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ORIGINAL ARTICLE

Basic Study

Murine study of portal hypertension associated endothelin-1 hypo-response

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Abstract

AIM: To investigate endothelin-1 hypo-responsive associated with portal hypertension in order to improve patient treatment outcomes.

METHODS: Wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice received partial portal vein ligation surgery to induce portal hypertension or sham surgery. Development of portal hypertension was determined by measuring the splenic pulp pressure, abdominal aortic flow and portal systemic shunting. To measure splenic pulp pressure, a microtip pressure transducer was inserted into the spleen pulp. Abdominal aortic flow was measured by placing an ultrasonic Doppler flow probe around the abdominal aorta between the diaphragm and celiac artery. Portal systemic shunting was calculated by injection of fluorescent microspheres in to the splenic vein and determining the percentage accumulation of spheres in liver and pulmonary beds. Endothelin-1 hypo-response was evaluated by measuring the change in abdominal aortic flow in response to endothelin-1 intravenous administration. In addition, thoracic aorta endothelin-1 contraction was measured in 5 mm isolated thoracic aorta rings *ex-vivo* using an ADI small vessel myograph.

RESULTS: In wild type and iNOS$^{-/-}$ mice splenic pulp pressure increased from 7.5 ± 1.1 mmHg and 7.2 ± 1 mmHg to 25.4 ± 3.1 mmHg and 22 ± 4 mmHg respectively. In eNOS$^{-/-}$ mice splenic pulp pressure was increased after 1 d (P = NS), after which it decreased and by 7 d was not significantly elevated when compared to 7 d sham operated controls (6.9 ± 0.6 mmHg and 7.3 ± 0.8 mmHg respectively, P = 0.3). Abdominal aortic flow was increased by 80% and 73% in 7 d portal vein ligated wild type and iNOS when compared to shams, whereas there was no significant difference in 7 d portal vein ligated eNOS$^{-/-}$ mice when compared to shams. Endothelin-1 induced a rapid reduction in abdominal aortic blood flow in wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ sham mice (50% ± 8%, 73% ± 9% and 47% ± 9% respectively). Following portal vein ligation endothelin-1 reduction in blood flow was significantly diminished in each mouse group. Abdominal aortic flow was reduced by 19% ± 9%, 32% ± 10% and 9% ± 9% in wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice respectively.

CONCLUSION: Aberrant endothelin-1 response in murine portal hypertension is NOS isoform independent. Moreover, portal hypertension in the portal vein ligation model is independent of ET-1 function.

Key words: Liver disease; Portal hypertension; Hyperdynamic circulation; Endothelin-1; Nitric oxide synthase isoforms

Core tip: Portal hypertension (PHT) is a complication associated with vascular derangements in response to liver disease and fibrosis. Perturbations of nitric oxide (NO) and endothelin-1 are believed to be interrelated and play a key role in PHT vasculopathy. This study investigates the importance of NO biosynthesis in endothelin-1 vasoconstriction hypo-response seen in patients with PHT. PHT was induced in wild type, $eNOS^{-/-}$ and $iNOS^{-/-}$ mice by partial portal vein ligation (PVL) and endothelin-1 contractile response was determined. Endothelin-1 (ET-1) induced contraction was significantly reduced following PVL in all mouse groups. Aberrant ET-1 function associated with PHT is NO independent.

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INTRODUCTION

Portal hypertension (PHT) is a life threatening complication of liver cirrhosis. Elevated portal venous pressure increases morbidity and mortality by promoting the formation and potential hemorrhage of gastric and esophageal varices[1,2]. PHT typically originates from underlying hepatic disease and is exacerbated by systemic and splanchnic vascular deregulation[3]. Hepatic injury stimulates liver stellate cell differentiation to adopt a smooth muscle cell phenotype resulting in sinusoidal contraction, increased sinusoidal perfusion resistance and increased portal pressure[4]. In addition, dilation of systemic and splanchnic vessels causes a hyperdynamic circulatory dysfunction characterized by increased cardiac output and hyperemia[5-8]. Consequently, although the underlying etiology of PHT is usually hepatic, clinical manifestations and intervention pertain more to vascular and cardiac control[9].

