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Article

Nitric oxide (NO) has been implicated in the arterial vasodilation and associated vascular hyporesponsiveness to vasoconstrictors observed in liver cirrhosis. Bacteria, potent activators of NO and TNF- α synthesis, are found in the mesenteric lymph nodes (MLNs) of ascitic cirrhotic rats. Here, we investigated the impact of bacterial translocation (BT) to MLNs on TNF- α production, vascular NO release, and contractility in the mesenteric vasculature of ascitic cirrhotic rats. Vascular response to the α -adrenoagonist methoxamine, which is diminished in the superior mesenteric arterial beds of cirrhotic rats, is further blunted in the presence of BT. BT promoted vascular NO release in cirrhotic rats, an effect that depended on pressure-induced shear stress and was blocked by the NO inhibitor N^{ω} -nitro-L-arginine. Removing the endothelium had the same effect. Endothelial NO synthase (eNOS), but not the inducible isoform (iNOS), was present in mesenteric vasculature of cirrhotic rats with and without BT, and its expression was enhanced compared with controls. TNF- α was induced in MLNs by BT and accumulated in parallel in the serum. This TNF- α production was associated with elevated levels of tetrahydrobiopterin (BH₄), a TNF- α -stimulated cofactor and enhancer of eNOS-derived NO biosynthesis and NOS activity in mesenteric vasculature. These findings establish a link between BT to MLNs and increased TNF- α production and elevated BH₄ levels enhancing eNOS-derived NO overproduction, further impairing contractility in the [...]

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Bacterial translocation in cirrhotic rats stimulates eNOS-derived NO production and impairs mesenteric vascular contractility

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Nitric oxide (NO) has been implicated in the arterial vasodilation and associated vascular hyporesponsiveness to vasoconstrictors observed in liver cirrhosis. Bacteria, potent activators of NO and TNF- α synthesis, are found in the mesenteric lymph nodes (MLNs) of ascitic cirrhotic rats. Here, we investigated the impact of bacterial translocation (BT) to MLNs on TNF- α production, vascular NO release, and contractility in the mesenteric vasculature of ascitic cirrhotic rats. Vascular response to the α -adrenoagonist methoxamine, which is diminished in the superior mesenteric arterial beds of cirrhotic rats, is further blunted in the presence of BT. BT promoted vascular NO release in cirrhotic rats, an effect that depended on pressure-induced shear stress and was blocked by the NO inhibitor N^ω-nitro-L-arginine. Removing the endothelium had the same effect. Endothelial NO synthase (eNOS), but not the inducible isoform (iNOS), was present in mesenteric vasculature of cirrhotic rats with and without BT, and its expression was enhanced compared with controls. TNF- α was induced in MLNs by BT and accumulated in parallel in the serum. This TNF- α production was associated with elevated levels of tetrahydrobiopterin (BH₄), a TNF- α -stimulated cofactor and enhancer of eNOS-derived NO biosynthesis and NOS activity in mesenteric vasculature. These findings establish a link between BT to MLNs and increased TNF- α production and elevated BH₄ levels enhancing eNOS-derived NO overproduction, further impairing contractility in the cirrhotic mesenteric vasculature.

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Introduction

Arterial vasodilation, particularly that occurring in the splanchnic circulation, is a major causative factor in the pathogenesis of the hyperdynamic circulatory syndrome that is known to occur in cirrhosis and portal hypertension (1–3). A classic expression of these hemodynamic abnormalities is an impairment in vascular contractility, with resulting hyporeactivity to vasoconstrictors (4–9). This has been demonstrated to be caused largely by overproduction of endothelium-derived vasodilators, mainly nitric oxide (NO) (5–7, 10, 11). Recent investigations have shown that endothelial NO synthase (eNOS) is the major enzymatic source for this NO overproduction (12–17).

However, in various pathologic entities, proinflammatory cytokines, endotoxins, and bacterial infections have also been associated with enhanced production of NO, hyporesponsiveness to vasoconstrictors, and development of a hyperdynamic state (18–20). These physiological similarities, and the fact that endotoxemia is a common finding in cirrhosis (21–24), led to the hypothesis proposed by Vallance and Moncada that bacterial endotoxin, directly or involving cytokines, may account for hemodynamic changes in advanced cirrhosis (25) by stimulating the

expression of inducible NO synthase (iNOS) and, thus, NO production.

The gut is a large reservoir of bacteria and endotoxins. Intestinal barrier failure can lead to passage of bacteria and microbial products through the epithelial mucosa to mesenteric lymph nodes (MLNs) and other organs, a process known as bacterial translocation (BT) (26, 27). In liver cirrhosis, intestinal bacterial overgrowth, impaired host defenses, and disruption of the gut mucosal barrier promote this process (28–32). In cirrhotic rats, BT has been reported to be increased after the development of ascites (33) and to occur with a frequency of 45–75% (34–36). Moreover, live bacteria have been isolated from MLNs in patients undergoing laparotomy – a situation associated with a significant increase in the development of postoperative sepsis (37). In experimental cirrhosis, the presence of BT has been shown to be directly correlated with the occurrence of severe bacterial infections, such as spontaneous bacteremia and spontaneous bacterial peritonitis (31, 38, 39).

TNF- α is a proinflammatory cytokine released by mononuclear cells in response to inflammatory stimuli (40). TNF- α blood concentration has been shown to be elevated in patients with chronic liver disease and in ani-

mals subjected to experimental portal hypertension (41–44). TNF- α is also known to be involved in the pathogenesis of the hyperdynamic circulatory syndrome in portal hypertension (42, 45). Finally, the gut and its associated lymphoid tissue, the largest immunologic organ of the body, has recently been shown to produce and release TNF- α in response to BT, even in the absence of portal or systemic spread of bacteria (46, 47).

We designed this study to determine (a) whether there is any relationship between BT to MLNs and vascular contractility in the superior mesenteric arterial bed in cirrhosis; (b) if so, whether this additional vascular impairment is also due to vascular NO release; and (c) if that is the case, what NOS isoform is responsible for this additional NO overproduction and by what mechanism this NOS isoform is upregulated; and (d) whether TNF- α is playing any role in this process.

