (16).

We hypothesized that long-term (1 wk) blockade of excessive vascular NO production, as demonstrated previously by normalization of cGMP and systemic hemodynamics, should be associated with improvement in sodium and water excretion. By using low doses of L-NAME as in our previous hemodynamic study, the goals of this study were: (a) to investigate the effects of 1 wk treatment with low doses of L-NAME on renal sodium and water excretion in cirrhotic rats with ascites; (b) to assess the effect of this NOS inhibition on the neurohumoral response regulating sodium and water retention, including plasma renin activity (PRA), aldosterone, arginine vasopressin (AVP), and atrial natriuretic peptide (ANP); and (c) to investigate the effect of one week NOS inhibition on cGMP concentration and endothelial NOS (eNOS) expression in selected vessels.

## Methods

**Cirrhosis induction.** Male Sprague-Dawley rats (Sasco, Omaha, NE), weighing between 150–200 g, were housed in a controlled environment using filter-top microisolators. The animals were allowed free access to food (Prolab 3000; Agway, Inc., Syracuse, NY; 22.5% protein and 0.44% sodium) and tap water. Cirrhosis was induced by weekly intragastric administration of carbon tetrachloride (starting with 40  $\mu$ l per week and increasing progressively up to 300–400  $\mu$ l per week) along with phenobarbital in the drinking water (0.35 g/liter) as previously described (17). The onset of ascites was heralded by a rapid weight gain associated with bulging flanks. When ascites developed, rats were given lower doses of CCl<sub>4</sub> (80  $\mu$ l per week) for 2 wk and then discontinued. This method was chosen to avoid the spontaneous disappearance of ascites that occurs if CCl<sub>4</sub> is stopped immediately after ascites appears (18). The experimental protocol started 7 d after the last dose of CCl<sub>4</sub>. Control rats were phenobarbital-treated and age-matched with cirrhotic rats.

**Study design.** Cirrhotic rats with ascites (CIR) and control rats (CTL) were divided in four groups: control rats treated with L-NAME (Bachem, Torrance, CA) (CTL-NAME,  $n = 8$ ) and cirrhotic rats treated with L-NAME (CIR-NAME,  $n = 14$ ), untreated cirrhotic rats (CIR,  $n = 12$ ), and untreated control rats (CTL,  $n = 8$ ). During the 7 d after the last dose of CCl<sub>4</sub> (days –6 to 0), rats were acclimated progressively to the metabolic cages. Based on our previous observation (14), a dose of 0.5 mg/kg per day of L-NAME was administered orally during 7 d (days 1 to 7). To ensure a constant amount of L-NAME the calculated dose was diluted in 15 ml of tap water and administered from 7 P.M. to 7 A.M. in a small bottle. When the rats had drunk the whole quantity, they were allowed free access to water during the rest of the day. The untreated cirrhotic and control rats had the same protocol but with only vehicle. In the balance experiments animals were housed individually in metabolic cages (Nalgene metabolic cages; Nalge Company, Rochester, NY) with pelleted chow. These cages provide a good separation of the urine and the feces with the combination of a collecting funnel and a separating cone in the lower chamber. Animals in metabolic cages were subjected to

12-h light–dark cycles. Measurements of water and food intake, body weight, and urine volume were undertaken at day 0 and day 7. Urine specimens were collected twice a day (7 A.M. and 7 P.M.) and frozen in –70°C for later analysis. Each urine sample was centrifuged to remove any solid matter before analysis. The rats were killed by decapitation to obtain the aorta and mesenteric artery and the truncal blood. During exsanguination blood samples were collected under ice in EDTA, sodium heparin and regular tubes, centrifuged at 4°C for 10 min and plasma was stored at –70°C until assayed. Ascites when present at laparotomy was aspirated with a 30-ml syringe and quantified. The arteries obtained were cleaned from adjacent tissue, rinsed in cold phosphate-buffered saline and snap-frozen in liquid nitrogen in less than 3 min (three samples for each rat). Tissue samples were stored at –70°C until protein extraction. Plasma and urine sodium levels were determined in the clinical laboratory using a Beckman CX3 (Beckman Instruments, Inc., Fullerton, CA). Plasma and urine osmolalities were determined by freezing point depression (Advanced Instruments Inc., Needham Heights, MA). Plasma and urine creatinine concentrations were determined using a Beckman creatinine analyzer 2 (Beckman Instruments, Inc.). 24-h creatinine clearance was used to estimate glomerular filtration rate (GFR). Sodium balance was calculated by subtracting the daily urinary sodium excretion from the sodium intake; fecal sodium was not measured. Water balance was calculated by subtracting the urine output from the water intake. Pulmonary and intestinal fluid losses were not taken into account. This explains the positive water balance obtained in all rats.