Vascular dilators and constrictors play a significant role in controlling blood flow and pressure *via* modulation of vascular resistance to flow[10-13]. Increased vascular resistance reduces flow and increases pressure whereas decreased resistance increases flow and lowers pressure. In patients with PHT reduced vascular resistance increases blood supply to the portal system, which is congested because of increased hepatic resistance, and increases portal pressure. Consequently, reducing cardiac output and increasing vascular resistance has the potential to reduce portal pressure and lower the risk of esophageal variceal hemorrhage. Primarily prophylaxis using β-blockers (*e.g.*, propranolol) to prevent/treat variceal bleeding in cirrhotic patients by the reduction of heart rate by 25% is the mainstay of treatment but does not work for all[14-16]. Moreover, the use of vasoconstrictors such as octreotide demonstrate the potential for vasoconstrictors despite limited reductions in bleeding and no improvement in overall outcomes when compared to direct endoscopic treatments to the varices (banding and sclerotherapy)[17,18]. The introduction of long-acting octreotide analogs may provide better results although care should be taken to avoid renal complications caused by renal hypertension[19,20].

Therefore, to date, options for patients with PHT, whom are at risk of variceal formation and hemorrhage, are limited. One reason for this deficiency in treatment options is due to our lack of knowledge relating to vascular aberrancies concomitant with PHT. Previous studies have demonstrated that PHT is associated with a significant diminution of reactivity to specific vasoconstrictors[21]. Endothelin-1 (ET1), a potent vasoconstrictor, is associated with vascular resistance and blood pressure[22]. Previous studies indicate that synthesis of the potent vasoconstrictor ET1 is increased contemporaneously with PHT[23,24]. Plasma ET1 concentrations are three times higher in patients with cirrhosis than in healthy controls[25]. Subsequent studies showed that despite increased ET1 levels vessels from PHT animals exhibited a markedly reduced contractile response to exogenous ET1[26,27]. In cirrhotic rats mesenteric arterial response to endothelin-1 is markedly reduced concomitant with a 5-fold reduction in ET1 cellular signaling[28]. This reduction in vasoconstrictor response has been linked to elevations in expression of vasodilators prostacyclin (PGI_2) and nitric oxide (NO) which counter ET1 induced vasoconstriction[29-32] because disruption of NO or PGI_2 biosynthesis reduces portal pressure in experimental models of PHT[33-35]. Suggesting that inhibition of vasodilators NO or PGI2 would also ameliorate ET-1 vasoconstriction in PHT animals.

Therein this manuscript examines the importance and etiology of NO biosynthesis in impaired ET1 vasoconstriction associated with PHT. This initial study utilizes the pre-hepatic murine portal vein ligation (PVL) model because PHT develops rapidly and vascular aberrancies have been reported in this model[30]. At present we do not fully know the importance and etiology of ET1 hypo-response in PHT. Previous studies have suggested that NO plays a significant role in ET1 hypo response[36,37]. This is based on data

that shows ET1 hypo-response is not observed when NO synthesis is inhibited[38-41] and the connection between ET1 signaling, *via* endothelin receptor A (ETA) and endothelin receptor B (ETB) receptors, and vasodilatory compounds. (Figure 1)[42] Vascular smooth muscle cell ETA and ETB promotes vasoconstriction *via* phospholipase C, phosphoinositide metabolism and increased Ca^{2+}[43]. Whereas, endothelial cell ETB are functionally coupled with NO and PGI2 biosynthesis and promote vasodilation[44-48]. Therefore, increased levels of NO biosynthesis *via* nitric oxide synthase isoforms could explain ET1 hypo-contractile response. In experimental animal studies ETB antagonism prevents hyperemia and PHT following portal vein ligation[49]. However, testing the role of ETA and ETB in PHT murine models is hindered because, homozygous ETA or ETB receptor gene knock out results in lethal developmental phenotypes in the mouse[50]. In contrast, $eNOS^{-/-}$ and $iNOS^{-/-}$ mice are viable and have previously been used to better understand the pathophysiology of PHT[34,35].

To examine the relationship between eNOS and ET-1 hypo-response this manuscript examines the development of ET1 hypo response and PHT in a murine PVL model of pre-hepatic PHT using $eNOS^{-/-}$ mice. If the hypothesis that eNOS is important to the development of ET1 hyper-response is correct then *eNOS* gene deletion will prevent aberrant ET1 function following PVL. In contrast, we observed that *eNOS* gene deletion enhanced ET1 contraction in sham mice and did not prevent ET1 hypo-response following PVL. In addition we found that aberrant ET1 contractility is not central to the development of PHT in the PVL model and that eNOS mediated hyperemia is key. This data does not negate the role and importance of ET1 in portal hypertension. Normalized ET1 contractility, potentially, would reduce portal venous flow, pressure and bleeding. Our data suggests that targeting of NO biosynthesis would not mediate this affect and alternate targeting and study is required.