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 liver cirrhosis in rats by CCl₄. Male Sprague-Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, Indiana, USA), weighing 100–125 g, underwent inhalation exposure to CCl₄ and had phenobarbital (0.35g/L) added to their drinking water, as described previously by ourselves and others (4, 5). This protocol produces a high yield of micronodular cirrhosis in about 12–16 weeks. 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 hydrochloride (100 mg/kg), and the abdominal skin was shaved and sterilized with an iodine solution. All of the following surgical procedures were performed under strict sterile conditions with sterile instruments. In cirrhotic animals, ascites was quantified. To exclude animals with systemic bacteremia for protocol 1 (see below), 3 cm³ of blood was withdrawn from the inferior vena cava and inoculated into aerobic and anaerobic Bactec culture bottles. The blood was incubated at 35 °C, and the growth value (a measurement of CO₂ production by bacteria) was continuously monitored for at least 7 days. None of these cultures showed bacterial growth, confirming that, in our laboratory, this model of CCl₄-induced liver cirrhosis is free of systemic bacteremia (33). Therefore, we did not test for bacteremia in the following protocols. The caudal and cranial MLNs were removed and weighed with an E400D scale from Ohaus Corp. (Florham Park, New Jersey, USA), which is accurate to ± 0.01 g. Tissues were then homogenized in a measured amount of saline, and 0.1-mL aliquots were plated onto blood, MacConkey, and phenylethyl alcohol blood agar plates (BBL Prepared Media; Becton Dickinson Microbiology Systems, Sparks, Maryland, USA). A swab of ascitic fluid from the peritoneal cavity was cultured. If this culture

showed bacterial growth, the animal was excluded from the study. Spleen and liver were removed and weighed. 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 the intestinal lumen.

In vitro perfusion. The in vitro perfusion system used was a partial modification of that originally described by McGregor (48); it has been used extensively in previous studies by our laboratory (4–7, 16, 17). Briefly, the superior mesenteric artery (SMA) was cannulated with a polyethylene PE-60 catheter, and then gently perfused with 15 mL of warm Krebs solution to eliminate blood. After isolating the SMA with its mesentery, the gut was cut off close to its mesenteric border. The arterial vasculature was then transferred to a 37 °C water-jacketed container and perfused with oxygenated (95% O₂, 5% CO₂) 37 °C Krebs solution, using a roller pump (Masterflex; Cole-Parmer Instrument Co., Barrington, Illinois, USA). The Krebs solution had the following composition (in mmol/L): NaCl, 118.0; KCl, 4.7; KH₂PO₄, 1.2; MgSO₄, 1.2; CaCl₂, 2.5; NaHCO₃, 25.0; disodium EDTA, 0.026; glucose, 11.0 (pH was 7.4). The effluent of the perfused tissue was continuously removed from the perfusing chamber. The perfusion pressure was measured with a H-P pressure transducer (Hewlett Packard, Andover, Massachusetts, USA) mounted on a side arm just before the perfusing cannula. Pressure was recorded continuously on a 7D polygraph inscriber (Grass Instruments, Quincy, Massachusetts, USA).

Experimental protocols

All cirrhotic rats were studied prospectively, because at the time of experimental procedures, it was not known whether the cirrhotic animals were positive or negative for BT. A total of 78 cirrhotic rats with ascites and 26 age-matched control rats were included in this study. In all animals (except in protocol 4), before starting procedures to assess BT, the left femoral artery was exposed and cannulated with a PE-50 catheter. Blood (1.5 cm³) was drawn into a pyrogen-free Vacutainer (Becton Dickinson) and centrifuged at 5,000 rpm for 15 minutes. The separated serum was stored immediately at –70 °C until analysis for NO metabolites (NO_x) and TNF- α . Mean arterial pressure (MAP) was evaluated by connecting the catheter to the H-P pressure polygraph inscriber, and was recorded on a model 7D inscription recorder (Grass Instruments). Measurements of MAP were performed before blood was drawn (and after its replacement with 1.5 cm³ saline). In the animals used in protocols 2 and 3, additional MLN homogenate was stored at –70 °C until measurement of NO_x and TNF- α levels.

Protocol 1. In 18 ascitic cirrhotic rats (with BT: $n = 10$; without BT: $n = 8$) and 10 control rats, in vitro perfusion of the mesenteric vessel bed was performed. Baseline perfusion at 4 mL/min was established for 60 minutes. At 30, 45, and 60 minutes, the perfusion solution from

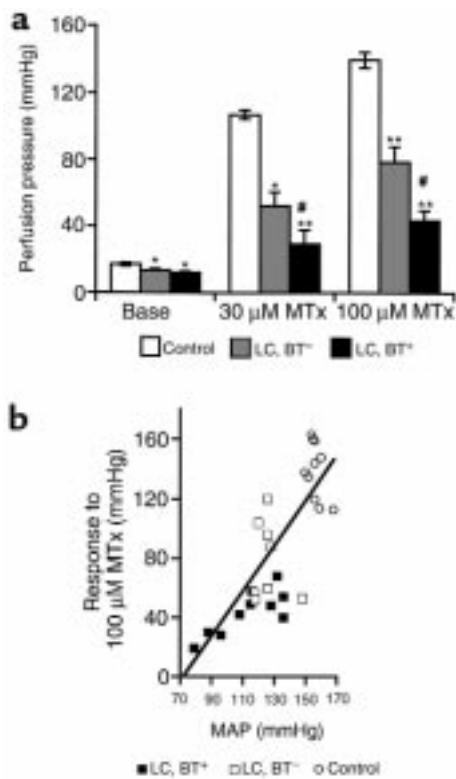


Figure 1
 (a) Perfusion pressure under basal conditions (Base) and during the administration of 30 μM and 100 μM MTx in the in vitro-perfused superior mesenteric arterial bed. * $P < 0.01$ vs. control. ** $P < 0.0001$ vs. control. # $P < 0.01$ vs. LC, BT⁻. (ANOVA for pressure-response periods: $P < 0.0001$ for control vs. LC, BT⁻ or LC, BT⁺ rats; $P < 0.01$ for LC, BT⁺ vs. LC, BT⁻ rats.) (b) Relationship between in vitro vascular responsiveness and systemic hemodynamic parameter MAP. A close correlation for in vitro pressure response to MTx (100 μM) and in vivo MAP was observed ($r = 0.797$, $P < 0.0001$).

the mesenteric tissue was collected into glass tubes for 1 minute. Then 2 doses of methoxamine (MTx; 30 and 100 μM) were administered noncumulatively by continuous infusion for 2 minutes (first perfusion cycle), and the pressure response was documented. Three perfusate samples were collected for each pressure-response period, beginning with the increase of perfusion pressure. Each sample was collected for 1 minute, frozen immediately, and stored at -30°C until the NO_x assay. After the first perfusion cycle was completed, each vessel preparation was perfused at the basal flow rate of 4 mL/min for 50 minutes (after that time, the perfusate NO_x concentration has returned to baseline levels; data not shown). Then the specific NOS inhibitor *N*^ω-nitro-L-arginine (L-NNA; 10^{-4}M ; Sigma Chemical Co., St. Louis, Missouri, USA) was added, and a second perfusion cycle was initiated after 20 minutes of incubation time. The NOS inhibitor was present at the same molar concentration in the perfusion system throughout the second perfusion cycle. Before initiation of the experiment, the Krebs solution that was circulated through the perfusion system (including the catheter, but not

mesenteric tissue) was collected for use in determining background NO_x levels. As shown previously, the perfusion system showed stable basal perfusion conditions and unchanged pressure response for the duration of this experiment (6, 7, 17).

