

Bacterial Translocation Up-regulates GTP-Cyclohydrolase I in Mesenteric Vasculature of Cirrhotic Rats

Reiner Wiest,¹ Gregory Cadelina,¹ Sheldon Milstien,³ Robert S. McCuskey,⁴ Guadalupe Garcia-Tsao,^{1,2} and Roberto J. Groszmann^{1,2}

In cirrhosis, arterial vasodilation and the associated hemodynamic disturbances are most prominent in the mesenteric circulation, and its severity has been linked to bacterial translocation (BT) and endotoxemia. Synthesis of nitric oxide (NO), the main vasodilator implicated, is dependent on the essential cofactor tetrahydrobiopterin (BH₄). The key enzyme involved in BH₄ synthesis is GTP-cyclohydrolase I (GTPCH-I), which is stimulated by endotoxin. Therefore, we investigated GTPCH-I activity and BH₄ biosynthesis in the mesenteric vasculature of cirrhotic rats with ascites, as well as their relationship with BT and endotoxemia, serum NO, and mean arterial pressure (MAP). GTPCH-I activity and BH₄ content in mesenteric vasculature was determined by high-performance liquid chromatography. BT was assessed by standard bacteriologic culture of mesenteric lymph nodes (MLNs). Serum endotoxin was measured by a kinetic turbidimetric limulus amoebocyte lysate assay, and serum NO metabolite (NOx) concentrations were assessed by chemiluminescence. BT was associated with local lymphatic and systemic appearance of endotoxin and was accompanied by increases in serum NOx levels. GTPCH-I activity and BH₄ content in mesenteric vasculature were both increased in animals with BT and correlated significantly ($r = 0.69$, $P < .01$). Both GTPCH-I activity and BH₄ levels significantly correlated with serum endotoxin and NOx levels ($r = 0.69$ and 0.54 , 0.81 and 0.53 , $P < .05$). MAP (a marker of systemic vasodilatation) correlated with endotoxemia ($r = 0.58$, $P < .03$) and with GTPCH-I activity ($r = 0.69$, $P < .01$). In conclusion, in cirrhotic animals BT appears to lead to endotoxemia, stimulation of GTPCH-I, increased BH₄ synthesis, and further enhancement of vascular NO production that leads to aggravation of vasodilatation. (HEPATOLOGY 2003;38:1508-1515.)

Bacterial translocation (BT) is defined as the passage of viable bacteria from the gut to mesenteric lymph nodes (MLNs) and/or other extraintestinal sites.¹ In experimental cirrhosis an incidence of BT up to

70% has been reported, and BT has been clearly related to the occurrence of severe infectious complications known to be associated with a poor prognosis.²⁻⁴ Moreover, BT has been shown to be increased in patients with advanced cirrhosis, indicating the clinical relevance of this phenomenon.⁵ BT is the primary event in the etiology of endotoxemia, which is also a common finding in cirrhosis, particularly with progressive severity of disease and predominantly in the portal circulation.^{6,7} However, the direct relationship between BT and endotoxemia has never been investigated.

We have recently reported that BT is associated with a worsening of arterial vasodilatation in cirrhotic animals with ascites.⁸ Nitric oxide (NO) is the main vasodilator mediating arterial vasodilation and the associated circulatory abnormalities in cirrhosis, and vascular NO overproduction has been shown to be predominantly of splanchnic origin.^{9,10} NO is synthesized by 3 different isoforms of NO synthase (NOS).¹¹ Of these the endothelial (eNOS) and neuronal NOS are expressed constitutively while the inducible isoform (iNOS) is synthesized

Abbreviations: BT, bacterial translocation; MLN, mesenteric lymph node; NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; BH₄, tetrahydrobiopterin; GTPCH-I, GTP-cyclohydrolase I; MAP, mean arterial pressure; NOx, NO metabolites; EDTA, ethylenediaminetetraacetic acid; TNF, tumor necrosis factor.

From the ¹Hepatic Hemodynamic Laboratory, Veterans Administration Medical Center, West Haven, CT, the ²Department of Medicine, Yale University School of Medicine, New Haven, CT, the ³National Institute of Mental Health, Bethesda, MD, and the ⁴Department of Cell Biology and Anatomy, College of Medicine, University of Arizona, Tucson, AZ.

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Address reprint requests to: Roberto J. Groszmann, M.D., Hepatic Hemodynamic Laboratory/111J, Veterans Administration Medical Center, 950 Campbell Avenue, West Haven, CT 06516. Fax: 203-937-3873.

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de novo after stimulation by bacterial lipopolysaccharide (LPS), endotoxins, and proinflammatory cytokines. So far, several studies have been unable to show vascular iNOS protein expression in the splanchnic vasculature of portal hypertensive animals, even in the presence of BT.^{8,12-14} Nonetheless, several studies have shown a correlation between serum endotoxin levels and NO in cirrhosis, suggesting a stimulatory role of endotoxemia in vascular NO overproduction.^{6,15,16} Therefore, an apparent paradox of enhanced vascular NO synthesis in conditions of endotoxemia and proinflammatory stimuli but in the absence of iNOS induction is present in cirrhosis.

In cirrhotic rats with BT, we recently observed a significant increase in eNOS activity and eNOS-derived NO overproduction in the mesenteric arterial bed, accompanied by an increase in tetrahydrobiopterin (BH₄).⁸ BH₄ is an essential and rate-limiting cofactor in the synthesis of NO,^{17,18} and can directly increase eNOS-derived NO bioavailability.^{18,19} This has been shown in cultured human endothelial cells, isolated rat aortas, and human primordial placenta.^{20,21} Furthermore, BH₄ has been shown to induce NO-mediated vasorelaxation in various arterial vascular beds and species.^{20,22,23} In addition, its endothelial concentration has been shown to correlate closely with intracellular cyclic guanosine monophosphate levels (cGMP),^{20,24} evidencing its vasodilative properties. However, the mechanism mediating the enhancement in BH₄ synthesis in the splanchnic vasculature in conditions of BT remains unclear.