MATERIALS AND METHODS

Pre-hepatic PHT model: Partial portal vein ligation

All studies were approved by the Indiana University committee for animal research and adhered to AAALC and federal guidelines for the humane care and treatment of animals. Mice were maintained in sterilized isolette cages on a 12-h light/dark cycle and were allowed access to food and water *ad libitum*. Mice were anesthetized using halothane inhalation. A midline laparotomy was performed and the portal vein was exposed. A blunt-ended 27-gauge needle was placed alongside the portal vein and a 4-0 silk suture was tied around the vein and needle, after which the needle was withdrawn, producing a standardized stenosis. In sham animals the procedure consisted of dissection and visual inspection of the portal vein without ligature. The abdomen was closed and the animals were allowed to recover under a heat lamp.

Physiological measurements

Physiological measurements were performed as previously described by Theodorakis *et al*[34] 2003. At the indicated times post sham-operation or PVL, animals were anesthetized and subjected to laparotomy to allow physiological measurements to be taken. Portal pressure was determined by measuring the splenic pulp pressure (SPP). We have previously shown that portal venous pressure and splenic pulp pressure are directly proportional[34]. To measure SPP, a microtip pressure transducer (ADI, CO) was inserted into the spleen pulp. Abdominal aortic flow (Qao) was measured by placing an ultrasonic Doppler flow probe (Transonic #11RB) around the abdominal aorta between the diaphragm and celiac artery. Flow rates were obtained with a Transonic T206 Blood Flow Meter (Transonic Instruments, NY) and recorded using ADI Chart 5 software. Aortic blood flows were standardized per gram of body weight. Fluorescent microspheres were used to assess the degree of portosystemic shunting as described previously[34]. 0-7 d after sham operation or PVL, mice were anesthetized and a laparotomy was performed as described earlier. Approximately 15 x 10^6 μm red polystyrene fluorescent microspheres (Molecular Probes, Eugene, OR) were injected into the spleen (red spheres). The liver and lungs were collected and placed in 20 mL of 2% sodium dodecyl sulfate, 0.1 mol/L EDTA, 10 mmol/L Tris, pH 8.0, and the tissue was homogenized. Proteinase K was added to 0.1 mg/mL, and the proteins were digested overnight at 45 ℃. Microspheres were collected by centrifugation at 1000 × *g*, washed in 0.2% Tween-80, centrifuged again, and re-suspended in 0.1 mL of 0.2% Tween-80. Microspheres were counted using a hemocytometer and a Nikon TE300 inverted microscope equipped for epifluorescence. The degree of shunting was calculated as the percentage of microspheres in the lungs compared to lung and liver combined.

Gene-deficient mice

Mice containing targeted mutations in the *nos2* gene (iNOS; strain B6,129P-*Nos2*^{tm1Lau},and the *nos3* gene (eNOS, strain C57BL/6J-*Nos3*^{tm1Unc}, were purchased from The Jackson Laboratory, ME. Age-matched mice from congeneic strains (B6, 129P or C57BL/6J) were used as wild type controls. Mice genotypes were confirmed by PCR on DNA isolated from tail samples using Qiagen Dneasy kit (Qiagen Inc, Stamford, CA), as per manufactures instructions. Gene-specific primers: nos3: 5'gtgtgaaggcaaccattctg 3'actcatccatgcacaggacc and nos2: 5'ggcttcacgggtcagagcca 3'tgcccattgctgggacagtc (cycle = 1 min each of 94 ℃, 60 ℃ and 74 ℃ x 25) are complementary to the site specific mutations previously published by Shesely *et al*[51] (1996) and Laubach *et al*[52] (1995).

