Inducible nitric oxide synthase and glomerular hemodynamics in rats with liver cirrhosis

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Porst, Markus, Andrea Hartner, Holger Krause, Karl F. Hilgers, and Roland Veelken. Inducible nitric oxide synthase and glomerular hemodynamics in rats with liver cirrhosis. Am J Physiol Renal Physiol 281: F293–F299, 2001.—This study was designed to test the hypothesis that glomerular de novo expression of inducible nitric oxide synthase (iNOS) contributes to renal hemodynamic abnormalities in liver cirrhosis developed 3 wk after common bile duct ligation (CBDL). De novo expression of iNOS mRNA was detected by RT-PCR in RNA extracts from isolated CBDL rat glomeruli whereas no iNOS mRNA was found in control rat glomerular RNA. Immunohistochemical staining for iNOS was negative in control animals whereas, in CBDL rats, positive iNOS staining was detected in an apparently mesangial pattern in all glomeruli. Western blots of protein extracts from isolated glomeruli of CBDL rats, but not control animals, showed a prominent iNOS band of 130 kDa. Application of 4 mg/kg (6)-(1-iminoethyl)lysine, a specific inhibitor of iNOS, reduced GFR and RPF significantly in CBDL rats, whereas control animals were not affected. Similar results were obtained with lipopolysaccharide (LPS)-pretreated animals, which were studied as a positive control. These findings suggest that glomerular de novo expression of iNOS detrimentally affects renal hemodynamics in rats with liver cirrhosis.

A COMMON CLINICAL PROBLEM of patients with liver cirrhosis is the disturbance of renal hemodynamics leading to increased sodium and volume retention, followed by ascites formation in the peritoneal cavity (42). Arterial vasodilation, particularly in the splanchnic vascular bed but also peripherally, seems to play a key role during this process (41), and evidence for a contribution of increased nitric oxide (NO) production to this phenomenon is accumulating (28, 29, 39). Studies in patients suffering from liver cirrhosis revealed an increased plasma level of NO (6, 50) and its metabolites (3, 18). In addition, the activity of nitric oxide synthase (NOS) in monocytes from these patients was elevated (13, 23). Similar results have been obtained in animal models when liver cirrhosis was induced by common bile duct ligature (CBDL) (12) or administration of carbon tetrachloride plus phenobarbital sodium (8, 28, 31). In these models, the nonspecific inhibition of the various NOS isoforms leads to a significant improvement in volume homeostasis (5, 10, 28), implying NO overproduction in the renal dysfunction. This phenomenon may be explained by the correction of exaggerated vasodilation (24, 33). However, NO is a multifunctional molecule acting as vascular and neuronal messenger and inhibitor of platelet aggregation. Moreover, NO might act as a mediator of tubuloglomerular feedback (32, 49) and may have a direct influence on sodium and water transport in proximal tubules (14, 16, 47) and collecting ducts (17, 46).

Despite systemic arteriolar vasodilation, renal vasocostriction may occur in cirrhotic states (41), leading to a decrease in glomerular filtration rate (GFR) (50). This observation indicates a high complexity of neurohumoral-vasodilator-vasoconstrictor interaction (4, 43). It has been hypothesized that NO in the kidney is acting as an antagonist of various vasoconstrictors such as angiotensin II or endothelin-1 (14), thus exhibiting beneficial effects similar to those seen after inhibition of the sympathetic nervous system (2, 45) or angiotensin II (15, 35).

In this study, renal hemodynamics were investigated, including the specific inducible NOS (iNOS) inhibitor L-NIL, to test the hypothesis that glomerular de novo expression of iNOS detrimentally affects renal hemodynamics in rats with liver cirrhosis. In addition, the glomerular expression of iNOS was studied by means of RT-PCR, Western blot analysis, and immunolocalization. We selected conditions leading to unchanged baseline renal hemodynamics to facilitate the study of intrarenal effects.

MATERIALS AND METHODS

Animals. Studies were performed in male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) of 230- to 290-g body weight. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
wt. All experiments in animals were conducted according to the guidelines of the American Physiological Society and were approved by the local government’s animal research ethics committee.

For the induction of liver cirrhosis by CBDL, rats were anesthetized intraperitoneally with methohexital sodium (Brevimytal, Lilly, Bad Homburg, Germany). Abdominal midline incisions were made, and the common bile duct was mobilized, ligated with a suture close to the liver, and cut (48). After 3 wk, animals were instrumented for measurements of renal hemodynamics or killed for further investigations. For Western blot analysis, an earlier time point (1 wk) was also included.