GTP cyclohydrolase I (GTPCH-I) is the key enzyme regulating the *de novo* synthesis of BH₄ from guanosine triphosphate by mediating the first of 3 enzymatic steps, cleavage of GTP yielding dihydroneopterin triphosphate.^{17,25} Recently, LPS and/or proinflammatory cytokines have been shown to induce GTPCH-I *in vivo* and *in vitro*, leading to enhanced intracellular levels of BH₄.²⁶⁻²⁸ The relationship between vascular GTPCH-I activity, BH₄ synthesis, and endotoxemia had not been investigated in cirrhosis. Thus, the goal of our study was to investigate (1) whether GTPCH-I is up-regulated in mesenteric vasculature of cirrhotic rats with ascites in the presence of BT, and (2) if so, to determine the interrelationship between GTPCH-I, endotoxemia, BH₄ biosynthesis, and augmented NO overproduction.

Materials and Methods

All experimental procedures in this study were conducted according to the American Physiological Society principles for the care and use of laboratory animals.

Induction of Cirrhosis by CCl₄. Male Harlan Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 100 to 125 g underwent inhalation

exposure to CCl₄. Briefly, phenobarbital (0.35 g/L) was added to the drinking water as previously described.²⁹ CCl₄ was used as a hepatotoxin, and phenobarbital was administered to shorten the time required to induce cirrhosis. After 1 week of treatment with phenobarbital, inhalation with CCl₄ was started. Rats were placed in a gas chamber (60 × 40 × 20 cm). Compressed air was passed via a flowmeter (1 L/min), bubbling through a flask containing CCl₄, into the chamber. Animals were exposed to this procedure 3 times per week, starting with 1 minute of bubbling air and 1 minute in the gas atmosphere. Afterward, the dosage was increased by 1 minute until 5 minutes of bubbling air and exposure in the gas atmosphere was reached. This protocol produces a high yield of micronodular cirrhosis in about 12 to 16 weeks of CCl₄ inhalation. Phenobarbital and CCl₄ exposure were stopped at least 6 days before the perfusion experiments. Normal sex- and age-matched untreated rats were used as controls.

Assessment of BT. On the study day, the animals were anesthetized with ketamine (100 mg/kg) and the abdominal skin was shaved and sterilized with an iodine solution. All surgical procedures were performed under strict sterile conditions. In cirrhotic animals, ascites was quantified. Because this model of cirrhosis has been shown to be free of systemic bacteremia,^{2,8} bacteriologic blood cultures were not performed. The caudal and cranial MLNs were removed and weighed with an Ohaus E400D scale (Ohaus Corp, Florham Park, NJ) with an accuracy of ±0.01 g. Tissues were then homogenized in a measured amount of saline, and aliquots of 0.1 mL were plated onto blood, McConkey, and phenylethyl alcohol agar plates (BBL Prepared Media; Becton Dickinson Microbiology Systems, Cockeysville, MD). Spleen and liver were removed and weighed, and liver slices were fixed in 10% neutral buffered formaldehyde. Solid culture media were examined and colonies were counted after 24 and 48 hours of aerobic incubation at 35°C. Any positive MLN cultures were considered indicative of BT from intestinal lumen.

Experimental Protocols. All cirrhotic rats were studied prospectively because, at the time of experimental procedures, it is not known whether BT is present or absent. A total of 34 cirrhotic rats with ascites were included in this study.

In protocol I (n = 16 cirrhotic rats with ascites) serum markers, mesenteric vasculature BH₄ content and GTPCH-I activity as well as mean arterial pressure (MAP) were assessed. Before starting procedures to assess BT, the left femoral artery was exposed and cannulated with a PE-50 catheter. A sample of 1.5 mL of blood was withdrawn in pyrogen-free Vacutainer (Becton Dickenson)

and centrifuged at 5,000 rpm for 15 minutes, and the separated serum stored immediately at -70°C until analysis for endotoxin and NO metabolites (NOx). MAP was evaluated by connecting the catheter to a Statham P-23-Db strain gauge transducer (Statham, Oxnard, CA) and recorded on a Grass model 7D inscription recorder (Grass Instrument Co, Quincy, MA). Measurement of MAP was performed before blood drawing and, once blood had been drawn, it was replaced with 1.5 mL of saline and MAP was remeasured. Homogenate from MLN was stored at -70°C until measurement of endotoxin and NOx.

In protocol II ($n = 18$ cirrhotic rats with ascites), *in vitro* perfusion of de-endothelialized mesenteric preparations were performed as control experiments evaluating potential iNOS-derived NO synthesis in cirrhotic rats with BT.

Levels of endotoxin in serum and MLN homogenates were assayed using the Kinetic Turbidimetric Limulus Amebocyte Lysate (LAL) Microtiter test. In an 8-channel photometer, the increase in optical density was registered at 380 nm. The maximum increase in optical density per minute of each reaction was computed. This procedure results in a standard endotoxin curve that is linear from 0.5 to 1,000 pg/mL. According to a mathematical model, the endotoxin concentration of each sample was determined, as well as factors interfering with the LAL-endotoxin reaction present in plasma or in protein-containing samples.

Determination of NOx concentrations in serum, MLN homogenates, and perfusate was performed using a Sievers Nitric Oxide Analyzer (Sievers Instruments, Boulder, CO) as previously described.⁸ In brief, this assay is based on spectrophotometric analysis after a chemiluminescent reaction between NO and ozone. 50 μL of each sample was placed into the purge vessel containing 3 mL of 0.1-mol/L vanadium chloride in 1 mol/L of hydrochloric acid at 95°C . The NO generated from nitrite and nitrate ions was carried into the analyzer by vacuum through the gas bubbler trap containing 5 mL 1 mol/L of NaOH. This analyzer quantitates dissolved NO and NO₂-derived NO that has been generated by acid and stripped from the solution by nitrogen gas. The NO then reacts with analyzer-generated ozone to form excited NO₂, which releases light in the red and near-infrared regions of the spectrum, and is detected by a thermoelectrically cooled red-sensitive photomultiplier tube. Serum and MLN homogenate were deproteinized before assay by treatment with 70% ethanol for 5 minutes. The lower limit of sensitivity for this machine is below 2 pmoles NO/second.