In another group of cirrhotic animals, the ability to excrete a water load was tested. 3 wk after the appearance of ascites, cirrhotic rats were subjected to a water load of 30 ml/kg by gavage under light methoxyflurane anesthesia (Metofane; Pittman-Moore, Inc., Mundelein, IL) and urine was collected for 3 h. The last urine was collected after a lower abdominal massage. Animals with an excretion of the water load between 20 and 50% after 3 h on day 0 were included in the same protocol of L-NAME treatment or vehicle ( $n = 6$  in each group). If water excretion was superior to 50%, the rats were treated one more week with low dose of CCl<sub>4</sub> and subjected to another water load 1 wk later. At day 7 of L-NAME or vehicle administration, animals had the same water load protocol.

The protocol was approved by the University of Colorado Health Sciences Center Animal Care and Use Committee.

**Determination of aortic cGMP concentration.** Concentration of cGMP in aortic tissue was measured as previously described (10). Briefly, the thoracic aorta was homogenized in 1 ml of 0.1 N HCl with an all-glass homogenizer at 4°C. Homogenates of aortic tissue were then centrifuged at 3,000 g for 60 min and the supernatant was stored at –20°C until assayed. Protein concentrations were determined using the Bradford method (Bio-Rad Laboratories, Richmond, CA). cGMP was measured by radioimmunoassay (RIA) (Amersham Corp., Arlington Heights, IL) using an acetylation protocol and expressed as femtomol per milligram of protein.

**Hormonal measurements.** After acetone–ether protein extraction, plasma AVP concentrations were measured by RIA as described earlier (19). The rat AVP antibody (No. 2849) for AVP RIA was generously provided by Dr. Jacques Durr (Bay Pines Veterans Affairs Medical Center, Bay Pines, FL). AVP was expressed as picograms per milliliter.

Plasma aldosterone concentrations were measured using a solid-phase RIA for measurement of aldosterone in unextracted serum (Coat-a-Count Aldosterone; Diagnostic Products Corp., Los Angeles, CA). Aldosterone was expressed in nanogram per deciliter. PRA was estimated by RIA of Angiotensin I generated (INCSTAR Corp., Stillwater, MN) and expressed as nanograms per milliliter per hour as described previously (14). Plasma ANP was measured by RIA with a rabbit anti-rat ANP (Peninsula Laboratories, Inc., Belmont, CA). Blood was collected in a chilled EDTA tube containing 500 KIU/ml of Aprotinin. Protein extraction was done using a C18 Sep Column extraction method according to the instructions of the manufacturer. ANP was expressed in picograms per milliliter.

**Western blotting.** Western Blots were performed as previously described (20). After pulverization, frozen arteries were glass homogenized in a lysis buffer (50 mM B-glycerophosphate, 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5% Triton X-100, 1 mM DTT) containing protease inhibitors (Pepstatin, 20  $\mu$ M; Leupeptin, 20  $\mu$ M; Aprotinin, 1000 U/ml; and PMSF, 1mM). The protein concentration was determined for each sample using the Bradford method (Bio-Rad Laboratories). The proteins were separated on denaturing SDS/7.5% polyacrylamide gels by electrophoresis. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) by wet electroblotting for 90 min. 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 with the specific anti-eNOS and anti-iNOS monoclonal antibodies (Transduction Laboratories, Lexington, KY). Blots were incubated with the first antibody, either anti-eNOS (1:1000) or anti-iNOS (1:1000) for 90 min at room temperature and washed. Subsequent detection of the specific proteins (140 kD for eNOS and 130 kD for iNOS) was carried out by enhanced chemiluminescence (ECL; Amersham Corp.), according to the manufacturer's instructions. Prestained protein markers (Sigma Chemical Co., St. Louis, MO) were used for molecular weight determinations. Proteins extracted from human aortic endothelial cells (Transduction Laboratories) were used as the positive control for eNOS and protein extracts from rat vascular smooth muscle cells treated for 24 h with lipopolysaccharide were used as the positive control for iNOS. ECL films were videoimaged (Videocamera; Sony Corp., Park Ridge, NJ) and quantitated using a software package (Image 1; Universal Imaging Corporation, West Chester, PA.). Bands from gels made with serial concentrations of proteins from aortas and mesenteric arteries were found to be linear over a wide range. For quantification of eNOS expression, 20  $\mu$ g of protein extract from aortas and 10  $\mu$ g from mesenteric arteries were chosen. Densitometry results are reported as integrated values (area  $\times$  density of the band) and expressed in percentage as compared with controls (100%).