As a positive control for iNOS expression in the kidney, rats were killed 16 h after an intraperitoneal injection of 3 mg/kg body wt lipopolysaccharides (LPS; serotype 0127:B8, Sigma, Deisenhofen, Germany) (44). Measurements of renal hemodynamics in LPS-pretreated rats were performed 16 h after LPS injection.

Extraction of RNA and protein from isolated glomeruli. Glomeruli were isolated by using a sieving technique as described previously (22). Glomerular RNA was extracted with TriFast reagent (Peqlab, Erlangen, Germany) by the method of Chomczynski (9).

Glomerular protein was extracted by incubating isolated glomeruli in RIPA lysis buffer ([in mM] 50 Tris, 10 EDTA, 150 NaCl, and 1 phenylmethylsulfonyl fluoride as well as 1% SDS, 1% NaCl, and 1% Triton X-100) for 10 min in an ultrasonic water bath. Insoluble debris was pelleted by centrifugation at 10,000 g for 10 min.

**RT-PCR.** For RT, 1 μg of total glomerular RNA was reverse-transcribed by Moloney murine leukemia virus RT (M-MuLV RT) by using oligo-dT primers (Boehringer, Mannheim, Germany). The reaction mixture contained (in mM) 50 Tris-HCl (pH 8.3), 50 KCl, 6 MgCl₂, 1 dithiothreitol, and 1 dNTP as well as 1.6 μg oligo-dT primer and 40 U M-MuLV RT in a total volume of 20 μl. The reaction mixture was incubated at 37°C for 60 min and then heated to 65°C for 10 min to denature the RT.

The PCR reaction was performed in a total volume of 20 μl using a PCR Core Kit (Boehringer) containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.5 U Taq DNA polymerase, 0.75 μM of the specific primer pair, and 4 μl of the RT product. Samples were cycled 34 times as follows: 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min. An additional incubation at 72°C for 7 min was executed after the last cycle. The nucleotide primers for iNOS (MWG-Biotech, Ebersberg, Germany) corresponded to 5'-AGC ATC ACC CCT GTG TTC ACC CC-3' (bp 1592–1613, sense) and 5'-TGG GCC AGT CTC CAT TGC CA-3' (bp 1979–1960, antisense), yielding a final PCR product of 388 bp (1). As a positive control and for evaluation of loaded amounts of RNA, primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-AAT GCA TCC TGC ACC ACC AA-3' (sense) and 5'-CTC ATT GAG ACC AAT GCC ACC-3' (antisense), were included as a positive control. Negative control RT-PCRs without RT were performed in parallel.

**Western blot analysis.** Protein content of the supernatant was determined with a protein assay kit (Pierce, Rockford, IL), adjusted to 1 μg/μl with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 20% SDS, 2% β-mercaptoethanol, and 0.01% Coomassie brilliant blue-G, 12% glycerol, f) and boiled for 5 min. Equal amounts of protein (50 μg/ lane) were loaded onto a denaturing 5–12% SDS-polyacrylamide gradient gel. Sigma 7B molecular weight markers (33–230 kDa) were used as standards. Proteins were electroblotted onto nitrocellulose (Hybond ECL, Amersham Pharmacia). To prevent nonspecific antibody binding, membranes were blocked with 2% BSA/3% skim milk powder in Tris-buffered saline (TBS) overnight at 4°C. Detection of iNOS protein was performed by incubating the membrane in a 1:2,500 dilution of a rabbit polyclonal anti-iNOS antibody (Transduction Labs, Lexington, UK). The secondary antibody used was an anti-rabbit horseradish peroxidase-conjugated IgG (Amersham Pharmacia) in a 1:10,000 dilution. Peroxidase labeling was detected with luminescence immunodetection (ECL; Amersham Pharmacia) according to the manufacturer’s recommendation. The resulting signal was captured on Kodak X-OMAT film.

**Immunolocalization.** Immunohistochemical analysis for iNOS in renal tissue sections was performed using the APAAP technique as described (27). Five-micrometer cryostat kidney sections were air-dried for 10 min, fixed in cold acetone for 10 min, washed twice in TBS/1% BSA, and incubated with 100% FCS for 30 min at 37°C. Sections were then layered with the primary antibodies and incubated overnight at 4°C. As a negative control, equimolar concentrations of preimmune rabbit IgG were used. After a rinse in TBS/1% BSA, the sections were incubated with secondary antibodies, mouse anti-rabbit and subsequently rabbit anti-mouse or only rabbit anti-mouse (M 737 and Z 456, both DAKO, Hamburg, Germany) for 1 h at 37°C each. Sections were then layered with the APAAP complex (D 651, DAKO) for 30 min at room temperature. A Neufuchsin kit (K 698, DAKO) was used as a chromogen. Finally, sections were counterstained with hematoxylin and embedded in Aquatex (Merck, Darmstadt, Germany).