BH₄ Assay. Frozen mesenteric vasculature was weighed, homogenized in 3 volumes of 50-mmol/L Tris-HCl (pH, 7.4) containing 1 mmol/L dithiothreitol and 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and centrifuged, and supernatant BH₄ levels were measured by high-performance liquid chromatography with fluorescence detection after oxidation as previously described.³⁰

GTPCH-I Activity Assay. Using gel-filtered extracts the product of the GTPCH-I reaction, dihydroneopterin triphosphate, was oxidized to neopterin triphosphate, which then was dephosphorylated by alkaline phosphatase, forming neopterin. Neopterin content was measured by high-performance liquid chromatography as the activity of GTPCH-I in the extract, with 1 unit being equivalent to the production of 1 pmol of dihydroneopterin triphosphate per minute. Data were expressed as [U/mg protein].

In Vitro Perfusion of De-endothelialized Mesenteric Vasculature. The *in vitro* perfusion system used was a partial modification of that originally described by McGregor and used extensively in previous studies from our laboratory.^{8,31} Briefly, the superior mesenteric artery was cannulated with a PE-60 catheter and gently perfused with 15 mL of warm Krebs solution to eliminate blood. Removal of the endothelium was achieved by a combined treatment with cholic acid (sodium salt) and distilled water.^{8,32} In brief, perfusion with cholic acid (0.5% / 1.5 mL for 10 seconds) was followed by flushing with 15 mL of Krebs solution to eliminate cholic acid. The gut was excised close to its mesenteric border, leaving the mesenteric vasculature in the tissue preparation, which then was transferred to the 37°C water-jacketed container and perfused with oxygenated 37°C Krebs solution (95% O₂, 5% CO₂; 4 mL/min for 10 minutes) using a roller pump (Masterflex; Cole-Parmer Co, Barrington, IL). After the mesenteric vasculature was relaxed, 37°C warmed distilled water was perfused for 10 minutes. A period of 30 minutes was allowed before starting any further study protocol. The Krebs solution had the following composition (mmol/L): NaCl, 118; KCl, 4.7; KH₂PO₄, 1.2; MgSO₄, 1.2; CaCl₂, 2.5; NaHCO₃, 25; disodium EDTA, 0.026; and glucose, 11.0; pH, 7.4. Next, over a period of 60 minutes triplet perfusate samples (for 1 minute each, 15 minutes apart) were collected, frozen immediately, and stored at -30°C until NOx assay. The perfusion pressure was measured with a P-23-Db strain gauge transducer (Statham, Oxnard, CA) on a side arm just before the perfusing cannula and recorded on a Grass 7D Polygraph inscriber (Grass Instruments Co, Quincy, MA). To assess whether the vessel was completely de-endothelialized and whether the smooth muscle function

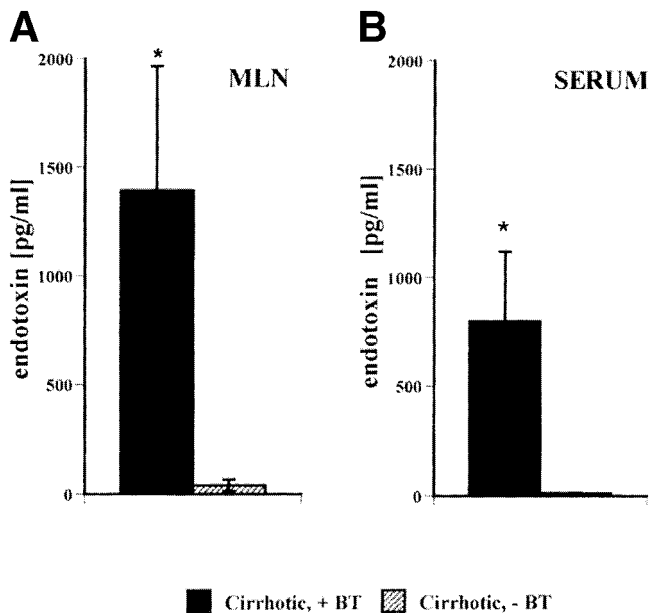


Fig. 1. Levels of endotoxin in serum and mesenteric lymph nodes. Endotoxin concentrations as determined by a kinetic turbidimetric limulus amoebocyte lysate microtiter test in MLN homogenates (A) as well as in serum (B) were significantly elevated in the presence of BT.

was maintained, the mesenteric preparation was precontracted with methoxamine (α_1 -agonist; methoxamine, 100 $\mu\text{mol/L}$) at the end of perfusate collection and dose-dependent vasorelaxation to the endothelium-dependent vasodilator acetylcholine (10^{-8} to 10^{-6} g, bolus of 0.1 mL) and the endothelium-independent vasodilator sodium nitroprusside (SNP) (10^{-6} to 10^{-5} g, bolus of 0.1 mL) was tested.

Statistical Analysis. Results were expressed as mean \pm SE. Statistical analysis was performed using ANOVA (2-way, with repeated measurements), paired and unpaired nonparametric tests when appropriate, with statistical significance set at $P < .05$. Each relationship of serum, tissue, and hemodynamic parameters was tested for regression by a simple regression analysis.

