Role of endothelial nitric oxide synthase in the development of portal hypertension in the carbon tetrachloride-induced liver fibrosis model

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Submitted 17 June 2009; accepted in final form 20 July 2009

Theodorakis NG, Wang YN, Wu J, Maluccio MA, Sitzmann JV, Skill NJ. Role of endothelial nitric oxide synthase in the development of portal hypertension in the carbon tetrachloride-induced liver fibrosis model. Am J Physiol Gastrointest Liver Physiol 297: G792–G799, 2009. First published July 23, 2009; doi:10.1152/ajpgi.00229.2009.—Portal hypertension (PHT) is a complication of liver cirrhosis and directly increases mortality and morbidity by increasing the propensity of venous hemorrhage. There are two main underlying causations for PHT, increased hepatic resistance and systemic hyperdynamic circulation. Both are related to localized aberrations in endothelial nitric oxide synthase (eNOS) function and NO biosynthesis. This study investigates the importance of eNOS and systemic hyperdynamic-associated hyperemia to better understand the pathophysiology of PHT. Wild-type and eNOS−/− mice were given the hepatotoxin CCl4 for 4–12 wk. Hepatic fibrosis was determined histologically following collagen staining. Portal venous pressure, hepatic resistance, and hyperemia were determined by measuring splenic pulp pressure (SPP), hepatic portal-venous perfusion pressure (HPVPP), abdominal aortic flow (Qao), and portal venous flow (Qpv). Hepatic fibrosis developed equally in wild-type and eNOS−/− CCl4-exposed mice. SPP, Qao, and Qpv increased rapidly in wild-type CCl4-exposed mice, but HPVPP did not. In eNOS−/− CCl4 mice, Qao was not increased, SPP was partially increased, and HPVPP and Qpv were increased nonsignificantly. We concluded that the systemic hyperemia component of hyperdynamic circulation is eNOS dependent and precedes increased changes in hepatic resistance. Alternative mechanisms, possibly involving cyclooxygenase, may contribute. eNOS maintains normal hepatic resistance following CCl4-induced fibrosis. Consequently, increased portal pressure following chronic CCl4 exposure is linked to hyperdynamic circulation in wild-type mice and increased hepatic resistance in eNOS−/− mice.

IN THE UNITED STATES, cirrhosis and chronic liver disease is the twelfth leading cause of death, accounting for more than 27,000 deaths in 2005 (20a). Portal hypertension (PHT) is a serious complication of liver cirrhosis, whereby the portal venous pressure is significantly elevated. This increase in pressure promotes the formation of varices, which are porto-systemic collaterals formed following dilation of preexisting vascular channels. About 25–40% of all cirrhotic patients have varices, of which one third will hemorrhage with a 20–30% mortality rate (24). In most patients with cirrhosis, increased resistance to portal outflow is almost always the initial pathophysiological event and is followed by an increased portal venous flow (Qpv) through a hyperdynamic splanchnic system. Increased hepatic resistance manifests via both liver fibrosis and hepatic sinusoidal cellular aberrations that promote constriction of the hepatic sinusoids. Hyperdynamic circulation is characterized by a decrease in systemic vascular resistance and arterial blood pressure and an increased cardiac index. The mechanisms underlying mesenteric arteriolar vasodilation, associated with a reduction in resistance, are not fully defined but probably include increased levels of vasodilators and decreased vascular responsiveness to endogenous vasoconstrictors. Consequently, vasodilation of systemic and splanchnic arteriolar vessels increases blood flow, elevates portal venous pressure, and is associated with the formation of varices.