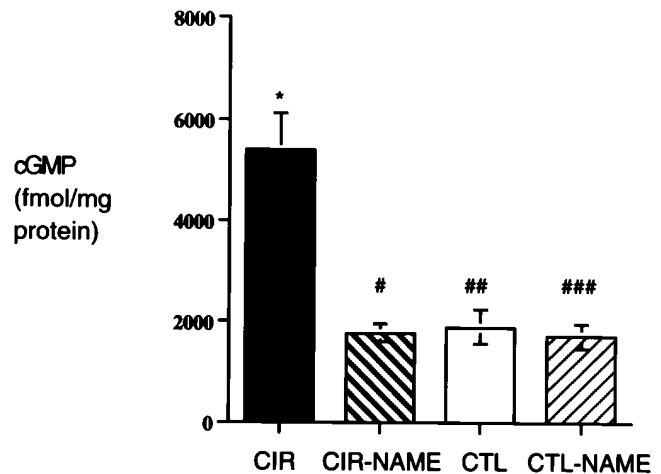
**Statistics.** Statistical analysis of cGMP data and densitometry results was performed using ANOVA, F-Scheffe post hoc tests, paired and unpaired Student's *t* test, Mann-Whitney test, and Wilcoxon test. Results are expressed as mean  $\pm$  SEM. *P* < 0.05 was considered significant.

## Results

The diagnosis of cirrhosis was confirmed by visual examination at laparotomy and by histological examination. One animal died during the protocol in the CIR-NAME group and two in the CIR group died. Cirrhotic rats were studied at 14.5  $\pm$  0.9 wk for the group treated with L-NAME (*n* = 13) and 12.8  $\pm$  0.9 wk for the group treated with vehicle (*n* = 10) after starting the cirrhosis-induction program. At the time of study all animals had marked ascites. Phenobarbital-treated control rats were studied after 14  $\pm$  1 wk of phenobarbital administration (*n* = 8 each group).

**Vascular cGMP concentration and NOS expression.** The aortic concentration of cGMP (5,669  $\pm$  483 fmol/mg of protein, *n* = 10) was markedly increased in cirrhotic rats with ascites and was significantly higher than the other three groups (*P* < 0.001). Aortic cGMP from cirrhotic rats with ascites treated with L-NAME was measured at 1931  $\pm$  231 (*n* = 13), a value not significantly different from the control rats (1845  $\pm$  218; *n* = 8). The control rats treated with L-NAME have a slight decrease of aortic cGMP (1635  $\pm$  166; *n* = 8) which was not significant (Fig. 1).

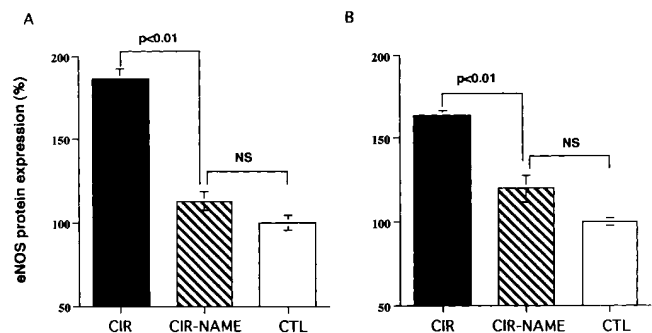
eNOS protein was upregulated in the aorta from cirrhotic rats with ascites (CIR 186  $\pm$  6%, *n* = 6 versus CTL 100  $\pm$  4.6%,



**Figure 1.** Aortic concentrations of cGMP (mean  $\pm$  SEM) in cirrhotic rats treated with L-NAME (CIR-NAME) as compared with untreated cirrhotic rats (CIR), controls treated with L-NAME (CTL-NAME) and control rats (CTL). \**P* < 0.001 versus CIR-NAME, CTL, and CTL-NAME. #, NS versus CTL and CTL-NAME. ##, NS versus CIR-NAME and CTL-NAME. ###, versus CIR-NAME and CTL. Aortic cGMP content in cirrhotic rats treated with L-NAME was similar to control rats and significantly lower than the untreated cirrhotic rats.

*n* = 6, *P* < 0.01) but this upregulation was totally suppressed by L-NAME treatment and was similar to the control rats (CIR-NAME 113  $\pm$  5.7%, *n* = 6 versus CTL 100  $\pm$  4.6%: NS) (Fig. 2). Similar results were found in mesenteric arteries (CIR 164  $\pm$  3%, *n* = 6 versus CTL 100  $\pm$  2.5%, *n* = 6, *P* < 0.01; CIR-NAME 120  $\pm$  8%, *n* = 6 versus CTL 100  $\pm$  2.5%: NS). iNOS protein was not detectable either in the aorta or mesenteric arteries (data not shown).