Results

The livers of all CCl_4 -treated rats (LC) were macroscopically cirrhotic. The weight of cirrhotic rats with BT was significantly lower than that of cirrhotic rats without BT (+BT, 317.8 ± 23.5 g vs. -BT, 399.4 ± 12.4 g). The weight of the spleen, expressed as a percentage of body weight, was not significantly different between study groups (+BT, 4.11 ± 0.49 g/kg body weight vs. -BT, 3.63 ± 0.24 g/kg body weight). Moreover, no significant difference in volume of ascites or in weight of MLN was found between cirrhotic rats with or without BT (+BT, 31.4 ± 10.6 mL and 0.11 ± 0.01 g vs. -BT,

12.6 ± 7.8 mL and 0.11 ± 0.01 g, respectively). Among the 34 ascitic cirrhotic rats studied, MLN culture was positive, *i.e.*, BT was present, in 21 animals (+BT, 61.7%), a rate very similar to what has been reported earlier in this model.^{2,8}

Levels of Endotoxin and NOx (Figs. 1 and 2). In cirrhotic rats without BT only low and in most cases almost negligible concentrations of endotoxin were detectable in the systemic circulation. In contrast, cirrhotic rats with BT showed marked systemic endotoxemia and elevated endotoxin concentrations in MLNs (Fig. 1A and B). Moreover, local mesenteric lymphatic endotoxin levels and systemic endotoxemia correlated significantly ($r = 0.61$, $P < .05$), indicating that the source of endotoxins is most probably the gut. NOx concentrations in MLNs were also significantly enhanced in the presence of BT (+BT, 34.0 ± 8.9 $\mu\text{mol/L}$ vs. -BT, 16.2 ± 1.3 $\mu\text{mol/L}$, $P < .01$) and correlated very closely with MLN endotoxin levels ($r = 0.83$, $P < .0001$), reflecting the stimulatory effect of endotoxins on local NO production. Furthermore, as reported earlier,⁸ NOx concentrations in serum were significantly elevated in rats with BT compared with those without BT (Fig. 2A). Finally, serum endotoxin levels significantly correlated with serum NOx levels ($r = 0.80$, $P < .01$, Fig. 2B).

GTPCH-I Activity and BH_4 Content (Figs. 3 and 4). In mesenteric vasculature of cirrhotic rats with BT, GTPCH-I activity and BH_4 content was significantly en-

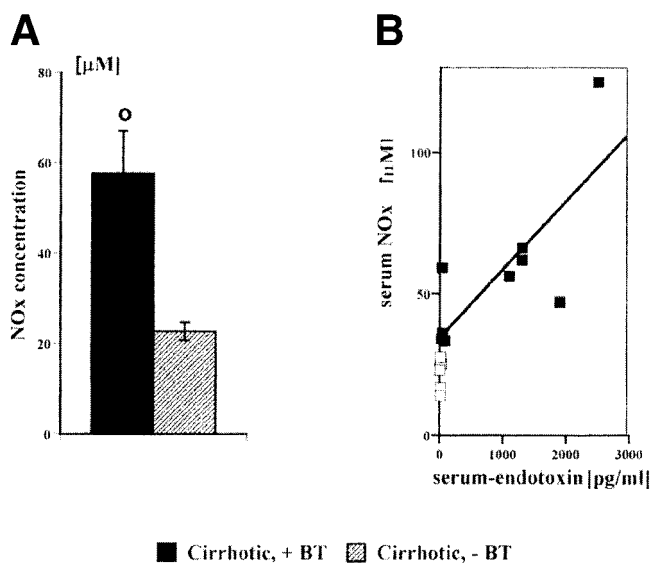


Fig. 2. Levels of NO metabolites in serum. Concentrations of NOx in serum as determined by chemiluminescence were significantly increased in animals with BT as compared with the absence of BT (A) ($P < .001$). This serum parameter was significantly correlated with the appearance of endotoxin in serum (B) ($r = 0.80$, $P < .01$), indicating a potential causative role of endotoxins for stimulation of NOS in cirrhotic rats with ascites.

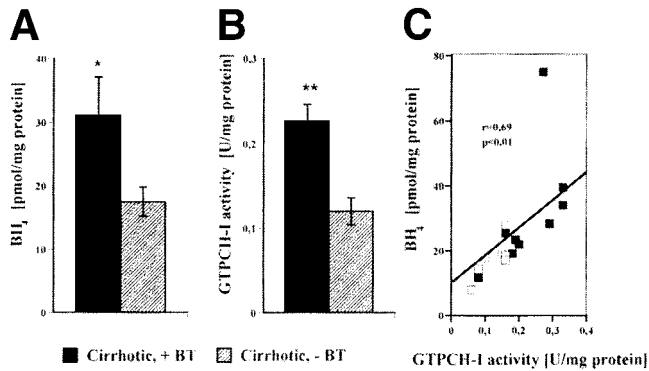


Fig. 3. GTPCH-I activity and BH₄ synthesis in mesenteric vasculature. The content of BH₄ (A) as well as the activity of GTPCH-I (B) in the mesenteric vasculature was markedly enhanced in animals showing bacterial translocation. (C) Concentrations of the essential cofactor BH₄ in the tested tissue were closely linked to the activity of the key enzyme GTPCH-I ($r = 0.69$, $P < .003$). * $P < .05$, ** $P < .01$ versus cirrhosis, -BT.

hanced as compared with animals without BT (Fig. 3A and B). A significant direct correlation was observed between GTPCH-I enzymatic activity and concentration of BH₄ in the splanchnic vasculature ($r = 0.69$, $P < .01$, Fig. 3C), reflecting the widely accepted role of GTPCH-I as key enzyme in the synthesis of BH₄. GTPCH-I activity also correlated with MLN endotoxin levels ($r = 0.53$, $P < .05$) and with serum endotoxin levels ($r = 0.69$, $P < .01$, Fig. 4A), indicating that the enzyme was stimulated by endotoxin. GTPCH-I activity was also found to correlate very closely with systemic NOx concentrations ($r = 0.81$, $P < .001$, Fig. 4B), emphasizing the important role of this enzyme in NO synthesis. Similarly, the essential cofactor BH₄ correlated directly with serum NOx levels as well as