Previous research has shown that nitric oxide (NO) is important to both increased hepatic resistance and hyperdynamic circulation-associated hyperemia. NO is a diatomic molecule that is freely permeable across cell membranes and causes rapid vasodilation via activation of soluble guanylyl cyclases and Ca2+. NO is synthesized by a family of enzymes known as nitric oxide synthase (NOS), of which there are three main isoforms: 1) neural (nNOS), 2) inducible (iNOS), and 3) endothelial (eNOS). In particular, eNOS has been shown to be key to both increased hepatic resistance and mesenteric hyperemia within experimental models (1, 32). These studies have shown localized modulation of eNOS expression in the development of PHT. Decreased NO production has been observed within cirrhotic livers and is associated with constriction of hepatic sinusoids and increased hepatic resistance to portal flow (12, 27, 32, 36, 40). Alternatively, increased eNOS expression has been reported within the mesenteric vasculature, causing dilation and increased Qpv (4, 5). These opposing events involving the same enzyme, but in differing locations, introduce a challenge in our ability to target eNOS in a PHT treatment paradigm. In experimental models, NOS gene transfer and the liver-specific NO donor, NX-1000, reduced portal pressure (7, 23, 38, 43). More recently, targeting of AKT and G protein receptor kinase-2 (GRK) regulation of hepatic eNOS activation has also been successful in reducing hepatic resistance (22, 26). On the other hand, nonspecific NOS inhibitors prevent systemic and mesenteric hyperemia and abrogate the development of PHT (8, 28). Therefore, both inhibition and stimulation of NO production ameliorate portal pressure.

At present, the pathophysiology of PHT in experimental models and its relevance to human disease pathology are poorly understood. Clinically, increased PHT is believed to manifest primarily from an increased hepatic resistance. This may not be the case in experimental models. We have previously shown in the PVL model of PHT that systemic hyperemia is eNOS dependent; moreover, in the absence of aortic hyperemia, PHT was not observed (37). In the PVL model, increased resistance to portal flow is compensated by the formation of a collateral circulation, and hyperemia maintains the elevated portal venous pressure. Therefore, experimental models may not mimic clinical etiology. In experimental models, vasculopathy may dominate and precede hepatic resis-
tance. To address this deficiency, within our knowledge, this study utilizes commercially available eNOS gene-deleted mice and the CCl$_4$-induced liver fibrosis and PHT model. To date the development of PHT in eNOS$^{-/-}$ mice following CCl$_4$ administration has not been reported despite the fact that the CCl$_4$ model has been extensively used in rats to generate precirrhotic liver fibrosis and PHT (32, 33). Previous investigations have used eNOS$^{-/-}$ mice in combination with other models of PHT (PVL and bile duct ligation). However, a clear consensus has not emerged pertaining to the role of eNOS in experimental PHT. Our observations using the prehepatic PVL model of PHT are in contrast to the findings of Iwakiri et al. (17) and Koshy et al. (20), who report elevated portal pressure in eNOS$^{-/-}$ mice following PVL and bile duct ligation. These opposing reports make it difficult to compose a unified understanding for the role of eNOS in PHT, especially in animal models. Therefore, additional studies are required to better understand the role of eNOS and the etiology of experimental PHT so that we can better treat the thousands of patients with cirrhosis who are at risk of PHT and variceal hemorrhage.

We present here data confirming that experimental PHT-associated hyperemia is eNOS dependent. In the absence of eNOS-dependent hyperemia, portal pressure is significantly reduced. Moreover, we present that, in mice, mild to moderate liver fibrosis induced by chronic CCl$_4$ administration was not associated with a significant increase in hepatic resistance. Therefore, in the murine CCl$_4$ model, vasculopathy dictates the initial elevation of portal venous pressure, and that inhibition of eNOS to prevent hyperemia would be efficacious to reduce portal venous pressure.

**MATERIALS AND METHODS**

All studies were approved by the Indiana University institutional animal care and use committee and adhered to AAALAC and federal guidelines for the humane care and treatment of animals. Mice were maintained in sterilized isolate cages on a 12-h light/dark cycle and were allowed access to food and water ad libitum.

Carbon tetrachloride induced hepatic fibrosis and PHT. Hepatic fibrosis was induced in 8–12-wk-old C57BL6/J male mice by intraperitoneal injection of 0.6 ml/kg CCl$_4$ twice weekly for 4–12 wk, as previously described by Kamada et al. (19). Vehicle control mice were given twice weekly intraperitoneal injection of 0.6 ml/kg vehicle (mineral oil) for 12 wk. The development of hepatic toxicity and fibrosis was monitored by determination of alanine aminotransferase (ALT) levels and hepatic histology. The development of PHT was determined by measuring portal and systemic hemodynamic physiological measurements. Serum was obtained by cardiac puncture and withdrawn into heparinized syringes. Serum NO ($\varepsilon$) and ALT levels were quantified using commercially available kits (Oxford Biomedical Research, Rochester Hills, MI and Thermo-Clinical, Pittsburgh, PA) per manufacturer’s instructions.