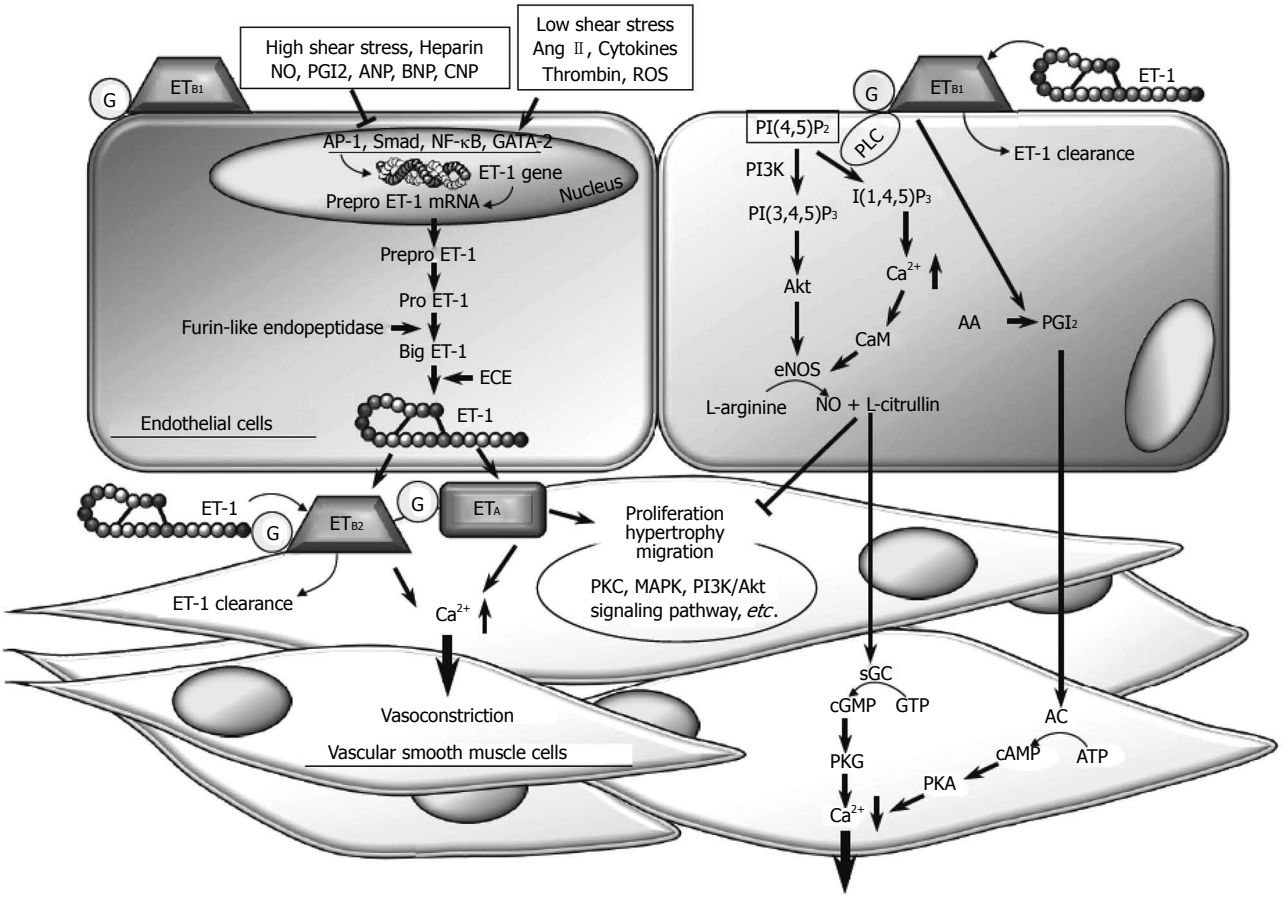


Figure 1 Illustration of interrelationship between Endothelin-1 induced vasoconstriction and nitric oxide mediated vasodilation. Endothelin-1, PGI2 and NO are closely related in relation to vascular smooth muscle cell tone. (Reprinted with permission Ohkita *et al*[42] 2002).

Endothelin-1 function

***In-vivo*:** Seven days Sham and PVL mice were anesthetized using halothane inhalation. A midline laparotomy was performed and the abdominal aorta was exposed. A Doppler flow probe was placed on the aorta between the diaphragm and celiac artery. Flow rates were allowed to stabilize and were monitored while the femoral vein was cannulated. Mice were discarded if Qao decreased by more than 20% during the cannulation. 10 μg/L (4 nmol/L) ET1 bolus (50 μL) was injected in to the femoral vein and the abdominal aortic flow was constantly recorded. The dose of ET1 (5 pmol/kg) was similar to that used in human studies[53]. Except ET1 was given as a bolus rather than *via* a peristaltic pump. Preliminary studies showed that 1hr following 5 pmol/kg ET1 injection plasma nitrite/nitrate (NOx) was increased 41%. Moreover, 4 nmol/L is significantly greater than normal murine plasma ET1 levels (1 pmol/L)[54]. This high dose will equalize out any differences in endogenous ET1 levels between PVL and sham mice.