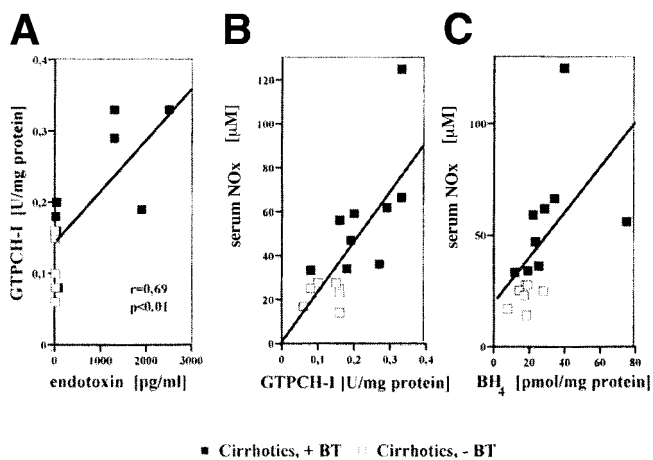


Fig. 4. Regression analysis for GTPCH-I activity and serum markers. GTPCH-I activity in mesenteric vasculature correlated with serum endotoxin levels (A) and serum NOx concentrations (B) ($r = 0.69$ and 0.81 , $P < .01$ and $.001$, respectively). For the essential cofactor BH₄, also a significant positive correlation with NOx serum concentration (C) is seen ($r = 0.53$, $P < .05$).

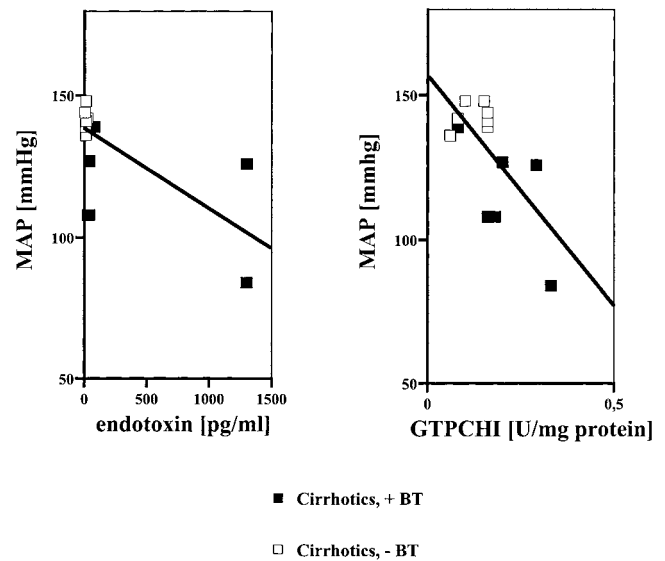


Fig. 5. Regression analysis for MAP showed correlations with serum levels of endotoxin (A: $r = 0.58$, $P < .03$) as well as GTPCH-I activity (B: $r = 0.69$, $P < .01$). $n = 6$ and 7 for cirrhotic rats with and without BT.

systemic endotoxemia ($r = 0.53$, $P < .05$, and $r = 0.54$, $P < .01$, respectively) but not with local mesenteric endotoxin levels.

MAP (Fig. 5). No differences in MAP were observed before and after blood withdrawal (and replacement with saline). As reported earlier,⁸ in the presence of BT, MAP was significantly lower as compared to animals without BT (+BT, 115.3 ± 7.9 mm Hg vs. -BT, 142.5 ± 1.7 mm Hg, $P < .005$). This circulatory dysfunction was linked to the occurrence of endotoxemia ($r = 0.58$, $P < .03$, Fig. 5A) and stimulation of GTPCH-I ($r = 0.69$, $P < .01$, Fig. 5B). Furthermore, as shown earlier,⁸ a significant correlation between MAP and content of BH₄ in the mesenteric vasculature was observed ($r = 0.63$, $P < .02$). Finally, systemic NOx levels also correlated with MAP ($r = 0.67$, $P < .05$), further supporting the importance of NO for arterial vasodilation seen in cirrhosis.^{8,16}

In Vitro Perfusion. Baseline perfusion pressure before removal of the endothelium was not significantly different between study groups (+BT, 12.8 ± 1.1 mm Hg vs. -BT, 12 ± 0.8 mm Hg). There were also no significant differences in baseline perfusion pressure after removal of the endothelium (+BT, 19.4 ± 1.1 vs. -BT, 19.4 ± 0.9 mm Hg). Vasodilator response to acetylcholine at the highest dose used was $5.6\% \pm 1.6\%$ and $7.7\% \pm 2.1\%$ in cirrhotic rats with and without BT, respectively (not significant), showing a sufficient de-endothelialization in the vasculature studied. Vasodilator response to SNP at the highest used dose was $91.5\% \pm 1.6\%$ and $84.7\% \pm 4.2\%$ for cirrhotic rats with and without BT,

respectively (not significant). This is evidence for functional integrity of the vascular smooth muscle in the vascular bed studied. NO_x was basically nondetectable in the perfusate of *in vitro* perfused de-endothelialized mesenteric vasculature of cirrhotic rats with ascites independent of the presence or absence of BT. This clearly shows the lack of vascular NO synthesis in the absence of endothelium.

Discussion

We have recently reported that in cirrhotic rats with ascites, BT promotes eNOS-derived NO overproduction in the mesenteric vasculature, aggravating arterial vasodilatation.⁸ This hemodynamic disturbance is associated with elevated mesenteric tissue levels of BH₄, a well-known stimulator of eNOS-derived NO biosynthesis. In this study, we expanded on these results and show that GTPCH-I, the key enzyme in the biosynthesis of BH₄, is markedly up-regulated in the mesenteric vascular bed of cirrhotic animals with BT and that the concentration of BH₄ correlates closely with GTPCH-I activity. Furthermore, BT is related to endotoxemia and this, in turn, also correlates with GTPCH-I activity. The stimulation of GTPCH-I is associated with an increase in serum levels of NO and is closely linked to the severity of arterial vasodilatation. These results extend our previous observations and clarify the pathway from BT, to endotoxemia, to up-regulation of GTPCH-I, increase in BH₄ biosynthesis, increase in eNOS-derived NO overproduction, and further vasodilatation.

