iNOS expression in vascular resident macrophages contributes to circulatory dysfunction of splanchnic vascular smooth muscle contractions in portal hypertensive rats

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The major clinical consequences of cirrhosis are impaired function of hepatocytes and an elevation of intrahepatic resistance (portal hypertension) (4, 15, 37, 38). Portal hypertension is defined as a pathological increase in the pressure gradient between the portal vein and inferior vena cava (>5 mmHg) and is characterized by the formation of portal-systemic collaterals that shunt the portal blood flow to the systemic circulation, bypassing the liver. The increased portal venous pressure also promotes the formation of esophageal and gastric varices. Cirrhotic portal hypertension is initiated by an elevation in hepatic circulatory resistance because of the activation of hepatic stellate cells, perisinusoidal space fibrosis, and node formation, resulting from hepatocyte necrosis and regeneration. Increased portal venous inflow, promoted by splanchnic vasodilation, is also important in the progression of systemic circulatory dysfunction in cirrhosis; i.e., patients with cirrhosis exhibit a characteristic hyperdynamic circulation with reduced systemic vascular resistance and increased cardiac output.

Several lines of evidence suggest that nitric oxide (NO) affects both the liver and systemic circulation. For example, endothelial NO synthase (eNOS) activity is impaired in the cirrhotic liver, which results in an increased intrahepatic vascular resistance (37). In contrast to the intrahepatic events, the increment in NO production due to eNOS activation has been suggested to induce systemic arterial vasodilation (32). Studies using eNOS knockout and inducible NO synthase (iNOS) knockout mice suggested that eNOS plays a key role in the pathogenesis of portal hypertension (42). However, there are several studies demonstrating the importance of iNOS in the hyperdynamic circulation of liver cirrhosis (9, 27, 42, 46).

The adventitial layer has attracted extensive attention in vascular inflammation because it contains many exogenous cell types, including various immune cells (such as T cells, dendritic cells, monocytes, and macrophages) and fibroblasts capable of phenotypically switching into migratory myofibroblasts (12, 19, 20, 22, 23, 48, 50). All of these cells have immune responsiveness and are believed to be involved in perivascular inflammation and other proliferative vascular diseases, as well as in the events after angioplasty.

The liver plays an important physiological role in lipopolysaccharide (LPS) detoxification in which hepatocytes are involved in the clearance of endotoxin of intestinal derivation. Therefore, endotoxemia is frequently seen in patients with cirrhosis (2). In addition, the increased level of blood LPS enhances gut permeability and bacterial translocation, which exerts continuous endotoxemia and worsens with disease progression. In view of these findings, we speculated that LPS might...
cause immune reactions in perivascular cells, which may subsequently cause vascular contractile dysfunction in cirrhosis.

The purpose of the present study was to examine the possible expression of iNOS in the macrophage lineage positioned in splanchnic vascular walls (mesenteric artery and extrahepatic portal vein) and its functional role in contractility of vascular smooth muscle in cirrhotic portal hypertension. For this purpose, we used bile duct ligated (BDL) cirrhosis rats and an in vitro organ culture system to determine the site of iNOS expression and contractility changes in vascular tissues. We provide evidence for the first time that the different responsiveness of the mesenteric artery and portal vein to NO, which is mainly produced by cells of vascular monocyte/macrophage lineage, may contribute to circulatory dysfunction in cirrhotic portal hypertension.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Yokohama, Japan) at 7 wk of age. Animal care and treatment protocols were approved by and conducted in conformity with the institutional guidelines of The University of Tokyo. Secondary biliary cirrhosis was induced by bile duct ligation and excision (BDL) using the standard method. In brief, common bile ducts of rats were exposed and ligated twice after median laparotomy. In each animal the segment between the ligations was resected, and the animals were sutured. Experiments were performed 4 wk after the BDL operation. Sham-operated rats served as controls. In these rats, the common bile duct was exposed, but no ligation or resection was performed. Surgical procedures were carried out under sterile conditions. Rats had free access to chow (CR-LPF; Oriental Yeast, Tokyo, Japan) and tap water.