Hemodynamic physiological measurements. Physiological measurements were performed as previously described by Theodorakis et al. (37). At the indicated times, animals were anesthetized and subjected to a laparotomy. Splenic pulp pressure (SPP) was measured as an index of portal venous pressure. To measure SPP, a microtip pressure transducer (no. SPR-839; Millar Instruments, Houston, TX) was inserted in to the spleen pulp. Abdominal aortic flow (Qao) and Qpv were measured by placing an ultrasonic Doppler flow probe (no. 11RB; Transonic Instruments, Ithaca, NY) around the abdominal aorta between the diaphragm and celiac artery or around the distal portal vein between the coronary vein and the portal venous bifurcation. Flow rates were obtained with a Transonic T201 Blood Flow Meter (Transonic Instruments). Aortic and portal venous blood flows were recorded and standardized per gram of body weight.

**Hepatic resistance.** The isolated hepatic portal venous perfusion pressure (HPVPP) was calculated as an index of hepatic resistance. Mice were anesthetized via inhalation with halothane, and body temperature was maintained at 37°C using a temperature controlled heating pad. After a midline incision and laparotomy, the portal vein was dissected and cannulated with P25 polyurethane tubing (ID 2.5 mm, and the inferior vena cava was severed. Oxygenated Krebs solution at 37°C (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.17 mM MgSO$_4$, 2.5 mM NaHCO$_3$, 1.18 mM KH$_2$PO$_4$, 27 µM EDTA, and 5.5 mM glucose) was perfused at 0.05 ml/min per gram of body weight using a high-performance liquid chromatography pump (W500 Waters, Milford, MA), and the perfusion pressure was monitored via an inline pressure transducer and recorded using a Millar MPV-400 pressure/volume recorder. Livers were perfused for 10 min before measurements to allow the pressure to stabilize.

**Gene-deficient mice.** Mice containing targeted mutation in the eNOS (nos3) gene were purchased from The Jackson Laboratory (Bar Harbor, ME). Age-matched mice from congenic strains (C57BL/6J) were used as wild-type controls. Mouse genotypes were confirmed by PCR using DNA isolated from tail samples using Qiagen DNeasy kit (Qiagen, Stanford, CA) per manufacturer’s instructions. Gene-specific primers were used to detect the targeted mutations in the eNOS gene (gttgtaaggcaaccattctg and actcatccatgcacaggacc) and the iNOS gene (ggctcaagggtagaacccttg and tccgattgagcaagctcg) (21, 34). PCR conditions were 30 cycles of 1 min each of 94°C, 60°C, and 74°C.

**Hepatic fibrosis.** Livers were harvested from halothane-anesthetized animals and fixed by immersion with 10% neutral-buffered formaldehyde overnight at 4°C or snap frozen and stored at −80°C. Masson’s trichrome staining for collagen. Following fixation, tissues were paraffin embedded, and 10-µm sections were cut. The development of fibrosis was monitored by Masson’s trichrome staining of collagen. The extent of fibrosis following CCl$_4$ was determined using a morphometric analysis on the basis of point counting following sample blinding and label randomization (35). Dewaxed sections stained with Masson’s trichrome stain were used. Transverse sections of the liver were selected. These were viewed by light microscopy with a ×400 flat-field objective and a squared lattice of 64 points with a surface area of 0.016 mm$^2$ superimposed on the tissue. Data were collected from a minimum of 25 fields. Three such linear series of 9 to 12 fields were measured per section, and two stained sections per liver were analyzed. The percentage points falling on stained structures were calculated in relation to the total number of points counted.