In cirrhosis, endotoxemia is a common finding and increasing levels are associated with hepatic failure, encephalopathy, and death.^{7,33} Moreover, cirrhotic patients with endotoxemia have lower systemic vascular resistance and higher cardiac output than cirrhotic patients without endotoxemia.⁶ BT to MLNs has been considered an important mechanism by which enteric organisms reach systemic sites and has been reported to correlate directly with the occurrence of bacterial infections in experimental cirrhosis.^{1,4} Recent evidence suggests that BT to MLN alone, in the absence of portal or systemic spread of bacteria, may lead to increases in systemic cytokines and indicates that the gut is a cytokine-releasing organ.^{34,35} In this study, we report that BT is strongly associated with the appearance of endotoxins in the mesenteric lymphatic tissue and in the systemic circulation, supporting previous findings^{1,36} and underscoring the importance of BT in the development of endotoxemia in cirrhosis.

In our study, BT was found to be associated with a marked enhancement in GTPCH-I activity that correlated closely with endotoxin levels in mesenteric lymphatic tissue and serum. These results are in line with

previous studies investigating the regulation of GTPCH-I.^{28,37} LPS has been shown to increase GTPCH-I activity in human umbilical endothelial cells and vascular smooth muscle cells without an effect on the activities of other enzymes involved in BH₄ synthesis.^{38,39} Treatment of rats with LPS has been shown to result in enhanced GTPCH-I mRNA expression as well as enzyme activity in several tissues.^{27,28} Moreover, a synergistic stimulatory effect between endotoxin and subsequently released proinflammatory cytokines has been shown^{21,26,40}; for example, while GTPCH-I activity is induced independently by interferon γ or tumor necrosis factor α (TNF- α), this effect is synergistic when combined with LPS.^{26,40} In fact, in our previous study we showed that cirrhotic rats with BT exhibited enhanced MLN and serum levels of TNF- α , which correlated directly with BH₄ levels in the mesenteric vasculature, indicating a contributory role of TNF- α in the induction of BH₄ synthesis.⁸ Considering the well-known stimulatory effect of endotoxin on TNF- α synthesis,⁴¹ it can be proposed that in cirrhotic rats with BT, the up-regulation of GTPCH-I is the result of both a direct stimulatory effect of endotoxins and stimulation by subsequently secreted cytokines, such as TNF- α .

GTPCH-I up-regulation in the mesenteric vasculature of cirrhotic rats with BT was accompanied by and correlated closely with tissue concentrations of BH₄. This is in accordance with well-documented data that show that regulation of BH₄ biosynthesis is almost exclusively dependent on the first and key enzyme, GTPCH-I.^{17,25} That GTPCH-I is essential for NO biosynthesis is shown by studies in which the enzyme is inhibited, thereby blocking the synthesis of BH₄ and leading to abrogation of basal and stimulated NO synthesis and cGMP formation.^{42,43} This is supported by our finding of a close correlation between GTPCH-I activity in mesenteric vasculature, the main site of altered vascular NO biosynthesis in portal hypertension,⁴⁴ and serum levels of NO metabolites.

Regarding the enzymatic source of NO overproduction, we have reported recently that, even in conditions of BT, cirrhotic rats do not overexpress iNOS-derived protein in the mesenteric vasculature but rather up-regulate eNOS-derived NO.⁸ This would seem to be at odds with the fact that iNOS is typically the NOS isoform induced by LPSs, endotoxins, and inflammatory cytokines. While iNOS is known to be predominantly expressed in vascular smooth muscle,¹¹ eNOS is expressed in the endothelium. Therefore, further confirmation of a lack of iNOS-derived NO in cirrhotic rats with BT derives from our finding that de-endothelialized arteries of these animals did not release a significant amount of NO_x. This lack of iNOS-derived NO production in rats with marked and

sustained endotoxemia and marked vasodilatation further supports previous findings by our group and others pointing against a role for iNOS in the circulatory abnormalities of portal hypertension.^{8,12,45} Studies showing an immediate enhancement in endothelial NO release by LPS mediating arterial vasodilation in the absence of iNOS induction were essential to understand this inconsistency.^{46,47} Our observed correlation between BH₄ and NO production, in the context of a deficiency of iNOS protein expression, further supports the view that intracellular concentration of BH₄ is important in determining eNOS-derived NO production. The precise molecular mechanism of this BH₄-dependent stimulation of eNOS is incompletely resolved. It has been suggested that BH₄ acts as an allosteric cofactor by stabilizing the dimeric state of the NOS enzyme, the only catalytically active structure.⁴⁸ However, it has been shown that structural changes alone cannot fully explain the absolute requirement of BH₄, and a strong line of evidence shows that BH₄ serves as redox-active cofactor.^{49,50}

The marked hemodynamic effect of increased BH₄ has been shown in various vascular beds and species.^{20,22,23} In our study, GTPCH-I activity and BH₄ concentration in mesenteric vasculature correlated inversely with MAP, that is, the higher the GTPCH-I activity and the BH₄ content in the mesenteric vasculature, the lower the MAP.

Therefore, our findings clearly suggest that, in advanced cirrhosis, BT induces endotoxemia, which would seem to up-regulate GTPCH-I-activity and subsequently BH₄ biosynthesis in the splanchnic circulation, leading to eNOS-derived NO overproduction and aggravation of arterial vasodilation. However, it is important to note that this study does not establish a causal interaction between LPS, GTPCH-1, BH₄, and the endothelial NO pathway. To achieve this goal it would be necessary to specifically block GTPCH-1 *in vivo* in the mesenteric vasculature of cirrhotic rats with BT. Unfortunately, available GTPCH-1 blockers will also block vital cell functions, and results obtained would be difficult to interpret and would not lead to valid and specific answers regarding BH₄'s impact on NO synthesis and its associated hemodynamic changes.