Tissue preparation and organ culture. Mesenteric arteries and extrahepatic portal veins were isolated from rats. After the fat tissue had been removed in normal physiological salt solution, each vessel was cut into rings ~1.0 mm wide for the mesenteric artery and 1.5 mm wide for the portal vein. In all experiments, the endothelium was removed by gently rubbing the intimal surface with forceps or a soft tissue. In the experiments shown in Figs. 1–4, we used tissues immediately after the isolation. For organ culture studies, mesenteric arteries and extrahepatic portal veins were isolated from healthy rats under sterile conditions. After the preparation procedure described above, the vascular rings were placed in Dulbecco’s modified Eagle’s medium at 37°C under an atmosphere of 95% O2-5% CO2 for 6 h (49). In preliminary experiments, we confirmed that organ culture with the serum-free condition for 6 h did not change the contractile activities in these vessels to high K+ (15–65 mM). We also confirmed that the tissues maintain contractile phenotype even after 6 h of culture through demonstrating the no changes of h-caldesmon content in the tissue (supplemental Fig. 1S).1

1 Supplemental material for this article is available at the American Journal of Physiology-Heart and Circulatory Physiology website.

Fig. 1. Electron micrographs of macrophages in the mesenteric artery and portal vein. A.a: mesenteric artery; several fibroblasts and their processes (F), nerve bundles (N), and a macrophage (M) were seen in the tunica adventitia. Macrophages were never seen in the smooth muscle (SM) layer of the media. OEL indicates the elastica externa. A.b: one macrophage (M) was seen within the adventitia. Nerve bundles (N) were seen nearby. B.a: portal vein; one macrophage (M) was seen in the connective tissue layer between the inner circular and outer longitudinal muscle layers. F, Fibroblast; SM, smooth muscle cells of the outer longitudinal muscle layer; V, vasa vasorum. A.b: two macrophages (M) were seen within the connective tissue septum of the outer longitudinal muscle layer. Nerve bundles (N) were also closely associated with macrophages.
Whole-mount fluorescent immunostaining. Whole-mount fluorescent immunostaining was performed as previously reported (13). Briefly, the mesenteric artery and extrahepatic portal vein were excised and opened. The endothelium was removed by gently rubbing the intimal surface with a soft tissue and fixed with 4% paraformaldehyde. The fixed preparations were incubated in 0.3% Triton X-100 and 5% skim milk dissolved in phosphate-buffered saline for 1 h and then probed with primary antibodies. Samples were incubated overnight at 4°C with a combination of anti-iNOS antibody (1:1,000 dilution; Transduction Laboratories, Lexington, KY) and anti-rat resident macrophage antibody (ED2, 1:500 in phosphate-buffered saline; Serotec, Oxford, UK). With the secondary reaction, ED2 was detected with FITC-conjugated goat anti-mouse IgG (1:200 dilution; Vector, Burlingame, CA) and iNOS was detected with Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:500 dilution; Invitrogen, Carlsbad, CA). The images were captured, and colocalization was analyzed using a Carl Zeiss confocal laser scanning microscope LSM510 imaging system (Tokyo, Japan).

Electron microscopy. Mesenteric arteries and extrahepatic portal veins were removed and placed in a fixative containing 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. Following fixation, the tissues were rinsed in the same buffer and postfixed in 1% osmium tetroxide for 2 h at 4°C. The tissues were then rinsed in distilled water, stained en bloc with 3% aqueous uranyl acetate overnight, dehydrated in a graded series of ethyl alcohol, and embedded in Epon epoxy resin. Ultrathin sections were cut using a Reichert microtome and double stained with uranyl acetate and lead citrate before viewing with JEOL JEM 1200EX II and Hitachi H7000 electron microscopes (Tokyo, Japan).

Semiquantitative reverse transcriptase-polymerase chain reaction. Total RNA was extracted from endothelium-free blood vessel preparations using the acid guanidinium isothiocyanate-phenol chloroform method, and concentrations of RNA were adjusted to 0.5 µg/µl with nuclease-free distilled water. Semiquantitative RT-PCR was performed as previously reported (13). Briefly, first-strand cDNA was synthesized using a random 9-mer primer and avian myeloblastosis virus (AMV) Reverse Transcriptase XL at 30°C for 10 min, 55°C for 45 min, and 99°C for 5 min.