***Ex-vivo*:** Seven days Sham and PVL mice were anesthetized using halothane inhalation. The abdominal aorta was carefully dissected and placed in oxygenated Krebs-Ringer solution (119 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl, 1.17 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 1.18 mmol/L KH_2PO_4, 27 μmol/L EDTA, 5.5 mmol/L glucose). ET1 contractility was measured on a small vessel myograph (610 M, ADI, CO) as per manufacturer's instructions. This instrument provides a temperature controlled oxygenated environment to measure vessel contractility/dilation. Vessels are attached to two wires (0.2 μm diameter). One wire is an anchor while the other is attached to a strain gauge. Segments were incrementally pre-tensioned by separating the two wires to 100 mmHg, as previously described and according to manufacturer's detailed instructions[55]. Vessel contractility was measured in response to 10^{-8}-10^{-4} mol/L ET1. Four vessel segments were analyzed per mouse, five mice per group.

Statistical analysis

The data shown are mean ± SE, with 5-7 animals per experimental group. Statistical significance was estimated using one-way ANOVA statistical analysis. A value of $P < 0.05$ was considered significant.

RESULTS

Plasma NOx

Plasma NOx and ET-1 were determined using commercially available assays (Oxford Biomedical, Oxford, MI) (R&D systems, Minneapolis, MN). Plasma

NOx and ET-1 were not significantly different between unadulterated C57B/6J (wild type), iNOS$^{-/-}$ and eNOS$^{-/-}$ mice. This is similar to previous studies[34,56]. Plasma NOx was increased 1 d following PVL in wild type (41%, P = 0.03) and iNOS$^{-/-}$ (35%, P = 0.02) but was not altered in eNOS$^{-/-}$ mice (11.7 ± 0.9 *vs* 9.9 ± 0.6 μmol/L, 1 d sham and 1 d PVL respectively, P = 0.31). Similarly serum ET-1 increased 1 d following PVL (1 ± 0.7 pg/mL *vs* 12 ± 3 pg/mL 1 d sham and PVL respectively, P = 0.02). After which levels returned to normal (2 ± 1.1 P = 0.23) and were not different to shams. In a similar manner serum ET-1 was increased 9 and 10 fold in eNOS$^{-/-}$ and iNOS$^{-/-}$ mice respectively 1 d following PVL.

Hemodynamics following PVL

The development of PHT was evaluated by recording (1) splenic pulp pressure; (2) aortic flow; and (3) portal systemic shunting as indices of portal pressure, hyperdynamic and collateral circulation respectively.

Splenic pulp pressure was quantified by placing a micro tip pressure transducer into the spleen: The spleen/body weight ratio was significantly increased in all wild type, iNOS, and eNOS mice 7 d following PVL (85%, 90% and 111% respectively). In wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice splenic pulp pressure was increased immediately following ligation of the portal vein. In wild type and iNOS$^{-/-}$ mice splenic pulp pressure increased from 7.5 ± 1.1 mmHg and 7.2 ± 1 mmHg to 25.4 ± 3.1 mmHg and 22 ± 4 mmHg respectively (Figure 2A and B). In eNOS$^{-/-}$ mice splenic pulp pressure was increased after 1 d (P = NS), after which it decreased and by 7 d was not significantly elevated when compared to 7 d sham operated controls (6.9 ± 0.6 mmHg and 7.3 ± 0.8 mmHg respectively, P = 0.3) (Figure 2C). This is similar to data reported previously[34].

Abdominal aortic flow (Qao) was measured by placing a doppler flow probe around the aorta between the diaphragm and celiac artery: Heart rate was not significantly altered by PVL in any mouse groups (data not shown). Immediately after portal vein ligation Qao reduced rapidly (0.15 ± 0.02 mL/min per gram *vs* 0.12 ± 0.01 mL/min per gram BW 1 d wild type sham and PVL respectively), decreasing by 20%, 22% and 30% in wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice respectively. Two days following PVL Qao had recovered and was greater in PVL mice when compared to shams in wild type and iNOS$^{-/-}$ mice (0.17 ± 0.02 mL/min per gram and 0.18 ± 0.04 mL/min per gram BW). By 7 d Qao had increased by 80% and 73% in wild type and iNOS (Figure 2D and E), whereas there was no significant difference in eNOS$^{-/-}$ mice between 7 d sham and 7 d PVL (Figure 2F).