Protocol 2. Endothelial denudation of the mesenteric vasculature was performed in 16 cirrhotic ascitic rats (with BT: $n = 8$; without BT: $n = 8$) and in 6 control rats by a combined treatment of cholic acid (sodium salt) and distilled water (4). In brief, after cannulation of the SMA and gentle flushing with 10 mL of warmed Krebs solution to eliminate blood, perfusion with 1.5 mL of 0.5% cholic acid (for 10 seconds) followed by 15 mL of Krebs solution (to eliminate cholic acid) was performed. The preparation was then transferred to a 37°C water-jacketed container and perfused with oxygenated 37°C Krebs solution (4 mL/min for 10 minutes). After the mesenteric vasculature was relaxed, it was perfused with 37°C distilled water for 10 minutes. A period of 45 minutes was allowed before noncumulative concentration-response curves to MTx (1–100 μM) were derived. To assess whether the vessel was completely de-endothelialized and whether smooth muscle function was maintained, the mesenteric preparation was kept precontracted with MTx (100 μM) at the end of each dose-response curve, while dose-dependent vasorelaxation response to the endothelium-dependent vasodilator acetylcholine (10^{-8} to 10^{-6} g; bolus of 0.1 mL) and the endothelium-independent vasodilator sodium nitroprusside (10^{-6} to 10^{-5} g; bolus of 0.1 mL) was tested.

Protocols 3 and 4. In protocol 3, a total of 25 cirrhotic ascitic rats and 10 control rats were used for performance of blotting and tetrahydrobiopterin (BH_4) measurements in the studied mesenteric vasculature. In protocol 4, a separate group of 19 cirrhotic ascitic animals were used for evaluation of NOS activity in mesenteric resistance vessels (MRVs).

Western blotting. SMA vessels and MRVs were analyzed for the presence of eNOS and iNOS protein. SMA vessels were harvested after removal of MLNs and freeing the artery from surrounding tissue over a length of 3–4 cm, starting at its aortic origin. Tissue for Western blotting of MRVs was harvested from a portion of the capillary bed situated between the MLNs and the small intestine. The tissue was cut off from the SMA at the level of second-order arteries and dissected from the intestine as close as possible to the gut wall. Vessels were washed in PBS and homogenized in a lysis buffer described previously (17). Protein supernatants were quantitated using the Lowry assay, and equal amounts of protein from each sample were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were probed with an mAb recognizing eNOS (Transduction Laboratories, Lexington, Kentucky, USA) and a polyclonal antibody recognizing iNOS (Affinity BioReagents Inc., Golden, Colorado, USA). The specificity of the eNOS and iNOS antibodies for rat tissue was established previously by using quies-

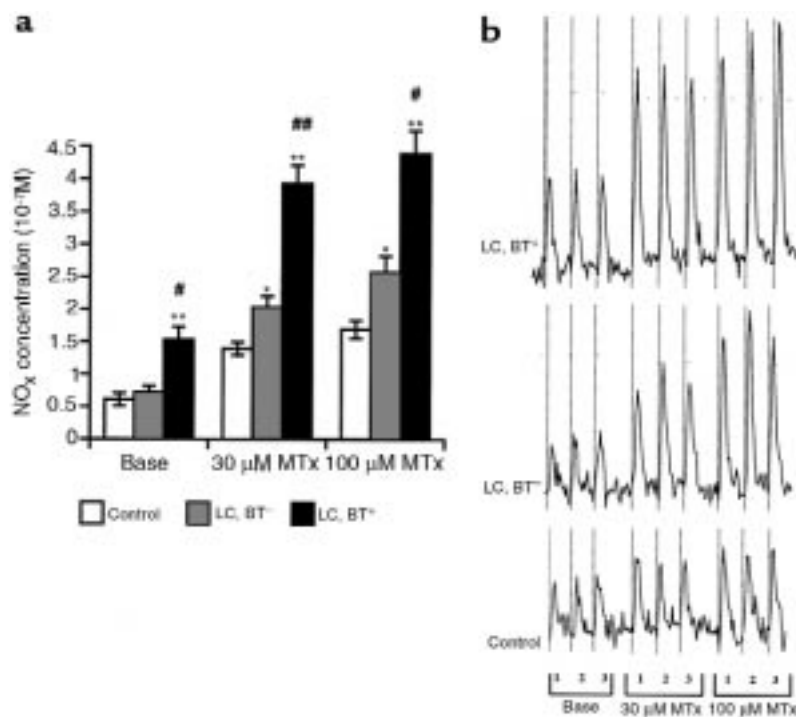


Figure 2

(a) Absolute perfusate NO_x concentrations under basal conditions and during pressure-response periods to MTx. **P* < 0.01 vs. control. ***P* < 0.0001 vs. control. #*P* < 0.001 vs. LC, BT⁻. ##*P* < 0.0001 vs. LC, BT⁻. (ANOVA for pressure-response periods: *P* < 0.0001 for control vs. LC, BT⁻ or LC, BT⁺ rats; *P* < 0.001 for LC, BT⁺ vs. LC, BT⁻ rats.) (b) Representative NO_x assays by chemiluminescence technique for rats of each study group. At baseline conditions and during pressure-response periods to 30 μM and 100 μM MTx, 3 perfusate samples (1, 2, 3) were collected, each for 1 minute. An increase in peak size (area under the curve) obtained in cirrhotic rats compared with that of controls and a further increase in peak size in readings from cirrhotic rats with BT is evident.

cent and activated rat sinusoidal endothelial cells, respectively, as positive controls. Enhanced chemiluminescence was used for protein detection.

BH₄ assay. Frozen mesenteric vasculature was weighed and homogenized in 3 volumes of 50 mM Tris-HCl (pH 7.4) containing 1 mM DTT and 1 mM EDTA. This mixture was centrifuged, and supernatant BH₄ levels were measured by HPLC with fluorescence detection after oxidation, as described previously (49).

NOS activity assay. NOS activity was measured by determining the conversion of ³H-labeled L-arginine to ³H-labeled L-citrulline, using a commercially available NOS assay kit (Calbiochem-Novabiochem Corp., San Diego, California, USA). Briefly, mesenteric tissue was harvested as described above, and then minced with fine scissors and homogenized in lysis buffer. Samples were incubated with a reaction buffer containing 1 mM NADPH, 3 μM BH₄, 100 nM calmodulin, 2.5 mM CaCl₂, 1 μM FAD, 1 μM FMN, and 1 μCi/μL of [³H]arginine or [¹⁴C]arginine. To determine NOS activity, duplicate samples were incubated in the presence and absence of N^ω-nitro-L-arginine methylester (L-NAME; 1 mM) or vehicle. The reaction was continued for 1 hour at 35 °C, and then stopped by the addition of 0.4 mL of cold stop buffer (50 mM HEPES and 5 mM EDTA; pH 5.5). After addition of 100 μL of equilibrated resin to each reaction mixture, samples were passed over prepared columns. The eluates were transferred into vials and analyzed using a liquid scintillation counter. Radiolabeled cpm of L-citrulline generation were measured.