Hot-start PCR amplification was performed using AmpliTaq Gold Polymerase. The oligonucleotide primers for iNOS were CTA CCT ACC TGG GGA ACA CCT GGG (forward) and GGA GGA GCT GAT GGA GTA GTA GCG G (reverse), and the size of the PCR product was 442 bp. The primers for IL-1β were CTC TGT GAC TCG TGG GAT GA (forward) and AGT TGG GGA ACT GTG CAG AC (reverse), and the product size was 400 bp. The primers for GAPDH were TAC CAG CCG GGG GAC CAC (forward) and CGA GCT GAC AGA GTA GTA (reverse), and the product size was 308 bp. After initial denaturation at 95°C for 10 min, 33 cycles of amplifications at 94°C for 40 s, 55°C for 60 s, and 72°C for 90 s were performed using a thermal cycler (Takara PCR Thermal

Fig. 2. Bile duct ligation (BDL)-induced increase in the number of resident macrophages and monocytes in mesenteric artery and portal vein vascular walls. Confocal micrographs of ED1 or ED2 immunopositive macrophages within the mesenteric artery (A) and portal vein (B) vascular walls are shown. Scale bar, 100 µm. The number of ED1-positive and ED2-positive cells was calculated (C). The data are expressed as means ± SE of 4 experiments. **P < 0.01 vs. sham-operated rats.
Cyclc MP, Takara Biomedicals, (Tokyo, Japan)]. PCR products were separated by electrophoresis on a 2% agarose gel containing 0.1% ethidium brodide. The possibility of DNA contamination was excluded by a PCR with total RNA without the reverse transcription step. Detectable fluorescent bands were visualized by UV-transilluminator using FAS-III (TOYOBO, Tokyo, Japan), and areas were measured using National Institutes of Health (NIH) image analyzing software ImageJ 1.42. The results were expressed as the ratio of the optical density of iNOS or TNF-α to that of GAPDH.

Measurement of released NO. Isolated and endothelium-denuded preparations were incubated in 100 μl Dulbecco’s modified Eagle’s medium for 4 h at 37°C under 95% O2-5% CO2. Incubated solution (90 μl) was removed for the measurement of released NO. Total nitrite was measured as an indicator of released NO by means of a fluorometric assay using 2,3-diaminonaphthalene (NO2⁻/NO3⁻ Assay kit-FX, Dijindo, Kumamoto, Japan). The released nitrite content was shown in millimoles per gram wet weight.

Measurement of muscle tension. The endothelium-free vessel rings were placed in normal physiological salt solution. The physiological salt solution (pH 7.4) was saturated with 95% O2-5% CO2 at 37°C. Muscle tension was recorded isometrically with a force-displacement transducer connected to a strain amplifier (model 3134 or 3170, Yokogawa, Tokyo, Japan) and an ink-writing recorder (model 3056 or 3711, Yokogawa) under a resting tension of 10 mN. At the end of all experiments, papaverine (100 μM) was added to determine the basal tone.

Myosin light chain phosphorylation measurement. Myosin light chain (MLC) phosphorylation was measured by the method previously described (36). Briefly, the endothelium-free strips were frozen by immersion in dry ice-acetone slurry containing 10% trichloroacetic acid. The strips were homogenized in a solution composed of 23 mM glycine, 10 mM DTT, 20 mM Tris, and 8 M urea, and the supernatants were subjected to glycerol-PAGE to separate phosphorylated MLC. Rabbit polyclonal antibody against bovine tracheal MLC (kindly donated by Dr. J. T. Stull, University of Texas Southwestern Medical Center, Dallas, TX), Alexa Fluor 680-conjugated goat anti-rabbit IgG polyclonal antibody, and Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) were used to detect MLC phosphorylated MLC. Myosin light chain phosphorylation measurement. Myosin light chain (MLC) phosphorylation was measured by the method previously described (31). Mesenteric arteries and portal veins were placed in glass tube containing the loading solution replacing a 2,3-diaminonaphthalene (NO2⁻/NO3⁻ Assay kit-FX, Dijindo, Kumamoto, Japan). The released nitrite content was shown in millimoles per gram wet weight.