**Immunoprobing for collagen-I.** Sections (10 µm) of liver cryostat were immunoprobed for collagen-I (rabbit anti-collagen-I Ab34-710; Abcam, Cambridge, MA). Primary antibody binding was revealed using a goat anti-rabbit CY5-conjugated antibody (Invitrogen, Carlsbad, CA). Sections were visualized using a Bio-Rad (Hercules, CA) confocal microscope using a Kr/AR laser (647 nm and 488 nm) for both CY5 (optimal excitation 650 nm) and autofluorescence. Computer imaging and analyses were obtained at 665 nm and 488 nm for both CY5 (optional excitation 650 nm) and autofluorescence. Computer imaging and analyses were obtained at 665 nm and 530 nm for CY5 and autofluorescence, respectively. Semi-quantitation of staining on each slide was obtained using the mean CY5 emissions per field (mV/µm$^2$) from at least 10 ×200 magnification fields. To ensure consistency, the photomultiplier voltages and signal offset levels were set using control tissues and were maintained for subsequent sections.

**Statistics.** The data shown are means ± SE, with 5–8 animals per experimental group. Statistical significance was estimated using one-way ANOVA statistical analysis. A value of $P < 0.05$ was considered significant.

**RESULTS**

**Genotype confirmation.** Mouse genotypes were confirmed using the PCR. Genomic DNA isolated from wild-type (C57BL/J) mice or mice containing targeted mutations in the
eNOS gene was amplified by PCR using primers that span a 500-base pair (bp) region of the iNOS gene and a 300-bp region of the eNOS gene. Both PCR products were formed when DNA from wild-type mice was used as a template. The 300-bp product was absent when DNA from eNOS−/− mice was used, whereas the 500-bp product for iNOS was formed. In total, 30 wild-type and 15 eNOS−/− mice were used.

Mortality. Wild-type and eNOS−/− mice were injected intraperitoneally twice weekly with CCl4 or vehicle (mineral oil) for 4–12 wk. There were no spontaneous deaths in the four groups.

Serum ALT and NOx. Serum ALT and NOx were determined twice monthly using commercially available kits. The average serum ALT level was consistently elevated in wild-type and eNOS−/− mice following CCl4 administration. Levels were increased from 25.8 ± 3 and 21.5 ± 2 U/ml in wild-type and eNOS−/− mice mineral oil controls, respectively, to 309.2 ± 12 and 299.2 ± 16 following 12 wk of CCl4 administration. There was no significant difference in ALT levels between comparable wild-type and eNOS−/− mouse groups. The average serum NOx level was significantly increased following 12-wk CCl4 treatment in wild-type (P = 0.047) and eNOS−/− (P = 0.02) mice compared with mineral oil-treated control levels. NOx levels increased 52.8 and 58% following 12-wk CCl4 administration to wild-type and eNOS−/− mice, respectively. These data are in correlation with previous studies that have shown that serum NOx levels (NO2 and NO3 oxidative products of NO) are increased in CCl4-treated animals that develop PHT (2, 25, 33, 39). Moreover, this datum suggests that the elevated serum NOx level in CCl4 mice is not eNOS dependent and thus is probably attributable to iNOS upregulation or a nonspecific increase in NOx intake from the diet.

Development of liver fibrosis. Evaluation of liver fibrosis was determined by monitoring collagen accumulation via Mason’s trichrome staining and immunofluorescence. In untreated wild-type and eNOS−/− mice, collagen staining, as determined by Mason’s trichrome staining, was localized to vascular structures and was absent within the parenchyma or lobule borders (Fig. 1A, i and ii). There was no significant change in collagen staining within livers from 12-wk mineral oil-treated wild-type or eNOS−/− mice compared with comparative 0-day controls (0.8 ± 0.3 and 0.9 ± 0.1, respectively). Following the administration of CCl4, increased perportal collagen staining was observed and normal hepatic architecture was lost, characteristic of bridging fibrosis (Fig. 1A, iii and iv). Semiquantitative point counting of Masons trichrome-stained sections recorded a progressive increase in scarring index with CCl4 treatment in both wild-type and eNOS−/− mice. Scarring index increased from 0.4 ± 0.4 and 0.7 ± 0.8 arbitrary units in 0-day controls to 5 ± 1.5 and 6 ± 1.6 following 12-wk administration of CCl4 to wild-type and eNOS−/− mice, respectively (Fig. 2, A and B).