Determination of NO_x concentration. The NO_x concentration of each perfusate and serum sample was measured using the Nitric Oxide Analyzer from Sievers Instruments (Boulder, Colorado, USA), as described

previously (16, 17). In brief, this assay is based upon spectrophotometric analysis after a chemiluminescent reaction between NO and ozone. Fifty microliters of each sample was placed into the purge vessel, which contained 3 mL of 0.1 M vanadium chloride in 1 M HCl at 95 °C. The NO generated from nitrite and nitrate ions was carried into the analyzer by vacuum through a gas bubbler trap containing 5 mL of 1 M 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. The lower limit of sensitivity for this machine is below 2 picomoles of NO per second. Because of this high sensitivity, it is necessary to make sure that exogenous contamination does not affect the results. Therefore, we conducted control experiments in which the perfusate was circulated through the perfusion system without tissue for the duration of the experiment. Under these circumstances, NO_x concentrations of the collected perfusate were low and practically the same at all timepoints. The displayed NO_x concentrations and related NO_x production rates were corrected for each experiment by subtracting background NO_x concentrations. The net NO_x release in response to MTx (Δ NO_x concentration) is calculated as the difference between NO_x concentration at baseline (without MTx) and after each pressure-response period. NO_x production rate was obtained by multiplying the perfusate flow rate by the concentration of NO_x in the perfusate, and is expressed in nanomoles per minute. For calcula-

tion of the regression coefficients between the amount of NO_x production and shear stress, the average NO_x concentration was calculated for each experimental period. Shear stress was calculated as described previously using the following equation (16, 17):

(Equation 1)

$$\tau = K\Delta P^{3/4}Q^{1/4}$$

where $K = (\eta/2\pi L^3)^{1/4}$ or $K = 1/2(8\eta/\pi L^3)^{1/4}$ = constant factor.

Assays. Concentrations of TNF- α in serum and MLN homogenate were measured in duplicate with a commercially available rat-specific ELISA (sandwich ELISA) kit (Endogen Inc., Woburn, Massachusetts, USA) according to the protocol supplied by the manufacturer. The lower limit of sensitivity for TNF- α in this assay was 3 pmol/mL. Statistical analysis

Results were expressed as mean \pm SEM. Statistical analysis was performed using 2-way, repeated-measures ANOVA and paired and unpaired Student's *t* tests, with statistical significance set at $P < 0.05$. Each relationship of hemodynamic, perfusate, serum, and lymph node parameters was tested for regression by a simple regression analysis. To increase the sensitivity of the regression analysis, statistical significance for correlations was set at $P < 0.01$.

Results

All CCl₄-treated rats (LC) used in these experiments showed macroscopically macro/micronodular cirrhosis of the liver. The weight of cirrhotic rats was found to be significantly lower than that of control rats (348.1 \pm 6.0 g vs. 397.6 \pm 10.3 g, respectively; $P < 0.0001$). This difference was more pronounced in animals that were also positive for BT (LC, BT⁺: 326.4 \pm 7.1 g; LC, BT⁻: 370.9 \pm 8.5 g; $P < 0.01$). The weights of the spleen and the mesenteric vasculature, expressed in grams per kilogram of body weight, were both significantly higher in cirrhotic rats than in control rats (LC: 3.70 \pm 0.09 g/kg and 15.4 \pm 0.5 g/kg; control: 1.94 \pm 0.06 g/kg and 8.5 \pm 0.5 g/kg, respectively; $P < 0.0001$). There were no significant differences between cirrhotic rats with or without BT. Similarly, no statistical difference in volume of ascites was found between cirrhotic rats with or without BT (LC, BT⁺: 19.6 \pm 2.5 mL; LC, BT⁻: 17.4 \pm 3.2 mL). MLN weight was not different among the study groups. BT to MLNs did not occur in any of the 26 normal rats. Among the 78 ascitic cirrhotic rats, MLN culture was positive for bacteria in 40 animals (51.3%).

Protocol 1

In vitro perfusion pressure in mesenteric arterial vessel bed. Removal of MLNs from the mesenteric vasculature before performance of in vitro perfusion did not change unstimulated or MTx-induced perfusion pressures (data not shown). As shown before, basal perfusion pressures were significantly lower in vessel prepa-

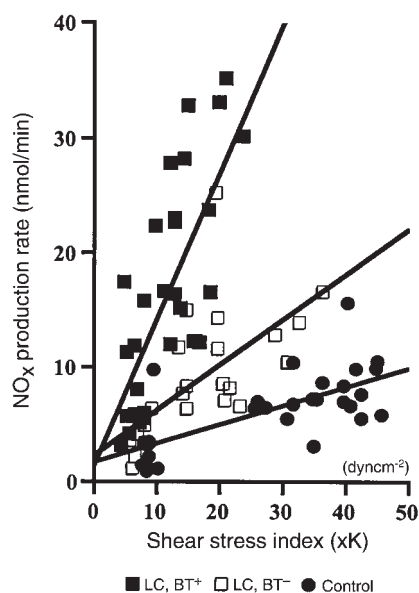


Figure 3

Regression analysis for NO_x production rate vs. applied pressure-induced shear stress index. A highly significant correlation was found for each experimental study group. LC, BT⁺: $r = 0.72$, $P < 0.0001$; LC, BT⁻: $r = 0.65$, $P < 0.001$; controls: $r = 0.69$, $P < 0.0001$. Note the markedly enhanced production of NO_x at given shear stress levels in rats with BT compared with animals without BT.

rations of cirrhotic rats than in control rats (4, 5). The mesenteric arterial beds of cirrhotic rats showed a significantly lower pressure response to MTx than control rats did ($P < 0.0001$ by ANOVA). This vascular hyporesponsiveness to MTx was significantly more pronounced in cirrhotic rats with BT ($P < 0.01$ by ANOVA; Figure 1a).