This study provides another link in explaining the paradox observed in cirrhosis of an augmented vascular NO production in the face of endotoxemia and a lack in iNOS-protein expression. The increase in BH₄ availability induced by endotoxins via stimulation of GTPCH-I may facilitate and augment the already-present and up-regulated eNOS machinery in the mesenteric endothelium in cirrhosis, making *de novo* synthesis of iNOS unnecessary. Therefore, the GTPCH-I/BH₄ pathway could become an important target for pharmacologic in-

terventions aimed at controlling excessive vasodilation in advanced cirrhosis. However, further studies on this proposed new pathway and its exact pathophysiological role in portal hypertension are required.

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References

1. Deitch EA. Bacterial translocation of the gut flora. *J Trauma* 1990;30: S184-189.
2. Garcia-Tsao G, Lee FY, Barden GE, Cartun R, West AB. Bacterial translocation to mesenteric lymph nodes is increased in cirrhotic rats with ascites. *Gastroenterology* 1995;108:1835-1841.
3. Llovet JM, Bartoli R, March F, Planas R, Vinado B, Cabre E, Arnal J, et al. Translocated intestinal bacteria cause spontaneous bacterial peritonitis in cirrhotic rats: molecular epidemiologic evidence. *J Hepatol* 1998;28:307-313.
4. Runyon BA, Squier S, Borzio M. Translocation of gut bacteria in rats with cirrhosis to mesenteric lymph nodes partially explains the pathogenesis of spontaneous bacterial peritonitis. *J Hepatol* 1994;21:792-796.
5. Cirera I, Bauer TM, Navasa M, Vila J, Grande L, Taura P, Fuster J, et al. Bacterial translocation of enteric organisms in patients with cirrhosis. *J Hepatol* 2001;34:32-37.
6. Lin RS, Lee FY, Lee SD, Tsai YT, Lin HC, Lu RH, Hsu WC, et al. Endotoxemia in patients with chronic liver diseases: relationship to severity of liver diseases, presence of esophageal varices, and hyperdynamic circulation. *J Hepatol* 1995;22:165-172.
7. Lumsden AB, Henderson JM, Kutner MH. Endotoxin levels measured by a chromogenic assay in portal, hepatic and peripheral venous blood in patients with cirrhosis. *HEPATOLOGY* 1988;8:232-236.
8. Wiest R, Garcia-Tsao G, Cadelina G, Das S, Shah V, Groszmann RJ. Bacterial translocation to mesenteric lymph nodes enhances eNOS-derived NO overproduction in mesenteric vasculature of cirrhotic rats: role for impairment in vascular contractility. *J Clin Invest* 1999;104:1223-1233.
9. Battista S, Bar F, Mengozzo G, Zanon E, Grosso M, Molino G. Hyperdynamic circulation in patients with cirrhosis: direct measurement of nitric oxide levels in hepatic and portal veins. *J Hepatology* 1997;26:75-80.
10. Wiest R, Groszmann RJ. The paradox of nitric oxide in cirrhosis and portal hypertension: too much, not enough. *HEPATOLOGY* 2001;35:478-491.
11. Sessa WC. The nitric oxide synthase family of proteins. *J Vasc Res* 1994; 31:131-143.
12. Martin PY, Xu DL, Niederberger M, Weigert A, Tsai P, St John J, Gines P, et al. Upregulation of endothelial constitutive NOS: a major role in the increased NO production in cirrhotic rats. *Am J Physiol* 1996;270:F494-499.
13. Morales-Ruiz M, Jimenez W, Perez-Sala D, Ros J, Leivas A, Lamas S, Rivera F, et al. Increased nitric oxide synthase expression in arterial vessels of cirrhotic rats with ascites. *HEPATOLOGY* 1996;24:1481-1486.
14. Heller J, Sogni P, Tazi KA, Chagneau C, Poirer O, Moreau R, Lebrec D, et al. Abnormal regulation of aortic NOS2 and NOS3 activity and expression from portal vein-stenosed rats after lipopolysaccharide administration. *HEPATOLOGY* 1999;30:698-704.
15. Guarner C, Soriano G, Tomas A, Bulbena O, Novella MT, Balanzo J, Vilardell F, et al. Increased serum nitrite and nitrate levels in patients with cirrhosis: relationship to endotoxemia. *HEPATOLOGY* 1993;18:1139-1143.
16. Chu CJ, Lee FY, Wang SS, Lu RH, Tsai YT, Lin HC, Hou MC, et al. Hyperdynamic circulation of cirrhotic rats with ascites: role of endotoxin, tumour necrosis factor and nitric oxide. *Clin Sci* 1997;93:219-225.
17. Werner ER, Werner-Felmayer G, Wachter H, Mayer B. Biosynthesis of nitric oxide: dependence on pteridine metabolism. *Rev Physiol Biochem Pharmacol* 1996;127:97-135.