Hepatic collagen-I staining was sparse in mineral oil-treated wild-type and eNOS−/− mice and was limited to the portal triad. Following chronic administration of CCl4 for 12 wk, collagen-I was increased and was observed surrounding the portal triad and within the parenchyma of wild-type and eNOS−/− mice (Fig. 1B, i–iv). Quantification of collagen-I staining using the CY5 emission intensity documented that collagen-I increased from 0.8 ± 0.3 and 0.9 ± 0.4 mV/μm² in mineral oil-treated wild-type and eNOS−/− mice to 10.4 ± 1 and 27.5 ± 3.8 mV/μm² in 12-wk CCl4-treated mice, respectively (Fig. 2C).

Hemodynamic alterations. Wild-type mice treated with twice weekly injections of CCl4 developed a significant elevation in Qao (0.26 ± 0.01 vs. 0.173 ± 0.03 ml/min per gram body weight, respectively, after 12-wk chronic administration) (Fig. 3B). Similarly, the Qpv was increased in 12-wk CCl4-treated mice compared with controls (74 ± 4 vs. 60 ± 3 μl/min per gram, respectively) (Fig. 3C). Qao was not increased in eNOS−/− mice 12 wk following CCl4 administration compared with mineral oil-injected controls (0.15 ± 0.01 vs. 0.14 ± 0.01 ml/min per gram body weight, respectively) (P = 0.39) (Fig. 3B). Qpv was not significantly increased in eNOS−/− mice following 12-wk CCl4 administration compared with mineral oil-treated controls (43 ± 2 vs. 51 ± 9 μl/min per gram) (Fig. 3B).

SPP increased within 2 wk of CCl4 administration (6.8 ± 0.03 vs. 7.3 ± 0.2 mmHg). SPP increased progressively over 12 wk in CCl4-treated wild-type mice compared with mineral oil controls (12.1 ± 0.2 vs. 7.1 ± 0.2, respectively, P < 0.01) (Fig. 4A). In addition, the spleen/body weight ratio was increased significantly 12 wk following CCl4 compared with mineral oil controls (6 ± 0.7 vs. 2.3 ± 0.3 mg/g, respectively, P = 0.01). In eNOS−/− mice, the SPP was increased in 12-wk CCl4-treated eNOS−/− compared with mineral oil controls (6.7 ± 0.24 vs. 9.1 ± 0.4 mmHg, respectively, P = 0.008) (Fig. 4B). However, the percentage increase of SPP in eNOS−/− mice (35%) was nearly half that seen in wild-type mice (70%), but the spleen/body weight ratio was increased in a similar manner to that seen in wild-types (5.6 ± 1.1 vs. 2 ± 0.6 mg/g for 12-wk CCl4-treated and 12-wk mineral oil-treated eNOS−/− mice, respectively).

Hepatic resistance. The isolated HPVPP was measured as an index of hepatic resistance. There was no statistical difference in the perfusion pressure between 12-wk CCl4-treated and mineral oil treated wild-type mice (3.8 ± 0.4 vs. 5 ± 0.5, respectively, P = 0.15). In control eNOS−/− mice, the perfusion pressure was higher than that seen in wild-type mice, but the increase was not statistically significant (7.1 ± 2.1 vs. 3.8 ± 0.4, respectively, P = 0.09). Following chronic CCl4 administration, the perfusion pressure increased to 13.5 ± 4, but this was not statistically significant (P = 0.12) (Fig. 4C). There was a greater variability in the perfusion pressure among both the eNOS−/− control and CCl4 groups compared with the corresponding wild-types. At this time, we do not know the reason for this. All experiments were standardized to ensure reproducibility. It is possible that eNOS compensatory mechanisms are variable within the eNOS−/− population, and thus additional numbers would be required to provide the statistical power necessary to detect significant differences.