Relationship between in vitro vascular contractility and in vivo MAP. No difference in MAP obtained before and after blood drawing was observed. Cirrhotic rats showed significantly lower MAP than did control rats (LC, BT⁺: 117.9 \pm 3.1 mmHg; LC, BT⁻: 133.1 \pm 2.1 mmHg; control: 153.8 \pm 1.6 mmHg; $P < 0.0001$). The presence of BT in cirrhotic rats was associated with a significant reduction in MAP ($P < 0.01$). Vascular responsiveness to MTx (100 μ M) was found to be significantly correlated with MAP ($r = 0.797$, $P < 0.0001$; Figure 1b), demonstrating the hemodynamic importance of in vitro vascular reactivity as a classic manifestation of the vasodilatation that characterizes the hyperdynamic circulatory syndrome in portal hypertension. Additionally, when both cirrhotic rats and control rats were included in the analysis, a significant correlation was observed between in vitro baseline perfusion pressures and in vivo MAP ($r = 0.537$, $P < 0.0001$). There was a similar correlation between in vitro baseline perfusion pressures and pressure response to MTx ($r = 0.656$, $P < 0.001$).

Perfusate NO_x concentrations. In a preliminary study, we collected the perfusate from the mesenteric arterial beds of the animals in each of the different study

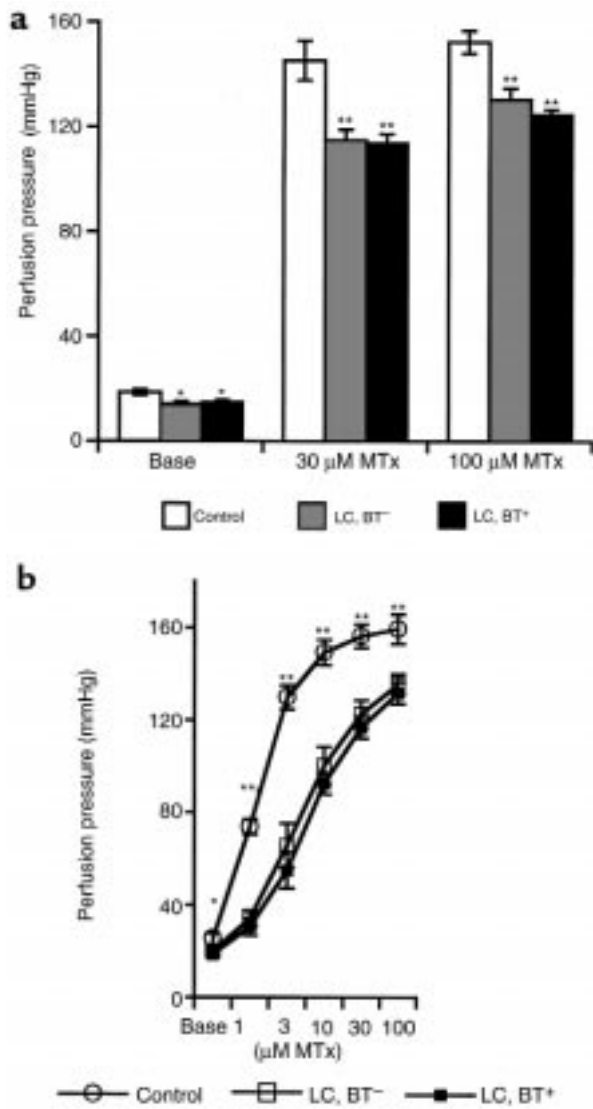


Figure 4 (a) In vitro perfusion pressures in superior mesenteric vasculature after inhibition of NO synthesis by incubation with L-NNA. * $P < 0.05$ vs. control. ** $P < 0.001$ vs. control. Blockade of NO formation corrected the hyporesponsiveness to MTx that is related to the presence of BT in cirrhotic rats. (b) Dose-response curves for MTx in de-endothelialized superior mesenteric vascular beds. * $P < 0.05$ vs. LC, BT⁻ and LC, BT⁺. ** $P < 0.001$ vs. LC, BT⁻ and LC, BT⁺. $P < 0.001$ for control vs. LC, BT⁻ and LC, BT⁺ (ANOVA). Note the lack of difference in vascular reactivity in mesenteric preparations from cirrhotic rats with and without BT.

groups under basal conditions at intervals of 15 minutes for 1.5 hours. In each study group, perfusate NO_x concentration was found to remain unchanged during this time (data not shown). As shown in Figure 2a, basal perfusate NO_x concentrations were significantly higher in cirrhotic rats with BT than in cirrhotic rats without BT and control rats. During MTx administration, NO_x concentrations in perfusate increased in all study groups and were significantly elevated in cirrhotic rats compared with control rats ($P < 0.0001$ by ANOVA). Cirrhotic rats with BT showed significantly

higher perfusate NO_x concentrations than did cirrhotic rats without BT ($P < 0.001$ by ANOVA). A representative spectrophotometric tracing, used to determine NO_x concentration in the perfusate from a typical experiment in each study group, is shown in Figure 2b. Regression analysis revealed that perfusate NO_x concentrations correlated with the in vitro vascular pressure response (e.g., at 100 μM MTx; $r = 0.507$, $P < 0.01$). That is, the higher the NO_x concentration, the lower the in vitro perfusion pressure. However, no correlation was found between basal perfusate NO_x concentration and baseline perfusion pressure (for all: $r = 0.16$, $P > 0.40$). Net NO_x release in response to MTx was significantly elevated in cirrhotic rats compared with control rats ($P < 0.001$ by ANOVA). In the presence of BT, an additional marked increase in NO_x release was found ($P < 0.01$ by ANOVA). In each study group, a significant regression correlation between NO_x production rate and shear stress index was found (LC, BT⁺: $r = 0.72$, $P < 0.0001$; LC, BT⁻: $r = 0.65$, $P < 0.001$; control: $r = 0.69$, $P < 0.0001$) (Figure 3). The slope of NO_x production rate vs. shear stress index was significantly higher in each group of cirrhotic animals than in the control animals (LC, BT⁺: 1.36 ± 0.10 ; LC, BT⁻: 0.535 ± 0.049 ; controls: 0.233 ± 0.018 ; $P < 0.0001$). Cirrhotic rats with BT showed the highest slope ($P < 0.0001$), demonstrating significantly more pronounced shear stress-dependent NO_x production by the mesenteric vascular endothelium in the presence of BT than in the absence of BT.

Effect of inhibition of NO synthesis by L-NNA on the pressure response to MTx. Incubation with L-NNA did not change baseline perfusion pressures. After inhibition of NO formation, no statistical difference in response to MTx could be observed between perfused mesenteric arterial beds of cirrhotic rats with and without BT. A significant hyporeactivity to MTx persisted in cirrhotic rats compared with control rats ($P < 0.01$; Figure 4a). Cirrhotic rats with BT had a significantly greater pressure response to L-NNA than did cirrhotic rats without BT ($P < 0.01$ by ANOVA). For example, pressure increases in response to treatment with 30 μM MTx were $236.5 \pm 27.1\%$ vs. $143.8 \pm 27.4\%$, respectively ($P < 0.01$), demonstrating that NO synthesis blockade with L-NNA led to a significantly greater response in vessel preparations of animals with BT.