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Intracellular [Ca2+]i measurements. Intracellular [Ca2+]i concentration ([Ca2+]i) was measured by the method previously described (31). Mesenteric arteries and portal veins were placed in a glass tube containing the loading solution replacing a 2,3-diaminonaphthalene (NO2⁻/NO3⁻ Assay kit-FX, Dijindo, Kumamoto, Japan). The released nitrite content was shown in millimoles per gram wet weight.

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Macrophages were also observed within the longitudinal muscle layer and outermost layer of the longitudinal muscle (data not shown). These cells were also characterized by vacuoles, lysosomes, coated vesicles within the cytoplasm, and many processes and by close association with nerve components.

**Increment in the number of ED1- or ED2-positive cells in vascular walls.** Using a whole-mount fluorescent immunostaining method, we next analyzed the change in the number and morphology of ED1-positive monocytes and ED2-positive resident macrophages within the vascular walls of mesenteric arteries and portal veins after BDL. In the mesenteric artery isolated from sham-operated rats, we observed a few round-shaped ED1-positive cells in the vascular wall (Fig. 2A). We also identified some ED2-positive resident macrophages in the vascular wall that displayed a ramified (dendritic) shape. ED1-positive and ED2-positive cells (Fig. 2B) were also identified in the portal vein. When compared with the mesenteric artery, ED1-positive cells in the vascular wall of the portal vein presented as small and round in shape, and ED2-positive cells were not characterized by as many projections (Fig. 2B).

After BDL, more ED1-positive and ED2-positive cells were detected in the mesenteric artery and portal vein compared with those of the sham-operated control. ED1-positive and ED2-positive cells were quantified, and significant increases were seen in both the mesenteric artery and portal vein (Fig. 2C). The absolute number of these cells in the portal vein was roughly two times greater than in the mesenteric artery. ED2-positive cells in mesenteric arteries changed from a ramified shape to round shape after the BDL operation.

**Change in iNOS expression in vascular walls.** It is well known that activated macrophages express iNOS and produce large amounts of NO, which may diminish vascular smooth muscle contractile function. In fact, in cirrhosis patients, as well as in rodent cirrhosis models, it is thought that iNOS contributes to the regulation of vascular tone (see Introduction). Therefore, we next investigated the change in expression of iNOS within the vascular wall in BDL-operated rats. We measured mRNA expression of iNOS using endothelium-denuded vascular walls. Only a small amount of iNOS mRNA was detected in the mesenteric artery and portal vein isolated from sham-operated rats. However, the levels of iNOS mRNA increased in BDL-operated rats.

**Fig. 4.** iNOS distribution in ED1-positive monocytes and ED2-positive resident macrophages in vascular tissues isolated from sham-operated and BDL rats. Confocal micrographs of iNOS immunopositive cells in the mesenteric artery (A) and portal vein (B) are shown. Top: iNOS (red)-positive cells were stained with ED1 (green). Bottom: iNOS (red)-positive cells were stained with ED2 (green). Representative results are shown from 4 experiments. No iNOS immunoreactivity was detected in the vascular tissues isolated from sham-operated rats. Scale bar, 100 μm.
were drastically increased in both vessels after the BDL operation (Fig. 3A). We further measured nitrates in the culture supernatant 4 h after the incubation of these tissues in normal medium. Nitrite production in these tissues was greatly increased in BDL rats compared with sham-operated rats (Fig. 3B).

To identify the cellular origin of iNOS within the vascular walls, we performed double immunostaining of iNOS-positive cells with ED1 or ED2 antibodies. In the sham-operated rats, we scarcely detected any iNOS signal within the vascular walls of the mesenteric artery and portal vein, which is consistent with the results obtained from mRNA expression experiments. On the other hand, we detected a number of iNOS-immunoreactive cells in vascular tissues isolated from BDL rats, but not sham-operated rats (Fig. 4A and B).