**Balance studies and renal function.** Table I shows the results of balance studies, plasma sodium concentration, plasma osmolality, and GFR in the four groups of rats studied. No dif-



**Figure 2.** Effect of chronic NOS inhibition on eNOS protein expression examined by immunoblots. Quantification of the scanned band density expressed in percent compared with control. (A) Aortas (20  $\mu$ g of protein extracts). eNOS protein was upregulated in the aorta from cirrhotic rats with ascites (CIR 186  $\pm$  6%, *n* = 6 versus CTL 100  $\pm$  4.6%, *n* = 6, *P* < 0.01) but this upregulation was totally suppressed by L-NAME treatment and was similar to the control rats (CIR-NAME 113  $\pm$  5.7%, *n* = 6 versus CTL 100  $\pm$  4.6%: NS). (B) Similar results were found in mesenteric arteries (10  $\mu$ g). (CIR 164  $\pm$  3%, *n* = 6 versus CTL 100  $\pm$  2.5%, *n* = 6, *P* < 0.01; CIR-NAME 120  $\pm$  8%, *n* = 6 versus CTL 100  $\pm$  2.5%: NS).

Table I. Balance Studies, Plasma Sodium Concentration, Plasma Osmolality and Glomerular Filtration Rate in the Four Groups of Rats at Day 0 and Day 7 of L-NAME (0.5 mg/kg per day) or Vehicle Administration

	Control		Control + L-NAME		Cirrhosis		Cirrhosis + L-NAME	
	Day 0 n = 8	Day 7 n = 8	Day 0 n = 8	Day 7 n = 8	Day 0 n = 10	Day 7 n = 10	Day 0 n = 13	Day 7 n = 13
Weight (g)	469±16	455±14	474±13	465±17	406±19*	413±16	424±8*	397±8‡
Food intake (g per day)	15.4±1.4	13.7±1.5	15.7±1.0	12.7±1.3	15.8±1.8	14.5±1.8	19.8±0.7*	19.2±1.5*
Sodium intake (mmol per day)	1.6±0.1	1.4±0.2	1.6±0.1	1.3±0.1	1.6±0.2	1.5±0.1	2.0±0.3*	2.9±0.2*
Sodium excretion (mmol per day)	1.4±0.1	1.3±0.3	1.4±0.1	1.2±0.2	0.7±0.9*	0.7±0.1*	0.6±0.1*	1.4±0.2‡
Sodium balance (mmol per day)	+0.2±0.2	+0.1±0.2	+0.2±0.1	+0.03±0.1	+0.9±0.1*	+0.8±0.1*	+1.2±0.1*	-0.5±0.2‡
Water intake (ml per day)	25.3±1.5	27.1±1.7	27.1±1.7	23.8±1.6	28.6±2.4	30.9±1.9*	34.3±2.5*	29.7±1.3*
Urine output (ml per day)	16.9±1.2	20.4±3.3	15.7±0.9	15.9±0.7	9.8±0.4*	10.5±0.7*	10.6±0.8*	14.7±0.9‡
Water balance (ml per day)	8.4±0.9	6.5±1.7	11.4±1.1	7.9±1.0	18.8±2.2*	20.4±2.5*	23.7±2.2*	15.0±1.4‡
P Na (mmol/liter)		139.2±1.1		141.0±1.0		136.6±1.1		140.0±1.0 <sup>  </sup>
P osm (mOsm/kg/H <sub>2</sub> O)		299.3±1.5		297.1±1.5		294.1±1.8 <sup>§</sup>		299.9±1.8 <sup>  </sup>
GFR (ml/min/100 g)		0.94±0.04		0.82±0.04 <sup>§</sup>		0.83±0.02 <sup>§</sup>		0.81±0.03 <sup>§</sup>

\**P* < 0.05 versus control day 0; †*P* < 0.05 versus cirrhosis + L-NAME day 0 and cirrhosis day 7; §*P* < 0.05 versus control day 7; ||*P* < 0.05 versus cirrhosis day 7.