**Renal hemodynamics.** Measurements of GFR and renal plasma flow (RPF) were performed as previously described (48). Briefly, femoral arterial and venous catheters, as well as a bladder fistula, were implanted under intraperitoneal anesthesia (50 mg/kg) anesthetics. The animals were then placed in a restrainer and allowed to recover for at least 4 h. In conscious rats, continuous infusions of inulin and para-aminohippurate in saline were started, and mean arterial pressure (MAP) was monitored by pressure transducers connected to a polygraph (Grass, Quincy, MA). The infusion rate was set at 62.5 μl/min. After 2 h, urine samples were collected for determination of flow rate. Urine volume was measured gravimetrically. Steady-state conditions were assumed when diuresis matched infusion rate during three subsequent 10-min intervals. After steady-state conditions were reached, arterial blood was drawn for measurements of hematocrit, sodium, potassium, and chloride as well as determination of GFR and RPF.

The first bolus dose of the iNOS inhibitor L-NIL (0.1 mg iv) was applied 10 min after steady-state conditions were reached. Arterial blood for inulin and para-aminohippurate measurements was drawn 30 min after the first L-NIL injection. After another 30 min, the second L-NIL infusion (1 mg iv) was given, and 30 min later arterial blood was again drawn for GFR and RPF determination.

**Statistical analysis.** Statistical analysis was performed by using the SAS software package (SAS Institute, Cary, NC). For normally distributed data, Student’s t-test was used. Data are given as means ± SE. A P value < 0.05 was considered significant.

**RESULTS**

One week after CBDL, the bile duct was visibly dilated and the liver was enlarged but otherwise appeared normal. No ascites was observed. Three weeks after the procedure the liver appeared yellowish and...
pale with a nodular surface. Ascites was evident in all bile duct-ligated animals.

In RNA extracted from isolated glomeruli, iNOS mRNA could be detected by RT-PCR 3 wk after CBDL, whereas no signal was apparent in control animals (Fig. 1). The size of the positive band for iNOS mRNA was 388 bp, as predicted from the cDNA sequence of rat iNOS and the chosen primer pair. As a control for RNA extraction and RT-PCR, the reverse transcription and amplification of GAPDH mRNA were performed and yielded a band of 455 bp, positive in control and cirrhotic animals. The incubation of RNA extracts from both groups of animals without RT did not produce any signal, indicating that the RT-PCR products were not derived from genomic DNA.

Western blots with the iNOS antibody confirmed the expression of iNOS protein in CBDL rat glomeruli 3 wk after the ligature of the common bile duct. Glomerular protein extracts from animals with only 1 wk of CBDL and control animals did not show a positive signal (Fig. 2). The specificity of the antibody was validated both by a commercially available positive control (lysate from stimulated mouse macrophages, cell line RAW 264.7) and by a glomerular protein extract from LPS-pretreated rats. All positive samples showed a prominent band of ~130 kDa and some faint unspecific bands of lower molecular mass, which were not investigated further. In glomerular protein extracts from control and 1-wk CBDL animals, the antibody detected neither the specific band of 130 kDa nor any of the unspecific bands.

Immunohistochemistry for iNOS protein clearly showed the glomerular de novo expression of iNOS in LPS-pretreated rats and in rats 3 wk after CBDL (Fig. 3). In LPS-pretreated animals, glomeruli, interstitium and infiltrating cells stained positive. iNOS staining in CBDL rats suggested a more mesangial expression pattern and was evident in all of the six animals of the cirrhotic group.