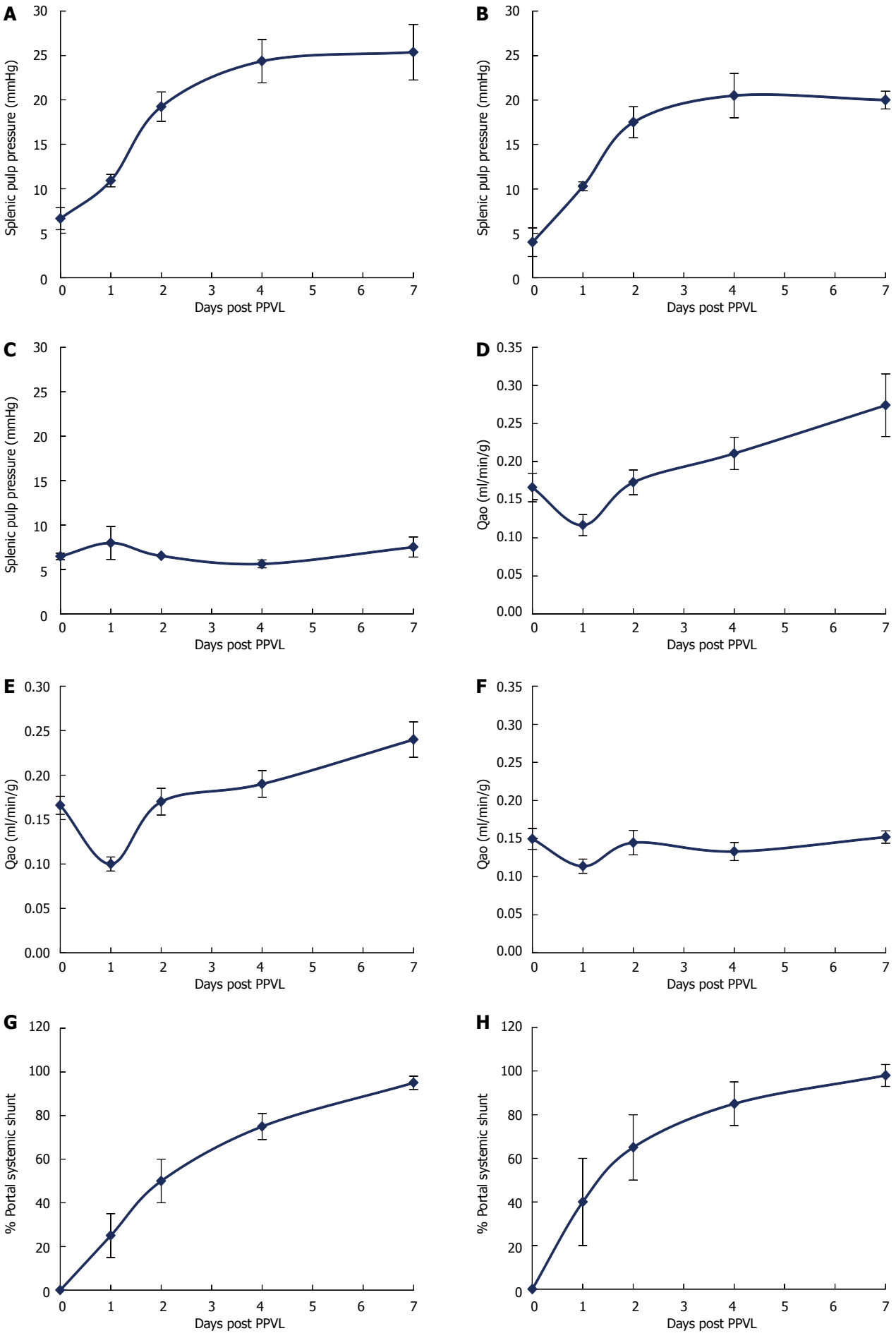
Portal systemic shunting was determined by injecting fluorescent microspheres in to the splenic vein *via* the spleen and monitoring the distribution of spheres between the lung and liver: In sham mice spheres were exclusively found in the liver, indicating normal circulation and no shunting. Following PVL sphere location changed from predominantly hepatic to predominantly pulmonary, indicative of collateral circulation. The rate of collateral circulation development was steady in wild type and iNOS$^{-/-}$ mice. (Figure 2G-H) However, in eNOS$^{-/-}$ mice the rate of collateralization was significantly slower, (Figure 2I) suggesting that acute collateralization is eNOS dependent but that alternate mechanisms are also involved.