ferences were observed among groups in food, sodium, or water intake either in baseline conditions (day 0) or at day 7, except for a greater food and water intake in the CIR-NAME group at both time points. Moreover, water intake was also greater in the CIR group at day 7, compared with control rats at day 0. In baseline conditions (day 0), CIR rats had significantly lower body weight, sodium excretion, and urine output as compared with CTL and CTL-NAME rats. CIR rats had a positive sodium balance and water balance as compared with both groups of control rats, indicating the existence of sodium and water retention consistent with the formation of ascites and/or edema. Similar findings were observed in baseline conditions in CIR-NAME rats. Cirrhotic rats not treated with L-NAME continued to have marked sodium and water retention with a positive sodium and water balance at the end of the experimental period (day 7). By contrast, the administration of L-NAME to cirrhotic rats with ascites was associated with a significant reduction in body weight and a significant increase in sodium excretion and urine output, in the absence of changes in food or water intake. Consequently, sodium balance became negative and water balance was markedly reduced, which was consistent with decreased ascites and edema. Consistent with these findings was the observation that CIR-NAME rats had markedly lower ascites volume as compared with CIR rats at the end of the treatment period (9.7±2.9 ml, range: 0–25 ml versus 38.1±5.9 ml, range: 21–75 ml, respectively; *P* < 0.001). Moreover, 6 out of 13 CIR-NAME animals did not have measurable ascites at the end of treatment. In contrast to the findings in cirrhotic rats, the administration of L-NAME to control rats was not associated with significant changes in any of the parameters studied.

At day 7, CIR rats had lower plasma sodium concentration and plasma osmolality as compared with CTL rats, whereas CIR-NAME rats had plasma sodium and osmolality similar to CTL rats and higher than CIR rats. GFR was lower in CTL-NAME than in CTL rats. By contrast, the administration of L-NAME was not associated with a reduction of GFR in cirrhotic rats.

**Water excretion.** Acute water excretion (3 h after a water load: 30 ml/kg) was significantly improved by L-NAME treatment (CIR-NAME: day 0, 28±5% versus day 7, 65±7% of water load excreted; *P* < 0.05) but not untreated cirrhotic rats (CIR: day 0, 32±10% versus day 7, 33±11%: NS) (Fig. 3). Minimal urine osmolality after water load was also significantly decreased in CIR-NAME from day 0, 385±36 to 217±28 mOsm/kgH<sub>2</sub>O on day 7 (*P* < 0.01) as compared with untreated cirrhotic rats (CIR: day 0, 375±33 to 447±44 mOsm/kg H<sub>2</sub>O on day 7, NS).

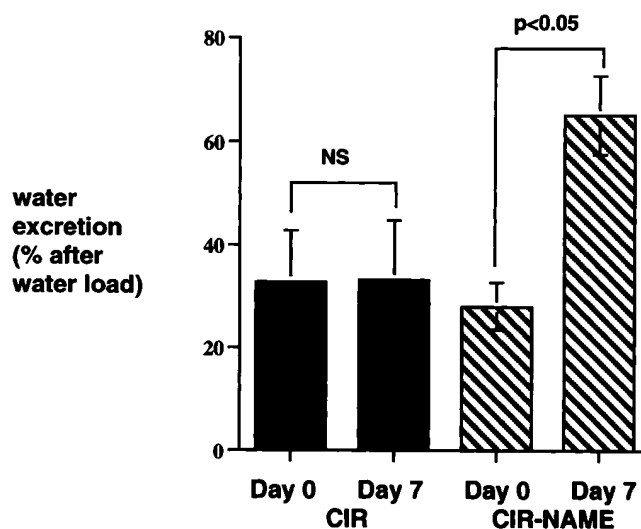


Figure 3. The ability to excrete water during the 3 h after a water load of 30 ml/kg was impaired in both treated (CIR-NAME, *n* = 6) and untreated (CIR, *n* = 6) cirrhotic rats with ascites at day 0. After 7 d of L-NAME a significant improvement in water excretion occurred in cirrhotic rats treated with L-NAME (CIR-NAME: day 0, 28±4.7% versus day 7, 65±7.5% of water load excreted; *P* < 0.05), but not in untreated cirrhotic rats (CIR: day 0, 32±9.7% versus day 7, 33±11.5%: NS).