Three weeks after CBDL, the animals exhibited severe liver cirrhosis and moderate ascites. Cirrhotic animals (108 ± 12 mmHg) did not show a significant difference in MAP compared with the control group (111 ± 14 mmHg), and the administration of L-NIL (up to 1 mg) also did not induce notable alterations of blood pressure (Fig. 4A). RPF of CBDL rats showed no significant difference from control animals (4,508 ± 233 vs. 4,900 ± 233 µl·min⁻¹·g·KW⁻¹, where KW is kidney wt) (Fig. 4B). The application of 0.1 mg L-NIL did not reveal a prominent effect on RPF, but 1 mg L-NIL significantly reduced RPF in CDBL (3,208 ± 452 µl·min⁻¹·g·KW⁻¹) rats, whereas RPF in the control group remained unaffected (4,920 ± 392 µl·min⁻¹·g·KW⁻¹). GFR was not different between CBDL rats and control animals (614 ± 55 vs. 598 ± 51 µl·min⁻¹·g·KW⁻¹) (Fig. 4C). Application of the low L-NIL dose did not show any effects, but the high dose of 1 mg decreased GFR in the CBDL animals (421 ± 52 µl·min⁻¹·g·KW⁻¹), whereas control animals did not show alterations (590 ± 48 µl·min⁻¹·g·KW⁻¹). A similar trend was seen in the LPS-pretreated animals. After 16 h of LPS injection, the application of 1 mg L-NIL had no effect on MAP (111 ± 11 vs. 110 ± 12 mmHg), but RPF (4,184 ± 323 vs. 1,936 ± 393 µl·min⁻¹·g·KW⁻¹) and GFR (918 ± 34 vs. 602 ± 48 µl·min⁻¹·g·KW⁻¹) were reduced significantly.

DISCUSSION

We demonstrated that glomerular de novo expression of iNOS occurred after the induction of liver cirrhosis by common bile duct ligature in rats. Because the specific inhibition of iNOS function did not influ-
ence MAP but decreased RPF as well as GFR in rats with liver cirrhosis, a contribution of NO formed by iNOS in glomeruli to the maintenance of renal perfusion and filtration seems likely.

For a better understanding of the role of NO, it is essential to identify its source. There are three isoforms of NOS, each showing a different expression pattern and particular functions (21). The availability of specific inhibitors of the isoenzymes (30) opens new experimental and therapeutic prospects and might help to elucidate NO formation and function in distinct pathological processes.

Because iNOS has been cloned from vascular smooth muscle cells (34), it was found in several tissues and cell types, and its expression after LPS stimulation was demonstrated in rat macrophages and glomeruli (12). In cirrhotic animals, iNOS mRNA expression was detected in thoracic and abdominal aorta and the mesenteric artery (31). Nevertheless, the results concerning renal expression of iNOS protein during cirrhosis are controversial. In some studies no iNOS mRNA was found in renal tissue extracts of cirrhotic animals (8), whereas other authors detected iNOS protein in isolated glomeruli from control animals as well as from cirrhotic animals by means of Western blot analysis (12). To assess iNOS expression in glomeruli during cirrhosis, we employed three different techniques for an unambiguous determination, and all of them clearly showed de novo expression of iNOS in rat glomeruli 3 wk after the ligature of the common bile duct.

The antibody used for Western blot analysis and immunohistochemistry has recently been tested for its specificity (11). Furthermore, we included two positive controls in our assay, both yielding the expected band of 130 kDa. Interestingly, samples from LPS-treated animals showed, beside the prominent band at 130 kDa, some faint bands of lower molecular mass not detectable in cirrhotic animals, suggesting some alternate mechanisms of induction and/or alternate splicing. A band at ~115 kDa appears in cell lysates from stimulated macrophages, LPS-pretreated animals, and cirrhotic animals and thus might be a degradation product of iNOS protein. However, sham-operated control animals did not show any bands. Neither were any bands detectable in glomerular protein preparations 1 wk after CBDL, indicating that the onset of cirrhosis preceded the upregulation of iNOS protein.

RT-PCR, the most sensitive technique used, showed no detectable amounts of iNOS mRNA in control samples but a marked signal in cirrhotic rats. There may be several reasons why other investigators (8) were unable to detect iNOS mRNA in kidneys of cirrhotic rats.

Fig. 3. Immunohistochemistry of cryostat kidney sections for iNOS protein. Positive staining is shown in red; counterstain is with hematoxylin. No glomerular iNOS staining was observed in sham-operated animals (A). Staining for iNOS was evident 3 wk after CBDL (B) and in LPS-pretreated rats (C). Glomerular staining pattern resembles residential glomerular cells rather than infiltrating macrophages and suggests a mesangial predominance. Lower magnification (D) illustrates a prominent glomerular iNOS induction in CBDL rats.
animals. First, these authors used a primer pair yielding a longer 473-bp fragment, which might be disadvantageous for reverse transcription. Second, RNA was extracted from whole kidney sections instead of isolated glomeruli; thus the amount of iNOS mRNA might have been below the detection limit. Finally, in this study cirrhosis was induced by CCl₄ treatment, and it cannot be ruled out that there is no glomerular iNOS expression in that model. At least for iNOS expression in the liver, it has been shown that the iNOS protein level varies temporally and spatially with the type of induction (38). In contrast to the CCl₄ model, endotoxin levels are elevated in the CBDL model, which may mediate iNOS induction (37). Alternatively, activation of the renin-angiotensin system may also contribute to iNOS induction (36).