In combination, these data show that the eNOS gene is essential for the development of hyperemia following CCl4-induced cirrhosis. However, in contrast to studies using the PVL model, SPP did increase in eNOS−/− mice following CCl4 administration, albeit at a much reduced level compared with comparable wild-type mice. Moreover, hepatic resistance was found to be eNOS dependent even though some differences were close to but not statistically significant. For this determination, HPVPP was the only method used. Alternate methods (intravitral microscopy) and increased numbers may have shown this more conclusively.
DISCUSSION

PHT is a complication of cirrhosis and leads to the formation and hemorrhage of gastric and esophageal varices. Twenty-five to forty percent of patients with cirrhosis have varices at the time of diagnosis, and, despite improvements in therapy, survival rates following an initial variceal bleed are poor. To date, treatment commonly involves nonselective β-blockers and/or endoscopic variceal ligation (EVL) to reduce the risk of bleeding. However, deaths from EVL are unavoidable, and β-blockers are associated with side effects necessitating withdrawal in ~20% of patients. Moreover, increased complications with the use of the β-blocker timolol have recently been shown. As such, the use of β-blockers is not recommended in patients without varices, and new therapies are required. Ultimately, the best way to prevent variceal formation and hemorrhage is to treat the underlying etiology of PHT by reducing hepatic resistance and correcting the systemic hyperdynamic circulation. Previous research has shown that the potent vasodilator NO and the enzyme eNOS are key to the development of PHT. However, the precise role of eNOS in the etiology of PHT is not fully understood. In this article, we examined the development of liver fibrosis, hepatic resis-

Fig. 1. Hepatic collagen staining increased following chronic CCl₄ administration in both wild-type and endothelial nitric oxide synthase (eNOS)−/− mice. A: livers were harvested from wild-type and eNOS−/− mice treated with either mineral oil (i–iv) or 0.6 ml/kg CCl₄ (v–viii) twice weekly for 12 wk. Formalin-fixed, paraffin-embedded liver sections were stained using the Masson’s trichrome protocol to reveal collagen (blue), cytoplasm (red), and nuclei (black). Normal murine hepatic histology was observed in livers from wild-type and eNOS−/− mice treated with mineral oil for 12 wk. Masson’s trichrome collagen staining was sparse and was limited to hepatic portal vein (HPV), bile ducts (BD), lymphatic vessels (LV), centrilobular vein, and hepatic artery (HA) (i–iv). 12-wk administration of CCl₄ to both wild-type and eNOS−/− mice significantly increased collagen staining within the lobule boundary and surrounding the hepatic portal vein, characteristic of precirrhotic bridging fibrosis. Normal portal triad architecture was lost, HA and BD were not identifiable, and the HPV was irregular (v and viii). There was no detectable collagen staining within the hepatic parenchyma, and there was no visually significant difference in collagen-1 staining between the wild-type and eNOS−/−-treated mice. Photomicrographs are ×200 or ×400. B: 10 μM unfixed hepatic cryostat sections were probed for collagen-1 using rabbit anti-collagen-1 and goat anti-rabbit CY5 antibodies. All photomicrographs are ×200 magnification and were obtained using a Bio-Rad MRC1024 laser scanning confocal microscope using a Kr/Ar laser. Collagen staining is shown in red (excitation at 647 nm and emission at 665 nm), whereas tissue autofluorescence is shown in green (excitation at 488 nm and emission at 530 nm). In mineral oil-treated mice, collagen-1 staining was minimal and was localized around the portal triads and centrilobular vein (i and iii). In CCl₄-treated mice, hepatic collagen-1 staining was markedly increased around the portal triads and the lobule border (ii and iv). There was little staining between hepatocytes in parenchyma. There was no visually significant difference in collagen-1 staining between the wild-type and eNOS−/−-treated mice.
Ohm’s law maintains that pressure is equal to resistance multiplied by flow. As such, amelioration of hepatic resistance and hyperdynamic circulation is equally important, assuming that both events occur evenly and simultaneously. This may not be the case, especially in animal models involving targeted gene deletions in which compensatory mechanisms can de-

![Collagen Staining](image1.png)