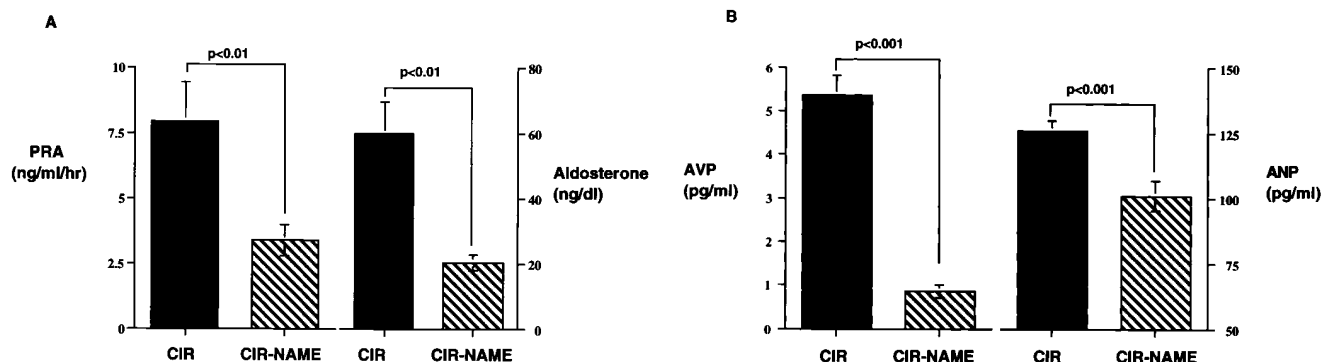


Figure 4. Plasma PRA and aldosterone (A), AVP and ANP (B) concentrations in cirrhotic rats with ascites after 7 d of L-NAME (CIR-NAME) versus untreated cirrhotic rats (CIR). With 7 d of L-NAME treatment plasma AVP, PRA and aldosterone decrease to concentrations not significantly different than those measured in control animals. Plasma ANP concentrations decreased but still remained above controls values.

**Hormonal results.** It was not possible to measure all hormones in each rat, therefore the numbers are different than the total number of rats studied. As depicted in the Fig. 4, A and B, PRA ( $n = 10$ ), aldosterone ( $n = 10$ ), and AVP ( $n = 7$ ) were significantly decreased in cirrhotic animals treated with L-NAME as compared with untreated cirrhotic rats: PRA ( $8.0 \pm 1.5$  versus  $3.4 \pm 0.6$  ng/ml per hour;  $P < 0.01$ ,  $n = 8$ ), aldosterone ( $60 \pm 9.3$  versus  $20.2 \pm 2.5$  ng/dl;  $P < 0.01$ ,  $n = 7$ ) and AVP ( $5.4 \pm 0.4$  versus  $0.9 \pm 0.1$  pg/ml;  $P < 0.001$ ,  $n = 6$ ). These results are in keeping with our previous study where a decrease in plasma PRA and AVP concentrations was observed (14); moreover, the values of cirrhotic rats treated with L-NAME were comparable to those in control animals (PRA: CIR-NAME  $3.4 \pm 0.6$  versus CTL  $2.6 \pm 0.4$  ng/ml per hour,  $n = 6$ ; NS; AVP: CIR-NAME  $0.9 \pm 0.1$  versus CTL  $1.0 \pm 0.3$  pg/ml; NS; aldosterone: CIR-NAME  $20.2 \pm 2.5$  versus CTL  $14.2 \pm 3.3$  ng/dl; NS). L-NAME treatment in CTL rats did not significantly change plasma aldosterone (CTL  $14.2 \pm 3.3$ ,  $n = 6$  versus CTL-NAME  $23.3 \pm 5.6$  ng/dl,  $n = 5$ ) or AVP concentrations (CTL  $1.0 \pm 0.3$ ,  $n = 7$  versus CTL-NAME  $0.8 \pm 0.2$  pg/ml,  $n = 6$ ).

Plasma ANP was also significantly decreased in cirrhotic rats with ascites treated with L-NAME ( $n = 7$ ) as compared with untreated cirrhotic animals ( $126 \pm 4$  versus  $101 \pm 6$  pg/ml;  $P < 0.001$ ,  $n = 7$ ), but the plasma ANP concentrations were still significantly higher than the values in control animals,  $n = 7$ , (CIR-NAME  $101 \pm 6$  versus CTL  $75 \pm 4$  pg/ml;  $P < 0.01$ ).

## Discussion

Peripheral arterial vasodilation has been well documented in cirrhosis and is known to contribute to the hemodynamic alterations of cirrhosis (2, 21). The cause of the renal sodium and water retention in cirrhosis is, however, not completely elucidated and there are different theories about its pathogenesis (2, 21–23). Recently, it has been proposed that the major initiating factor is the peripheral arterial vasodilation in the splanchnic circulation that activates baroreceptor-mediated vasoconstrictor, antinatriuretic, and antidiuretic responses to counterregulate the relative underfilling of the arterial circulation (2). This counterregulation in compensated cirrhosis is associated with an increased plasma volume but without ascites. However, when the cirrhosis progresses, decompensation occurs as defined by ascites formation.