Protocol 2

Effect of endothelium removal on the in vitro pressure response to MTx. De-endothelialized mesenteric vasculature in all groups practically eliminated vasodilatory response to acetylcholine, indicating an appropriate removal of the endothelium (data not shown). Dose-dependent relaxation responses to sodium nitroprusside proved the viability of the smooth muscle, and were found to be significantly elevated in cirrhotic rats with BT compared with control rats ($P < 0.05$ by ANOVA). Removal of the endothelium significantly increased baseline perfusion pressures and response to MTx in all study groups ($P < 0.01$). However, a significantly lower base-

line perfusion pressure, as well as hyporesponsiveness to MTx, persisted in cirrhotic rats compared with control rats ($P < 0.05$). Endothelial denudation completely abolished the difference in vascular responsiveness to MTx that was observed in intact mesenteric vasculature between cirrhotic rats with and without BT.

Protocol 3

Western blot analysis. Protein expression of eNOS was evident in each vasculature examined. In cirrhotic rats without BT, eNOS protein levels in SMA and MRV tissue were markedly increased compared with those in control animals. In cirrhotic rats with BT, expression of eNOS protein in the studied vasculature appeared to be additionally enhanced. There was no detectable iNOS protein in SMA or MRVs from cirrhotic or control rats, whereas iNOS was detected in cell lysates from activated sinusoidal endothelial cells from rat liver and in MLNs from LPS-treated (6 mg/kg for 12 hours) rats, which served as positive controls (Figure 5).

TNF- α , NO $_x$, and BH $_4$ levels. TNF- α levels in MLN homogenate and serum were significantly elevated only in cirrhotic rats with BT ($P < 0.0001$; Figure 6, a and b). In cirrhotic rats without BT, slight (not significant) increases in TNF- α concentrations in serum and MLNs were noted. A significant correlation between TNF- α levels in serum and MLN homogenate was observed ($r = 0.667$, $P < 0.0001$; Figure 6c). NO $_x$ concentrations in serum were significantly elevated in cirrhotic rats compared with control rats ($P < 0.0001$; Figure 7a). Significantly higher serum NO $_x$ levels were detected in the presence of BT than in the absence of BT ($P < 0.0001$). Serum NO $_x$ concentrations were found to correlate significantly with levels of TNF- α in MLN homogenate ($r = 0.561$, $P < 0.0001$; Figure 7b) and serum ($r = 0.529$, $P < 0.0001$), suggesting a prominent role for TNF- α (particularly that of mesenteric origin) in NO $_x$ production. In MLN homogenate, NO $_x$ concentrations were significantly increased in cirrhotic rats with BT compared with cirrhotic rats without BT and control rats ($P < 0.0001$), and correlated significantly with local TNF- α concentrations ($r = 0.450$, $P < 0.001$). BH $_4$ concentrations in the mesenteric vasculature were found to be significantly increased in cirrhotic rats with BT compared with cirrhotic rats without BT and controls ($P < 0.05$; Figure 7c); no difference in BH $_4$ levels was observed between cirrhotic rats without BT and controls. BH $_4$ levels in the mesenteric vascular bed were positively correlated with TNF- α concentrations in MLN homogenate ($r = 0.524$, $P < 0.01$; Figure 7d) and serum ($r = 0.631$, $P < 0.001$), suggesting the induction of BH $_4$ synthesis by TNF- α .

Relationship with hemodynamics. MAP was found to be inversely correlated with serum levels of NO $_x$ and TNF- α ($r = 0.675$, $P < 0.0001$ and $r = 0.575$, $P < 0.0001$, respectively). TNF- α levels in MLN homogenate tended to correlate inversely with MAP ($r = 0.36$, $P < 0.01$). Moreover, BH $_4$ levels in the studied mesenteric vasculature closely correlated inversely with MAP ($r = 0.627$, $P < 0.01$). The pressure response to MTx (100 μ M) was

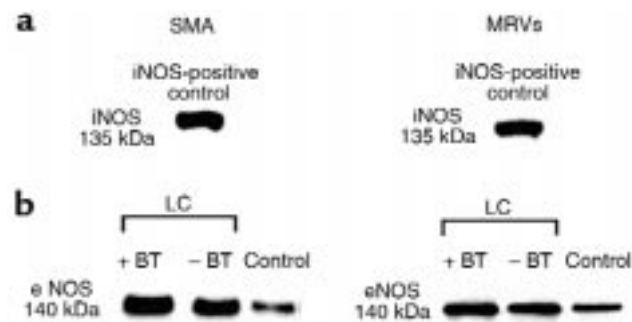


Figure 5

Expression of eNOS and iNOS protein in arterial vessels of control and cirrhotic ascitic rats with and without BT. The molecular weights of eNOS and iNOS are 140 and 135 kDa, respectively. Each lane was loaded with 100 mg of protein, and eNOS and iNOS protein was detected by enhanced chemiluminescence. (a) Western blotting for iNOS. No induction of iNOS was found in vessels from control or cirrhotic rats, including animals with BT. In iNOS-positive controls, strong iNOS bands detected with recombinant iNOS protein can be appreciated. (b) Western blotting for eNOS. For SMA, tissue was pooled from 3 animals per group; a representative blot from 3 independent experiments is shown. For MRVs, individual blots for each animal were performed, and a representative blot from 6 independent experiments is shown. Higher immunoreactivity in cirrhotic rats (LC) than in controls can be seen.

inversely correlated with NO $_x$ concentrations in serum ($r = 0.746$, $P < 0.0001$), and tended to correlate inversely with serum TNF- α levels.

Protocol 4

NOS activity in mesenteric vasculature. NOS catalytic activity (assessed as L-NAME-inhibitable conversion of 3 H-labeled L-arginine to 3 H-labeled L-citrulline) was significantly increased in mesenteric vasculature from cirrhotic rats with BT compared with animals without BT (1.4 ± 0.2 vs. 0.5 ± 0.2 pmol/mg protein, respectively; $P < 0.01$).

Discussion

This study provides direct evidence that there is a greater impairment in vascular reactivity in cirrhotic rats with BT to MLNs than in rats without BT, and that this additional impairment is largely mediated by endothelial overproduction of NO. We also demonstrate that, contrary to what may have been expected, this endothelial NO overproduction is shear stress-dependent and eNOS-derived and seems to be mediated by TNF- α through BH $_4$.