**Fig. 2.** Collagen staining increased in both wild-type and eNOS−/− mice following chronic administration of CCl4. A and B: Masson’s trichrome collagen staining was semiquantified for hepatic fibrosis using a point-counting procedure. Hepatic collagen staining was increased significantly by chronic CCl4 administration compared with mineral oil (MO) controls in both wild-type and eNOS−/− mice. There was no statistical difference in collagen staining between wild-type and eNOS−/− mice when treated with MO or CCl4. C: unfixed liver cryostat sections from 12-wk MO- and CCl4-treated mice were immunoprobed for collagen-I. Staining was quantified using the fluorescent Cy5 emission intensity at 665 nm after excitation at 647 nm. Hepatic collagen staining was significantly increased in CCl4-treated wild-type and eNOS−/− mice compared with MO-treated mice. Data represent means ± SE, n = 6 per experimental group and >10 fields of view per section (*P < 0.05, MO vs. CCl4).

![Increased Abdominal Aortic Flow](image2.png)

**Fig. 3.** Increased abdominal aortic flow in wild-type (WT) but not eNOS−/− mice following CCl4-induced fibrosis. Blood flow was determined by placing a Doppler flow probe around the abdominal aorta between the diaphragm and the celiac artery or around the portal vein between the coronary vein and the portal venous bifurcation (means ± SE). A: abdominal aortic flow in wild-type mice 0–12 wk following twice weekly administration of mineral oil (open bars) or 0.6 ml/kg CCl4 (shaded bars). Abdominal aortic flow increased progressively over time in the CCl4-treated mice compared with mineral oil-treated shams and was maximal after 12 wk of CCl4 treatment. B: abdominal aortic flow in wild-type and eNOS−/− mice following 12-wk twice weekly administration of mineral oil (open bars) or 0.6 ml/kg CCl4 (shaded bars). 12-wk CCl4 treatment did not increase the abdominal aortic flow in eNOS−/− mice compared with mineral oil controls. C: portal venous flow was significantly increased 12 wk following chronic CCl4 administration in wild-type mice compared with mineral oil controls. There was no significant increase in eNOS−/− mice treated similarly. Data represent means ± SE, n = 5 mice per group. P < 0.05 was considered significant.
develop. In these circumstances, continued validation of the model is required. We found that CCl4-induced fibrosis and hepatic damage in mice are not eNOS dependent. There was no significant difference in collagen accumulation or ALT levels between the wild-type and eNOS−/− experimental groups. This asserts that any disparity in HPVPP, Qao, Qpv, or SPP between wild-type and eNOS−/− mice is not the result of a differential development of fibrosis and increased hepatic resistance. This is because increased hepatic resistance is one of the key drivers of PHT.

In wild-type mice, we found no elevation in HPVPP following chronic CCl4 exposure despite moderate fibrosis. In contrast, the HPVP was increased in eNOS−/− mice treated with CCl4, albeit the increase was not statistically increased compared with comparable mineral oil-treated eNOS−/− mice (P = 0.12) but was significantly increased compared with comparable wild-type CCl4-treated mice (P = 0.012). Therefore, eNOS may potentially be playing a significant role in maintaining normal hepatic resistance during the development of mild/moderate hepatic fibrosis. This goes against previous reports that have documented a reduction in NOS activity in the rat CCl4 model and have argued that reduced eNOS in fibrotic livers increases sinusoidal constriction and increases hepatic resistance. Possible explanations for this disparity include 1) eNOS microlocalization, 2) the possible development of compensatory mechanisms, 3) unmodified endothelin-1 responsiveness, and 4) species and technical differences between this study and others performed in rats.