**Fig. 5.** Effect of LPS on mRNA expression of inflammation-related molecules and NO production in the mesenteric artery and portal vein. Isolated healthy vascular rings without endothelium were exposed to LPS (1 or 10 μg/ml) for 6 h. A: mRNA expression of iNOS was measured by semiquantitative RT-PCR analysis. mRNA expression of iNOS was normalized to GAPDH. B: the amount of released NO from vessels treated with or without LPS (1 or 10 μg/ml) for 6 h. After incubation, 90 μl of the incubated solution were removed for measurement of released NO. C: mRNA expression of TNF-α, IL-1β, and IL-10 was measured by semiquantitative RT-PCR analysis. Results are expressed as means ± SE of 5 to 6 experiments. *P < 0.05 and **P < 0.01 vs. control.
iNOS-positive cells were double stained with ED2 in the mesenteric artery. In the portal vein, 88 ± 11% of iNOS-positive cells were also positive to ED2. We also observed several iNOS-positive, but ED2-negative, cells (Fig. 6A, green cells in the merged picture; and Fig. 6B, red cells in the merged picture) in the LPS-treated tissues.

**Change in contractile function in LPS-treated vascular tissues.** To evaluate the effect of NO produced by iNOS on vascular contractile function, we measured vascular tissue contractile tension after a 6-h incubation with LPS. The LPS-treated mesenteric artery showed an impaired response to phenylephrine (0.01–10 μM) compared with the control (Fig. 7A). The reduced contractility was almost completely restored by treatment with the NO synthase inhibitor L-NAME (300 μM), indicating that the inhibitory effect of LPS treatment is mostly due to NO. Interestingly, in contrast to the results in the mesenteric artery, phenylephrine-induced (0.01–10 μM) portal vein contractile tension was scarcely affected by LPS treatment (Fig. 7B).

**Low sensitivity of portal vein to NO.** As demonstrated in Fig. 7, the portal vein contractile response was less sensitive to LPS treatment compared with the mesenteric artery. To explain this difference in responsiveness, we next examined the effect of NO-donor SNP on these vascular tissues. SNP was cumulatively added (0.0001–10 μM) to vascular preparations that had been precontracted by phenylephrine (10 μM) or KCl.
mM). SNP inhibited the contractions to a greater extent in the mesenteric artery than in portal vein (Fig. 8A). We further evaluated the downstream pathway following NO stimulation. 8-Br-cGMP, a nonhydrolyzable and cell-permeable analog of cGMP (1 nM–10 μM), was cumulatively added to vascular tissues precontracted by 35 mM KCl. As expected, the portal vein was less sensitive to 8-Br-cGMP (Fig. 8B).

We also examined the change in MLC phosphorylation levels after the SNP treatment in both vessels. The endothelium-denuded tissue strips were treated with phenylephrine (10 μM) for 1 min, and SNP (10 μM) was then added for 1 min. In mesenteric artery, SNP treatment significantly reduced MLC phosphorylation almost to the basal level. On the other hand, in the portal vein, the inhibition of MLC phosphorylation by SNP was obviously less than in the mesenteric artery (Fig. 8C).

In smooth muscle cells, cGMP-dependent protein kinase (PKG) phosphorylates various targets that lead to the inhibition of MLC phosphatase and phosphorylation of MLC (28, 41). Activated PKG also phosphorylates large conductance Ca²⁺-activated K⁺ channels, thereby inhibiting Ca²⁺ entry (16). As a result, the contractile activity of smooth muscle cells is diminished in a [Ca²⁺]i-dependent manner.

Therefore, we measured [Ca²⁺]i of smooth muscle tissues using the fluorescent Ca²⁺ indicator fura-2. The endothelium-denuded tissue strips were pretreated with phenylephrine (10 μM) or KCl (35 mM), and SNP (0.1 nM–10 μM) was then cumulatively added. SNP treatment resulted in a large reduction in [Ca²⁺]i, in the mesenteric artery pretreated with phenylephrine but not in the portal vein (Fig. 9, A and B). In the case of 35 mM KCl-pretreated tissues, [Ca²⁺]i was slightly decreased in the mesenteric artery, but almost no change was observed in the portal vein (Fig. 9, C and D).