NO is synthesized by different isoforms of NOS. Two such isoforms have been investigated extensively in the vasculature (50–52). NOS 2, or iNOS, is induced by LPS, endotoxins, and inflammatory cytokines, and produces large amounts of NO for extended periods of time. This enzyme is not stimulated by mechanical factors. NOS 3, or eNOS, releases NO for short periods, in response to physical stimuli such as flow- or pressure-induced shear stress — a specific and unique function of this enzyme (53, 54). In our model, we assessed endothelial NO release in response to MTx-induced

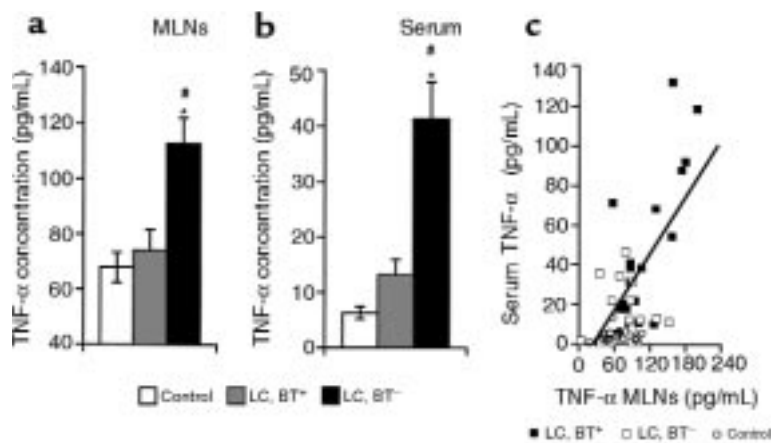


Figure 6 TNF- α concentrations in MLNs (a) and serum (b). * $P < 0.0001$ vs. control. # $P < 0.001$ vs. LC, BT⁺. (c) Observed correlation between TNF- α concentrations in MLNs and serum ($r = 0.67$, $P < 0.0001$).

vasoconstriction and the resulting increase in pressure and shear stress. In cirrhotic rats without BT, we found that in the mesenteric vessels NO was overproduced in response to vasoconstrictors, and that this vascular NO release was shear stress-dependent. This directly shows, in a functional manner, the involvement of eNOS-derived NO release in the observed vascular hyporesponsiveness found in cirrhotic rats without BT. Additionally, Western blotting with eNOS antibodies showed a consistent augmentation in the eNOS immunoreactivity in the mesenteric vasculature of animals without BT. These results are consistent with recent reports demonstrating NO-mediated vascular hyporesponsiveness (5–7, 10, 55) and eNOS upregulation (12–17) in portal hypertensive animals.

However, the novel finding of this study is that BT to MLNs was associated with an additional 45% decrease in pressure response to MTx in the superior mesenteric arterial bed. This vascular hyporeactivity in rats with BT was associated with significantly enhanced vascular NO release compared with that occurring in the absence of BT. Blockade of NO formation by L-NNA induced a significantly greater pressure response in cirrhotic rats with BT than in those without BT, thereby abrogating the difference in vascular responsiveness between the 2 study groups. This demonstrates that NO is the main causative vasodilator mediating the more pronounced impairment in vascular contractility in animals with BT. Additionally, de-endothelialization reversed the impairment in vascular reactivity observed in cirrhotic rats with BT, indicating that the vasoactive factor acting as the source of NO is the endothelium, not the vascular smooth muscle. The observation that a blunted response to MTx persisted after de-endothelialization or incubation with L-NNA has been made before (4, 56). This reduced response is probably due to structural changes in the vessel wall, which are known to be induced by the chronic high blood flow and plasma-volume expansion (57) conditions present in cirrhosis and portal hypertension. At basal conditions, the vasculature in our in vitro technique expresses no active vascular tone; therefore, basal perfusion pressure is determined solely by the flow

resistance caused by the structure of the vessel wall (6). Basal perfusion pressure was significantly lower in cirrhotic rats than in controls, a phenomenon found to be independent of BT. The close correlation observed between basal perfusion pressures and pressure response to the highest MTx concentration used indicates that the vessel structure, at least in part, defines the maximum vasoconstrictive response to MTx that can be achieved in these vessels. Moreover, baseline pressures correlated significantly with MAP, suggesting that worsening of the hyperdynamic circulatory syndrome and subsequent increase in blood flow induces progressive vascular remodeling. Finally, the tissue weight of mesenteric vasculature was significantly higher in cirrhotic rats than in control rats, and there were no differences between animals with and without BT. This indicates vascular hypertrophy or angiogenic alterations in the mesenteric vasculature (or both), as has been recently reported in prehepatic portal hypertensive rats (58). Nonetheless, we cannot completely exclude the possibility that other vasoactive substances besides NO are playing a minor role in the observed vascular hyporeactivity in cirrhotic rats.

The additional enhancement in NO overproduction in conditions of BT was found to be clearly shear stress-dependent. This is shown in the significantly larger NO release in response to MTx-induced increase in perfusion pressure and in the highly significant regression coefficient for the relationship of NO release vs. shear stress in the animals (Figure 3). Additionally, in the studied vasculature, eNOS protein expression was slightly accentuated and enzyme activity was significantly enhanced in cirrhotic rats with BT compared with cirrhotic animals without BT. This marked upregulation of eNOS in the studied vessels probably explains the elevated perfusate NO_x concentrations detected at basal conditions in cirrhotic rats with BT, because it leads to a higher sensitivity in mechanotransduction mechanisms that mediate shear stress-induced endothelial signaling (59). In contrast, expression of iNOS protein was not detectable in SMA or MRVs, indicating that the presence of bacteria and a local inflammatory process in the mesen-

tery of rats with BT does not lead to a significant induction of iNOS and associated iNOS-derived NO synthesis in the splanchnic circulation of cirrhotic rats. In fact, NO production mediated by iNOS has been shown to be independent of physical factors such as flow and shear stress (50, 52, 60). Moreover, iNOS is known to be predominantly expressed in vascular smooth muscle (51), which is not the site of NO production in the studied arteries, as shown by the endothelium dependence of that production. This lack of induction of iNOS protein expression, even in rats with BT, further supports findings against a role for iNOS in the circulatory abnormalities in portal hypertension (55, 61–63). The explanation for this deficit in response to inflammatory stimuli remains to be elucidated. Taken together, the presence of BT is associated with significant enhancement of eNOS-derived NO overproduction in the mesenteric arterial bed in cirrhotic rats, aggravating vascular hyporesponsiveness to vasoconstrictors (Figure 1a) and arterial hypotension (Figure 1b). It is important to

emphasize that the severity of arterial hypotension has been shown to be a strong independent predictor of survival in cirrhotic patients (64).