Cytosolic eNOS may indeed be reduced following chronic CCl4 exposure, whereas membrane-associated eNOS may be unaltered. eNOS translocation from the cytosol to the cell membrane is characteristic for eNOS phosphorylation and activation (18, 22). Once at the membrane, eNOS associates with HSP90 and caveolin-1, whereby it can be activated via AKT phosphorylation. Furthermore, eNOS activity is further regulated by the availability of key cofactors. In particular, eNOS requires tetrahydrobiopterin as an electron donor to produce NO. In the absence of BH4, eNOS becomes “uncoupled” and produces free radicals. Matei et al. recently demonstrated that hepatic BH4 is reduced in CCl4-treated rats and that supplementation of BH4-augmented acetylcholine induced reduction of hepatic resistance (24a). At present, we do not suggest by what mechanism eNOS is influencing hepatic resistance. NO may directly relax differentiated hepatic stellate cells, which take on a smooth muscle cell phenotype in cirrhotic livers. Alternatively, it may be more complicated and linked to its role in free radical scavenging.

As with all gene deletions, there is a potential for alternative protein expression to counteract gene deletion and provide an alternative phenotype. Moreover, eNOS-null mice may manifest adaptive effects such that observed effects are not directly related to an absence of eNOS. For example, cyclooxygenase-1 gene expression is markedly increased in the portal vein of unadulterated eNOS−/− mice compared with wild-type controls (data not shown). Addition of eNOS inhibitors to confirm observations in eNOS−/− mice would strengthen conclusions made in this study. Developed in the Sessa laboratory, cavatrin, a caveolin-1-derived peptide, reportedly acts as a selective eNOS inhibitor and inhibits tumor microvascular permeability and progression with little effect on iNOS (3, 10). Moreover, they found that cavatrin-treated mice had similar responses compared with eNOS−/− mice. However, caveolin-1 is an important component in caveoli formation, and as such cavatrin has the potential to disrupt other signaling processes. Moreover, others have suggested problems with cavatrin permeability because of its relative size (~3 kDa) (13). For these
reasons, cavitratin was not used and this study focused on the genetic abrogation of eNOS. Given the positive data obtained, future studies will evaluate potential of cavitratin to prevent PHT and cavitratin selectivity in eNOS $^{-/-}$ mice and also identify and correct compensatory mechanisms in eNOS $^{-/-}$ mice (such as cyclooxygenase-1).

Endothelin-1 has repeatedly been linked with the development of PHT (31). In experimental models of cirrhosis, a vascular hyporesponsiveness to endothelin-1 has been reported despite an increase in endothelin receptors (ET-A, ET-B) (5, 14). The development of this hyporesponsiveness has been linked to NO expression and increased blood flow (15, 29, 30). Feng et al. (6) recently studied the role of endothelin receptor antagonists in the CCl$_4$ mouse model. They found that intravenous infusion of ET-B receptor antagonism increased portal pressure, whereas ET-A receptor antagonists and mixed endothelin receptor antagonists induced a reduction in portal pressure. In eNOS $^{-/-}$ mice, modified endothelin-1 expression and signaling potentially would increase hepatic stellate cell contractility and hepatic resistance.

Whereas we perfused the livers at a physiological flow rate (50 $\mu$L/min per gram), other studies used a flow rate nearly threefold greater than normal rat Qpv (175 vs. 60 $\mu$L/min per gram, respectively). In our experience of PHT models, hyperemia is limited to one fold increase. This may have serious consequences because flow is known to increase eNOS and NO production (16). Hyperdynamic hepatic perfusion of CCl$_4$-treated mice may overwhelm residual NO production and normal physiological hepatic sinusoidal regulation that would be sufficient under normal perfusion rates. Therefore, changes in resistance would reflect basic changes in fibrosis and would negate any physiological control. This elevation would exaggerate differences seen between CCl$_4$- and vehicle-treated mice (such as cyclooxygenase-1). These observations suggest that, in the CCl$_4$ model, the early development of PHT is primarily driven by eNOS-dependent vascular aberrations concurrent with normal hepatic resistance. Although this model does not directly relate to the perceived etiology of human PHT, it does highlight the importance of cirrhosis-associated vasculopathy. Targeted treatment of these aberrations would reduce portal pressure and collateral circulations. At present, we know that eNOS is a key mediator and that inhibition has the potential to provide significant improvements. However, additional mechanistic studies are required to better understand the trigger, signaling, and mechanisms by which eNOS plays such an integral role in experimental and clinical PHT. These studies would help improve our understanding and potentially advance present treatment paradigms.

REFERENCES