**DISCUSSION**

In the present study, we tested the hypothesis that iNOS expressed by macrophages positioned in splanchnic vascular walls is involved in contractile dysfunction of vascular smooth muscles in cirrhotic portal hypertension. Recent studies have revealed the identity of the adventitial cell population, which includes fibroblasts, monocytes, dendritic cells, T lymphocytes, and macrophages in arterial vascular smooth muscle tissues (12, 19, 23, 48, 50). It is also recognized that the development of muscle layers is different in arterial and venous blood vessels where the artery media is composed only of circular smooth muscle but that of vein is composed of circular and longitudinal muscles. Therefore, it is likely that the composition and distribution of the interstitial cell population is...
different in these tissues. In this study, we initially attempted to identify the exact anatomical location of resident macrophages in the mesenteric artery and extrahepatic portal vein by transmission electron microscopy. In the mesenteric artery, macrophages were found in the connective tissue of the tunica adventitia; however, these cells were never observed in the tunica media. Unlike in the mesenteric artery, ultrastructural analysis of the portal vein revealed that macrophages were distributed within the musculature; i.e., they were positioned within the connective tissue layer between inner circular and outer longitudinal muscle layers, within the connective tissue septa of the outer longitudinal muscle layer, and within the longitudinal muscle layer and outermost layer of the longitudinal muscle. Given the difference in the anatomical location of resident macrophages in the mesenteric artery and portal vein, it is expected that these cells may control smooth muscle contractile functions differently. In this study, we further showed that these macrophages are situated closest to nerve components, both in the mesenteric artery and portal vein. Such close contact to nerve components has been reported in resident macrophages in the intestinal smooth muscle layers (14).

In BDL cirrhosis model rats, we demonstrated an increase in the number of ED2-positive resident macrophages in these tissues compared with that in sham-operated rats. We also observed that the number of ED1-positive monocytes was increased, indicating that monocytes migrate into the vascular wall of BDL-operated rats following an activation of resident macrophages. An increase in the number of ED2-positive macrophages may be attributable to the conversion of recruited ED1-positive monocytes to ED2-positive, resident-type macrophages (14).

Activated macrophages and monocytes are known to express iNOS and produce NO under many inflammatory conditions including cardiovascular disorders (5, 11, 26, 33, 45). On the other hand, although most studies on portal hypertension have focused on the mechanisms and pathways through which eNOS activity is altered in the development of circulatory dysfunction (see Introduction), some studies have demonstrated the importance of iNOS in hyperdynamic circulation of liver cirrhosis (3, 9, 24, 27, 46). Thus these findings suggest the possibility of the involvement of iNOS expression in the vascular monocyte/macrophage cell lineage. The present study clearly demonstrated that iNOS was largely expressed in ED1-positive monocytes and ED2-positive macrophages of mesenteric artery and portal vein tissues after BDL. In vascular tissues, iNOS can also be expressed in smooth muscle cells. In the present study, immunohistochemical analysis indicated that iNOS reactivity was not observed in the spindle-shaped, smooth muscle-like cells. However, we cannot discard the involvement of iNOS expression in other immune cells, such as neutrophils, infiltrated into the vascular walls after BDL.

In the gastrointestinal tract, macrophages that reside in the muscle layer play important roles in the regulation of smooth muscle...
muscle contraction under several inflammatory conditions (13, 14, 18, 30, 39). For instance, macrophage-derived NO, prostaglandins, and proinflammatory cytokines (such as TNF-α, IL-1β, and IL-4) contribute to the dysfunction of gastrointestinal smooth muscle contraction, either in a stimulatory or inhibitory manner (1, 13, 17, 29, 39, 43, 44). Analogous to the function of macrophages in the gastrointestinal tract, it is likely that macrophages residing in the vascular wall may also affect smooth muscle contraction. In this study, we used an in vitro organ culture system to elucidate the role of activated vascular resident macrophages in contractile functions. It is recognized that bacterial toxins, particularly endotoxins such as LPS, can activate macrophages to induce iNOS and cyclooxygenase-2.