TNF- α is a multifunctional endogenous proinflammatory cytokine that has been demonstrated to be involved in the pathogenesis of the hyperdynamic circulatory syndrome in portal hypertension (antagonism of TNF- α with anti-TNF antibody or inhibition of TNF- α synthesis by thalidomide blunts the development of the hyperdynamic circulation in portal vein-ligated and cirrhotic rats; refs. 42, 45). Moreover, in patients with chronic liver disease and in animal models of portal hypertension, elevated blood concentrations of TNF- α have been reported, compared with normal controls (41–44). TNF- α is produced by mononuclear cells upon activation by infectious microorganisms or their cell-wall products, such as endotoxin (40). BT can be considered a primary event in the etiology of endotoxemia, as reflected by significant portal-to-peripheral venous gradients in endotoxin blood levels of patients with cirrhosis who are undergoing shunt surgery; such gradients demonstrate relatively high levels of endotoxin in the portal circulation (24). Moreover, it has been proposed that macrophages play a key role in the process of BT, as transporters of intestinal bacteria (65). The gut and its associated lymphoid tissue, the largest immunologic organ of the body, contains numerous mononuclear cells (66). Concordantly, we find increased levels of TNF- α in MLNs only in cirrhotic rats with BT. Local production of TNF- α correlated closely with corresponding serum levels that were also found to be significantly increased only in the presence of BT. This suggests that lymph node-derived TNF- α contributes to the associated systemic levels (e.g., by drainage into the systemic circulation). In fact, Mainous et al. demonstrated enhanced TNF- α bioactivity in efferent mesenteric lymph in conditions of zymosan-induced BT that appeared to correlate with the magnitude of inflammatory insult and was not different from systemic levels (46). Moreover, even in the absence of portal and systemic bacteremia, elevated portal concentrations of TNF- α were found in rats with shock-induced intestinal injury with BT (47). These findings demonstrate, in different models of BT, that the gut and its associated lymphoid tissue is capable of becoming a cytokine-releasing organ contributing to enhanced serum levels of TNF- α . This even occurs in conditions where BT is limited to MLNs (46, 47). Thus, our results suggest that the significantly increased systemic levels of TNF- α in cirrhotic rats with BT are, at least in part, due to enhanced mesenteric release of TNF- α .

The importance of this enhanced TNF- α production for NO synthesis in cirrhotic rats is reflected in the significant correlation observed between serum concentrations of TNF- α and NO. The significant elevation of serum NO levels in the presence of BT is specifically interesting in regard to results reported by Guarner et al. demonstrating a relationship between serum endotoxin levels and serum NO levels in patients with liver

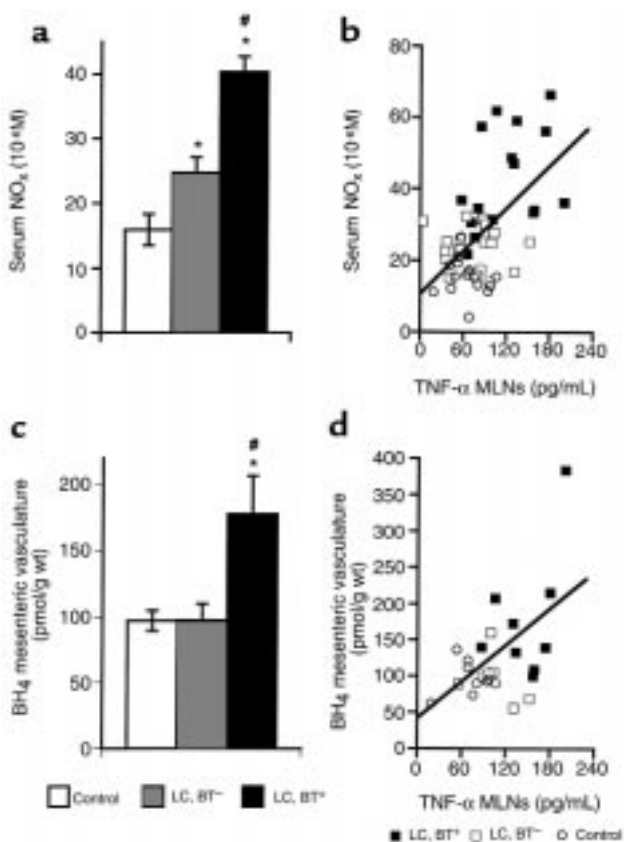


Figure 7

(a) Serum NO $_x$ concentrations. * $P < 0.0001$ vs. control. # $P < 0.0001$ vs. LC, BT $^{-}$. (b) Serum NO $_x$ levels correlated significantly with TNF- α concentrations in MLNs ($r = 0.561$, $P < 0.0001$). (c) BH $_4$ concentrations in mesenteric vasculature. * $P < 0.05$ vs. control. # $P < 0.05$ vs. LC, BT $^{-}$. (d) Levels of BH $_4$ in the studied vasculature were significantly correlated with TNF- α concentrations in MLNs ($r = 0.524$, $P < 0.01$), indicating an induction of BH $_4$ synthesis by BT and an associated increase in TNF- α synthesis.

cirrhosis, particularly in those with ascites (21). That this increase in serum concentrations of TNF- α and NO in the presence of BT has a crucial impact on systemic hemodynamics and vascular contractility can be seen in the inverse correlations found between these two serum markers and in vivo MAP as well as in vitro pressure response to MTx.

Although TNF- α and endotoxins are widely known as stimulators of iNOS induction in vitro (67), this study failed to detect iNOS protein expression. BH₄ is an endogenously synthesized essential cofactor for NO biosynthesis (68) and has been shown to be rate-limiting for in vivo NO activity (69). Moreover, BH₄ can directly increase eNOS-derived NO bioavailability (69, 70). TNF- α and LPS have been demonstrated to stimulate gene expression and activity of the key enzyme for regulation of BH₄ biosynthesis, GTP-cyclohydrolase I, in endothelial cells (71–74). Thus, TNF- α and LPS can directly increase the activity of eNOS in the absence of any induction of iNOS by upregulating production of BH₄ (73, 75). BH₄ was found to be increased in the studied vasculature only in cirrhotic rats with BT. The important role of BH₄ for NO synthesis in conditions of BT and associated TNF- α production can be seen in the positive correlation between serum levels of NO and TNF- α . The close relationship between systemic NO levels and TNF- α concentrations in MLNs particularly emphasizes the importance of TNF- α of mesenteric origin. Furthermore, induction of BH₄ by TNF- α is supported by significant correlations between TNF- α concentrations (in MLNs and serum) and levels of BH₄ in mesenteric vasculature. Finally, the hemodynamic impact of this increase in splanchnic BH₄ is apparent in its significant correlation with MAP, pointing out the special importance of the splanchnic bed for the circulatory abnormalities in liver cirrhosis, as has been suggested before (2).

In conclusion, BT to MLNs in cirrhotic rats is accompanied by a significant elevation in local and systemic concentrations of TNF- α . This is associated with increased BH₄ levels and enhanced eNOS-derived NO overproduction by the mesenteric vascular endothelium, further impairing vascular contractility in this vascular bed. Our results indicate that BT may play a predominant role in the severity of arterial vasodilation observed in advanced liver cirrhosis by stimulating a novel pathway for eNOS upregulation mediated by TNF- α .

Acknowledgments

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