ET1 induced aortic contractility

To better understand the interrelationship between ET1 and NO we initially performed a dose response experiment to optimize ET1 induced NO synthesis. This would confirm ETB binding and investigates vessel ET1 pressor response in the face of both constrictive and dilatory mechanisms. *In-vivo* ET1 dose was determined by monitoring plasma NOx levels following exogenous 50 μL bolus (0-20 μg/L) ET1 IV injection. Plasma NOx was increased 1hr following 2.5 (23%, P = 0.1) and 5 pmol/kg (41%, P = 0.04) ET1 IV injection. In contrast, IV injection of 10 pmol/kg ET1 reduced NOx 55% (P < 0.01). 5 pmol/kg ET1 was subsequently used for *in-vivo* experiments. Aortic response to ET1 was determined by (1) *in-vivo* monitoring of aortic blood flow; and (2) *ex-vivo* monitoring of isolated aorta segment contractility.

Aortic *in-vivo* response to ET1 was determined by monitoring abdominal aortic blood flow following IV injection of 50 μL 10 μg/L ET1 *via* the femoral vein: Bolus IV injection of 50 μL 10 μg/L (4.8 pmol/kg) ET1 to wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice induced a rapid reduction in abdominal aortic blood flow (50% ± 8%, 73% ± 9% and 47% ± 9% respectively). The ET1 induced reduction in flow was significantly greater in eNOS$^{-/-}$ mice compared to both wild types and iNOS$^{-/-}$ mice (P = 0.02) (Figure 3A). 7 d following PVL ET1 induced reduction in blood flow was significantly diminished in each mouse group. Abdominal aortic flow was reduced by 19% ± 9%, 32% ± 10% and 9% ± 9% in wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice respectively (Figure 3B). Aberrant ET1 function was significantly greater in iNOS$^{-/-}$ mice (81%) when compared to wild type (62%) or eNOS$^{-/-}$ (67%). The dose of ET1 is non-physiological and was used because it elicited a detectable increase in plasma NOx indicative of ET-B activation. Moreover, the dose given is equivalent to that used in human studies (4.8 pmol/kg)[53]. No change in heart rate was observed following ET1 injection.

Vessel *ex-vivo* response to ET1 was determined in isolated abdominal aortic segments attached


A
Splenic pulp pressure (mmHg)
Days post PPVL
B
Splenic pulp pressure (mmHg)
Days post PPVL
C
Splenic pulp pressure (mmHg)
Days post PPVL
D
Qao (ml/min/g)
Days post PPVL
E
Qao (ml/min/g)
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F
Qao (ml/min/g)
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G
% Portal systemic shunt
Days post PPVL
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% Portal systemic shunt
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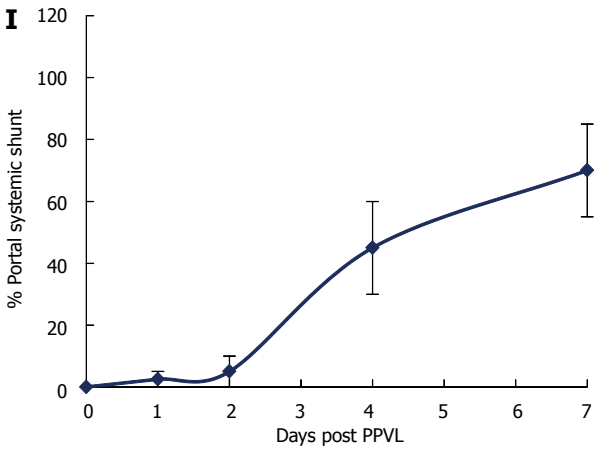