A role for NO in the arterial vasodilation of cirrhosis has emerged recently (24). The cause of the increased vascular production of NO is not completely known but its role in the vascular hyporeactivity of cirrhosis has been incriminated. Specifically, the pressor resistance to vasoconstrictors such as epinephrine (8), angiotensin II (25), endothelin (26), and vasopressin (27) in experimental cirrhosis can be reversed by non-specific NOS inhibitors. The increased NO production derives primarily from the endothelium, since removal of the endothelium abolishes the hyporesponsiveness of the cirrhotic vessels to vasoconstrictors (8, 11, 28, 29). Furthermore, the eNOS isoform has been shown to be upregulated in experimental cirrhosis (20, 30). Using aortic cGMP as an index of the vascular production of NO (10, 31), we have used low doses of L-NAME during 7 d to normalize the vascular cGMP content to the same level as the control rats (14). We have demonstrated that this normalization of vascular cGMP is accompanied by a reversal of the hyperdynamic circulation, namely, a correction of the low blood pressure and systemic vascular resistance, and the high cardiac index (14).

The goal of this study was to investigate the effect of the normalization of vascular cGMP concentration on the abnormal renal sodium and water excretion in cirrhotic rats with ascites. By using the same dose of L-NAME that reverses the hyperdynamic circulation of cirrhosis (14), aortic cGMP content in the cirrhotic rats treated with L-NAME was at the same level as the controls. In addition, upregulation of eNOS protein in aorta and mesenteric artery of cirrhotic rats, which is thought to play a major role in the increased NO production (20, 30), was suppressed in cirrhotic rats treated with L-NAME to a level comparable to control rats. Our results with L-NAME demonstrate a marked improvement in renal sodium and water excretion that was associated with a significant negative sodium balance and diminished ascites in the cirrhotic rats. Although quantification of the amount of ascites in cirrhotic rats was not done before the treatment, at the end of the study protocol the quantity of ascites measured at laparotomy was significantly less in the cirrhotic rats treated with L-NAME than in the untreated cirrhotic animals.

Claria et al. (5) have demonstrated that intravenous administration of pressor doses of another NOS inhibitor *N*<sup>ω</sup>-nitro-L-arginine (NNA), for a short period of time (25 min), acutely increases sodium excretion in CCl<sub>4</sub> cirrhotic rats with ascites.

Lee et al. (6) also demonstrated in partial portal vein-ligated rats (PVL) that intravenous administration of NNA, which increased mean arterial pressure to hypertensive values (150 mmHg), prevented the increase in plasma volume and extracellular sodium space observed during the 4 d after portal vein ligation. Both studies suggested that inhibition of NOS could be beneficial for the renal sodium excretory function. However, the acute effect of NOS inhibition (5) can not be extrapolated to a prolonged effect because of the complexity of vasoactive and neurohormonal interactions regulating chronic renal sodium and water handling. Moreover, the chronic effect of NOS inhibition also must be dissociated from a natriuretic effect of secondary hypertension (6). The CCl<sub>4</sub> cirrhotic model is associated with ascites whereas the PVL model is not (6). Vascular NO production is also more pronounced in the cirrhotic rat model than in the PVL model of portal hypertension (32). This study demonstrates that chronic treatment with an oral NOS inhibitor in cirrhotic rats with ascites improves sodium and water excretion and leads to a significant decrease in ascites. The dose and duration of the NOS inhibition normalizes systemic hemodynamics (14). The results therefore support a major role of excessive vascular NO production in the pathogenesis of sodium and water retention of cirrhosis. Moreover, normalization rather than complete suppression of aortic cGMP concentration improves sodium and water retention in cirrhotic rats with ascites, thus avoiding the vasoconstrictor and hypertensive effects of complete NOS inhibition (33).

The improved sodium and water excretion in cirrhotic rats with NOS inhibition no doubt involves several tubular factors independent of GFR. A change in GFR was not a contributor since the decreased GFR in cirrhotic rats was not improved by L-NAME treatment. Tubular sodium reabsorption is under the influence of several factors (34). The renin-angiotensin-aldosterone axis, which is known to be activated in cirrhosis, plays a major role in the control of tubular sodium reabsorption. Angiotensin II exerts a vasoconstrictor effect on efferent arterioles, thus resulting in enhanced peritubular interstitial oncotic pressure with subsequent increased proximal tubular sodium reabsorption (35). Angiotensin II not only modifies sodium reabsorption through peritubular hemodynamic changes, but also by a direct effect on sodium transport in the proximal tubules mediated via AT<sub>1</sub> receptors (36). In addition, aldosterone promotes sodium reabsorption in the cortical and medullary collecting ducts and has been shown to contribute substantially to the sodium retention in cirrhosis. Specifically, antagonism of the tubular action of aldosterone with high dose of spironolactone (300 mg per day) results in natriuresis in 90% of nonazotemic cirrhotic patients with ascites (37). In this study, PRA and plasma aldosterone were both decreased by L-NAME treatment in cirrhotic rats to concentrations comparable to the control rats. Thus, this hormonal suppression during L-NAME no doubt contributed to the improved sodium excretion.