Published results concerning renal hemodynamics in cirrhotic rats are rather conflicting and, because of diverse methods, hardly comparable. The overexpression of NO has mostly been implicated in pathological processes, confirmed by the beneficial effects of unspecific NOS inhibition (28, 33). The availability of an inhibitor 30-fold more specific for iNOS (30) than for other NOS isoforms opened the possibility to test the hypothesis that iNOS expression detrimentally affects renal hemodynamics (44). The data we obtained strongly argue against a beneficial effect of iNOS inhibition as GFR and RPF declined significantly after L-NIL application in cirrhotic rats, whereas control animals remained unaffected. To explain these conflicting results, one has to consider the differences in models and methods. The vast majority of investigations have been performed in anesthetized animals. Most of the anesthetics, like ketamine (8, 31) or inactin (12, 44), have an immediate influence on MAP in cirrhotic animals. Therefore, the drop in GFR seen in those studies (12, 44) more likely reflects a pressure effect. The increased sensitivity of cirrhotic animals can be explained by their reduced ability to break down these compounds because of their liver dysfunction. Moreover, reduced body fat in cirrhotic rats may alter the metabolism and distribution of those anesthetics. In agreement with this notion, LPS does not produce a reduction in blood pressure in awake animals (44). We cannot exclude the possibility that postoperative stress might influence our results. Chronic catheterization was not feasible in many CBDL rats due to a decreased immune response. We wanted to avoid anesthetics that could lead to a systematic difference between CBDL and control rats. Data collected in our study are solely from conscious rats, which might explain why neither MAP, GFR, nor RPF decreased in our setting, even 3 wk after induction of cirrhosis.

Another reason for conflicting results concerning the effects of iNOS inhibition might be the time point of inhibition. As iNOS expression was not detectable after 1 wk of CBDL, we studied LPS-pretreated animals as a model for recent onset of iNOS induction. At first glance, our results with L-NIL application after LPS pretreatment seem to contradict previous reports of a restoration of renal hemodynamics, accompanied by a 30- to 40-mmHg rise in blood pressure by iNOS blockade (44). However, there are several other reports that iNOS blockade restores systemic hemodynamics but not renal impairment after LPS administration (51) and that iNOS inhibition causes greater afferent than efferent vasoconstriction (20, 51). Clearly, factors such as time after LPS administration and state of anesthesia are important (25, 40). In addition, the Escherichia coli serotype of LPS may play a role: some authors used the 0111:B4 serotype, whereas we used the 0127:B8 serotype. The few studies that compared both sero-

![Fig. 4. Effects of the iNOS inhibitor L-N-(6)-(1-iminoethyl)lysine (L-NIL) on mean arterial pressure (MAP; A), renal plasma flow (RPF; B), and glomerular filtration rate (GFR; C). Administration of 1 mg L-NIL did not affect MAP but significantly decreased RPF and GFR in rats 3 wk after CBDL, whereas none of the parameters was affected in the control group. Open bars, sham operated; shaded bars, CBDL rats (n = 6). KW, kidney wt. *p < 0.05 vs. baseline values.](http://ajprenal.physiology.org/)

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types described greater impairment of renal perfusion with the O111:B4 serotype, which was not due to iNOS (19, 26). We purposely selected conditions (LPS serotype 0127:B8, 16 h before the experiment) under which LPS application did not cause hypertension and renal hyperperfusion. Under these conditions, as in cirrhotic animals, iNOS blockade impaired renal perfusion.

The fact that L-NIL does not alter any of the measured parameters in control animals and that GFR and RPF decline significantly in cirrhotic animals, whereas their MAP remains unaltered, suggest a role for iNOS in the maintenance of glomerular function, possibly by opposing exaggerated vasoconstriction. Our results cast doubt on the therapeutic potential of iNOS inhibition in liver cirrhosis and add to the growing body of evidence that iNOS-mediated NO generation may be helpful or protective under certain circumstances (7).

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