On the other hand, in cirrhosis or the hyperdynamic status of portal circulation in acute inflammatory liver diseases, it is known that intestinal permeability is increased following increased portal pressure, which results in an influx of gut flora-derived LPS (6, 7, 21, 40). In this study, we found that exposure of vascular tissues to LPS increased iNOS mRNA expression and the inflammation-related cytokines, such as TNF-α, IL-1β, and IL-10. In addition, we showed an increase in NO metabolites in the incubation medium with LPS. We also observed that the α1-adrenoceptor agonist-induced contraction of the mesenteric artery, in vitro, was significantly inhibited after LPS treatment. The reduced contractility was restored by L-NAME (NO synthase inhibitor), suggesting that the increased production of NO is responsible for the reduced smooth muscle contractility. Taken together, these results suggest that NO, which mainly originated from monocytes/macrophages, plays an important role in reducing the vascular smooth muscle contractile functions. In an organ culture study, we demonstrated that the majority of iNOS protein expression appeared to be limited to monocytes/macrophages after LPS treatment, as in the case of in vivo BDL model. However, the results additionally indicated that a small number of ED2-negative cells also expressed iNOS proteins in LPS-treated tissues. As judged from the cellular morphology, these cells may be adventitial myofibroblasts (not smooth muscle cells), which are considered to be involved in the perivascular inflammation responsible for fibroproliferative vascular remodeling (8, 23, 47).

Another important finding of this study is that although LPS upregulated iNOS mRNA and protein, and subsequent NO production, in both mesenteric artery and portal vein, portal vein contractility was less affected by LPS. To understand the lower responsiveness of portal vein contraction to NO, we examined the difference in the sensitivity of these tissues to NO/cGMP pathways (10). SNP (NO donor) and 8-Br-cGMP (membrane permeable cGMP analog) inhibited contraction to a greater extent in the mesenteric artery than the portal vein. Consistent with this, [Ca\textsuperscript{2+}] increase and MLC phosphorylation of mesenteric artery stimulated with α-adrenergic agonist was also more greatly inhibited by SNP compared with the portal vein. These results suggest that, after NO stimulation, the cGMP-dependent pathway appears to contribute less to contractile function in the portal vein than in the mesenteric artery. Taken together, we speculate here that circulatory dysfunction in cirrhotic portal hypertension, characterized by increased hepatic vasconstriction and systemic arterial vasodilation, can be explained, at least in part, by the differential sensitivity of vascular smooth muscles to NO/cGMP-depen-

dent pathways. Meanwhile, it has been reported that cGMP inhibits smooth muscle contraction not only by reducing [Ca\textsuperscript{2+}], and Ca\textsuperscript{2+} sensitivity of MLC phosphorylation but also by thin filament inactivation due to heat shock protein (HSP) 20 phosphorylation without a reduction of [Ca\textsuperscript{2+}] (34, 35). As for the mechanism responsible for the lower sensitivity of the venous smooth muscle to NO, Brophy and coworkers (25) have suggested that the difference in the expression of HSP27 (carotid artery > saphenous vein), an inhibitor protein of phosphorylation of HSP20, may contribute to the different responsiveness of these tissues to cGMP. In the present study, however, we found a close correlation between the changes in the levels of MLC phosphorylation and contraction in mesenteric artery and portal vein to NO.

In conclusion, our findings provide experimental evidence for the essential role of iNOS induction in splanchic vascular walls and the subsequent production of NO in circulatory dysfunction in cirrhosis. The imbalance in the responsiveness to NO between the mesenteric artery and portal vein contributes to increased portal venous flow in cirrhotic portal hypertension. The present study further addresses the importance of the monocyte/macrophage lineage, which predominantly expresses iNOS in the vascular wall.

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**DISCLOSURES**

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