Among neurohumoral systems capable of influencing renal function, renal sympathetic activity has been demonstrated to have an antinatriuretic effect independent of changes in GFR and renal hemodynamics (38), this appears to be a direct effect on the proximal tubule sodium transport through  $\alpha$ 1 adrenergic receptors (39). An increase in renal sympathetic activity is present in cirrhotic rats and contributes to the renal sodium retention as demonstrated by the natriuretic effect of renal denervation (40). Although renal sympathetic activity was not

measured in this study, it is likely that the increase in arterial pressure secondary to L-NAME administration loaded high pressure arterial baroreceptors and led to a reflex suppression of renal sympathetic nervous activity (41). The reduced renal sympathetic nerve activity would then promote natriuresis both by a direct effect to reduce tubular sodium reabsorption and an indirect effect via suppression of renin release (42). The increase in arterial pressure would also contribute to natriuresis by increasing renal perfusion pressure (33).

ANP is a major counterregulator to vasoconstrictor and antinatriuretic systems and plays a pivotal role to maintain GFR and natriuresis in sodium-retaining states (43, 44). In cirrhotic rats, the majority of the increased ANP production comes from the ventricles rather than the atria (45). In this study, plasma ANP was increased in cirrhotic rats with ascites and was decreased by the chronic treatment of L-NAME. The decrease of ANP may be due to the decrease in cardiac output (14) and angiotensin II (46) that followed chronic treatment with L-NAME. However, the plasma ANP was still elevated after L-NAME as compared with the control rats; moreover, an increase in distal delivery of sodium has been shown to reverse the resistance to ANP in cirrhotic patients (47). As discussed above, there are several factors which would be expected to increase distal sodium delivery in the cirrhotic animals during L-NAME treatment. Thus, enhanced sensitivity to ANP associated with increased distal delivery can not be excluded as a contributor to the natriuretic response to L-NAME.

In this study water excretion was also improved by L-NAME in cirrhotic rats. Hypersecretion of arginine vasopressin (AVP) is an important factor in the water retention of cirrhotic rats (48) and is not suppressed by the hyponatremia which accompanies advanced stages of cirrhosis. V<sub>2</sub> receptor AVP antagonists have been shown to increase water excretion in cirrhotic rats (49) and V<sub>1</sub> receptor AVP antagonists have been shown to decrease blood pressure in cirrhotic animals (50). In this study the decrease of plasma AVP concentration observed in the cirrhotic rats treated with L-NAME was associated with an increase in water excretion and reversal of the hyponatremia and hypoosmolality present in the cirrhotic rats with ascites. In addition to the decreased plasma AVP, other factors could participate in the enhanced water excretion in the L-NAME treated cirrhotic rats, such as an increase in distal fluid delivery.

Finally, it is important to point out that NO synthesis inhibition for 1 wk was not associated with a reduction in GFR in cirrhotic rats with ascites, while GFR decreased significantly in control rats. This observation is in keeping with previous studies showing that the acute inhibition of NO synthesis in cirrhotic rats with ascites does not produce changes in GFR and increases renal prostaglandin production (5, 51). By contrast, when both NO and prostaglandin synthesis are inhibited simultaneously a marked reduction in GFR occurs, which suggests that prostaglandins maintain renal hemodynamics during NO synthesis inhibition in cirrhosis (51).

In conclusion, the results of this study demonstrate that normalization of aortic cGMP concentration and eNOS expression in the aorta and mesenteric arteries by prolonged NOS inhibition is associated with improvement in renal water and sodium excretion and a decrease of ascites in cirrhotic rats. These results raise the possibility of using long term NOS inhibition in patients with cirrhosis and ascites. If methods recently applied to assess NO production in human cirrhosis, such as

measurements of NO production in peripheral blood neutrophils or monocytes or of NO in exhaled air (52, 53), prove to be an accurate index of increased NO production in the peripheral arterial (splanchnic) vasculature, then NOS inhibitors could be titrated to normalize NO production. Our findings may thus lead to a more rational therapeutic approach for sodium and water retention, and the associated sequelae, in human cirrhosis.

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