18. Stroess E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, Luscher T, et al. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest* 1997;99:41-46.
19. Wever RMF, Van Dam T, Van Rijn HJM, DeGroot F, Rabelink TJ. Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. *Biochem Biophys Res Commun* 1997;237:340-344.
20. Van Amsterdam JG, Werner J. Tetrahydrobiopterin induces vasodilation via enhancement of cGMP level. *Eur J Pharmacol* 1992;215:349-350.
21. Rosenkranz-Weiss P, Sessa WC, Milstien S, Kaufman S, Watson CA, Pober JS. Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells. Elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity [see comments]. *J Clin Invest* 1994;93:2236-2243.
22. Schaffner A, Blau N, Scheemann M, Steurer J, Edgell CS, Schoedon G. Tetrahydrobiopterin as another EDRF in man. *Biochem Biophys Res Commun* 1994;205:516-523.
23. Rosenblum WI. Tetrahydrobiopterin, a cofactor for nitric oxide synthase, produces endothelium-dependent dilation of mouse pial arterioles. *Stroke* 1997;28:186-189.
24. Schoedon G, Blau N, Schneemann M, Flury G, Schaffner A. Nitric oxide production depends on preceding tetrahydrobiopterin synthesis by endothelial cells: selective suppression of induced nitric oxide production by sepiapterin reductase inhibitors. *Biochem Biophys Res Commun* 1994;199:504-510.
25. Auerbach G, Nar H. The pathway from GTP to tetrahydrobiopterin: three-dimensional structures of GTP cyclohydrolase I and 6-pyruvoyl tetrahydropterin synthase. *Biol Chem* 1997;378:185-192.
26. Katusic ZS, Stelzer A, Milstien S. Cytokines stimulate GTP cyclohydrolase I gene expression in cultured human umbilical vein endothelial cells. *Arterioscler Thromb Vasc Biol* 1998;18:27-32.
27. Hattori Y, Oka M, Kasai K, Nakanishi N, Shimoda S. Lipopolysaccharide treatment in vivo induces tissue expression of GTP cyclohydrolase I mRNA. *FEBS Lett* 1995;368:336-338.
28. Werner-Felmayer G, Prast H, Werner ER, Philippu A, Wachter H. Induction of GTP cyclohydrolase I by bacterial lipopolysaccharide in the rat. *FEBS Lett* 1993;322:223-226.
29. Sieber CC, Lopez-Talavera JC, Groszmann RJ. Role of nitric oxide in the in vitro splanchnic vascular hyporeactivity in ascitic cirrhotic rats. *Gastroenterology* 1993;104:1750-1754.
30. Tsutsui M, Milstien S, Katusic ZS. Effect of tetrahydrobiopterin on endothelial function in canine middle cerebral arteries. *Circ Res* 1996;79:336-342.
31. McGregor DD. The effect of sympathetic nerve stimulation on vasoconstrictor responses in perfused mesenteric vessels of the rat. *J Physiol (Lond)* 1965;177:21-30.
32. Atucha NM, Shah V, Garcia-Cardena G, Sessa WE, Groszmann RJ. Role of endothelium in the abnormal response of mesenteric vessels in rats with portal hypertension and liver cirrhosis. *Gastroenterology* 1996;111:1627-1632.
33. Bigatello LM, Broitman SA, Fattori L, DiPaoli M, Pontello M, Bevilacqua G, Nespoli A. Endotoxemia, encephalopathy, and mortality in cirrhotic patients. *Am J Gastroenterol* 1987;82:11-15.
34. Mainous MR, Ertel W, Chaudry IH, Deitch EA. The gut: a cytokine-generating organ in systemic inflammation? *Shock* 1995;4:193-199.
35. Deitch EA, Xu D, Franko L, Ayala A, Chaudry IH. Evidence favoring the role of the gut as a cytokine-generating organ in rats subjected to hemorrhagic shock. *Shock* 1994;1:141-145.
36. Mainous MR, Tso P, Berg RD, Deitch EA. Studies of the route, magnitude, and time course of bacterial translocation in a model of systemic inflammation. *Arch Surg* 1991;126:33-37.
37. Thöny B, Auerbach G, Blau N. Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem J* 2000;347:1-16.
38. Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Schmidt K, Weiss G, et al. Pteridine biosynthesis in human endothelial cells. Impact on nitric oxide-mediated formation of cyclic GMP. *J Biol Chem* 1993;268:1842-1846.
39. Hattori Y, Gross SS. GTP cyclohydrolase I mRNA is induced by LPS in vascular smooth muscle: characterization, sequence and relationship to nitric oxide synthase. *Biochem Biophys Res Commun* 1993;195:435-441.
40. Hattori Y, Nakanishi N, Kasai K, Shimoda S. GTP cyclohydrolase I mRNA induction and tetrahydrobiopterin synthesis in human endothelial cells. *Biochim Biophys Acta* 1997;1358:61-66.
41. Old LJ. Tumor necrosis factor (TNF). *Science* 1985;230:630-632.
42. Schmidt K, Werner ER, Mayer B, Wachter H, Kukovetz WR. Tetrahydrobiopterin-dependent formation of endothelium-derived relaxing factor (nitric oxide) in aortic endothelial cells. *Biochem J* 1992;281:297-300.
43. Bune AJ, Brand MP, Heales SJ, Shergill JK, Cammack R, Cook HT. Inhibition of tetrahydrobiopterin synthesis reduces in vivo nitric oxide production in experimental endotoxic shock. *Biochem Biophys Res Commun* 1996;220:13-19.
44. Wiest R, Groszmann RJ. Nitric oxide and portal hypertension: its role in the regulation of intrahepatic and splanchnic vascular resistance. *Semin Liver Dis* 1999;19:411-426.
45. Fernandez M, Garcia-Pagan JC, Casadevall M, Bernadich C, Piera C, Whittle BJ, Pique JM, et al. Evidence against a role for inducible nitric oxide synthase in the hyperdynamic circulation of portal-hypertensive rats. *Gastroenterology* 1995;108:1487-1495.
46. Salvemini D, Korb R, Anggard E, Vane JR. Immediate release of a nitric oxide-like factor from bovine aortic endothelial cells by *Escherichia coli* lipopolysaccharide. *Proc Natl Acad Sci U S A* 1990;87:2593-2597.
47. Szabo C, Mitchell JA, Thiemermann C, Vane JR. Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxin shock. *Br J Pharmacol* 1993;108:786-792.
48. Gross SS, Jones CL, Hattori Y, Raman CS. Tetrahydrobiopterin: an essential cofactor of nitric oxide synthase with an elusive role. *Nitric Oxide Biology and Pathobiology*. San Diego: Academic; 2000:167-187.
49. Raman CS, Li H, Martasek P, Kral V, Masters BS, Poulos TL. Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell* 1998;95:939-950.
50. Wei C, Wang Z, Meade A, McDonald J, Stuehr D. Why do nitric oxide synthases use tetrahydrobiopterin? *J Inorg Biochem* 2002;91:618.