Figure 2 Partial portal vein ligation induces chronic hyperemia and persistent portal hypertension in wild type and iNOS$^{-/-}$ mice but not eNOS$^{-/-}$ mice. Wild type (A, D, G), iNOS$^{-/-}$ (B, E, H) and eNOS$^{-/-}$ (C, F, I) mice were subjected to partial portal vein ligation surgery. 0-7 d thereafter-splenic pulp pressure (A-C), aortic blood flow (D-F) and portal systemic shunting (G-I) were determined. A-C: Splenic pulp pressure was increased acutely in all mouse groups following ligation (0-1 d). After which pressure was increased further in wild type and iNOS$^{-/-}$ but not in eNOS$^{-/-}$ mice; D, E: Aortic flow was significantly reduced in wild type, iNOS$^{-/-}$ and eNOS$^{-/-}$ mice (0-1 d). In wild type and iNOS$^{-/-}$ mice this low blood flow converted to hyperemia and increased steadily. In eNOS mice flow returned to pre-surgical baseline and was not increased; G-I: Portal systemic shunting increased steadily in wild type and iNOS$^{-/-}$ mice (G, H). There was a significant delay in the development of collateral circulation in eNOS$^{-/-}$ mice (I).

to a force transducer within an isolated tissue bath: Four segments per mouse and 5 mice per group were assayed. Vessels were pre- tensioned to 100 mmHg and equilibrated for 20 min. Vessels were exposed to logarithmic increases in ET1 (10^{-8}-10^{-4} mol/L). Maximal vessel contractility was recorded. Wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mouse aortic segments increased tension steadily in response to ET1 (Figure 3C). There was no significant difference between the ET1 aortic contractile response of wild type, eNOS and iNOS mice at doses between 10^{-8} and 10^{-5} mol/L. However, at increased doses aortic vessels from unadulterated eNOS$^{-/-}$ mice contracted significantly greater (43%, P = 0.03) then wild-type controls. 7 d following PVL contraction to 10^{-4} mol/L ET1 was reduced from 4.2 ± 0.32 to 1.7 ± 0.7 mmol/L in wild type mice, 6 ± 0.6 to 1.8 ± 0.1 mmol/L in eNOS$^{-/-}$ mice and 4.9 ± 0.7 to 1.5 ± 0.2 mmol/L in iNOS mice$^{-/-}$ (Figure 3D).

DISCUSSION

The study described in this manuscript focuses on the role of eNOS in ET-1 hypo response associated with PHT. Previous reports introduced the hypothesis that ET1 hypo-response was linked to NO and/or hyperemia[40,49,57]. This study tests this hypothesis and the potential of targeting NO biosynthesis to reduce portal pressure, variceal formation and hemorrhage. This is important because eNOS and NO are known to be important to PHT and are the basis for intervention in numerous studies[11,58,59]. To investigate the role of eNOS in PHT we hypothesized that eNOS is important to the development of ET1 hypo response and that eNOS gene deletion would prevent aberrant ET1 function in murine models. The hypothesis was challenged using the well-established pre-hepatic partial PVL of PHT and targeted eNOS and iNOS gene deleted mice. The PVL model was used because reduced extra-hepatic arterial ET1 contractile response is known to develop rapidly in this model in the absence of the milieu of inflammatory and cytokine changes associated with the carbon tetrachloride or bile duct ligation models of intra-hepatic PHT[60]. This allows us to focus on the ET1 vasculopathy in isolation from hepatic pathology. iNOS$^{-/-}$ mice were included as an isoform and gene deletion control. In both the PVL and CCl_4 models of PHT iNOS$^{-/-}$ mice develop PHT similar to wild type controls, including increased plasma NOx, hyperemia, and increased splenic pulp pressure. In contrast, eNOS$^{-/-}$ mice didn't develop hyperemia or PHT 7-14 d following PVL.

In this study we found that eNOS gene deletion increased ET1 contractility. This increase in ET1 contraction in sham eNOS$^{-/-}$ mice is probably due to absent eNOS mediated NO biosynthesis and dilation to counteract ET1 contraction *via* ETA receptor activation. However, contrary to our hypothesis we observed ET1 hypo response in eNOS$^{-/-}$ mice following PVL, suggesting that ET1 hypo-response in murine models of PHT it is a parallel occurrence rather than a pivotal component of PHT and has no distinguishable role in hyper-dynamic associated hyperemia. Therefore, our hypothesis that *eNOS* gene deletion would prevent aberrant ET1 function was false. At this point we suggest that alternative explanations for the development of ET1 hypo-response in eNOS$^{-/-}$ mice include: (1) reduced blood flow, observed immediately following PVL (0-1 d), may increase ET1 expression and modify ET1 response. Previous studies have shown increased ET1 expression following occlusion of portosystemic shunts in cirrhotic patient[61]; (2)

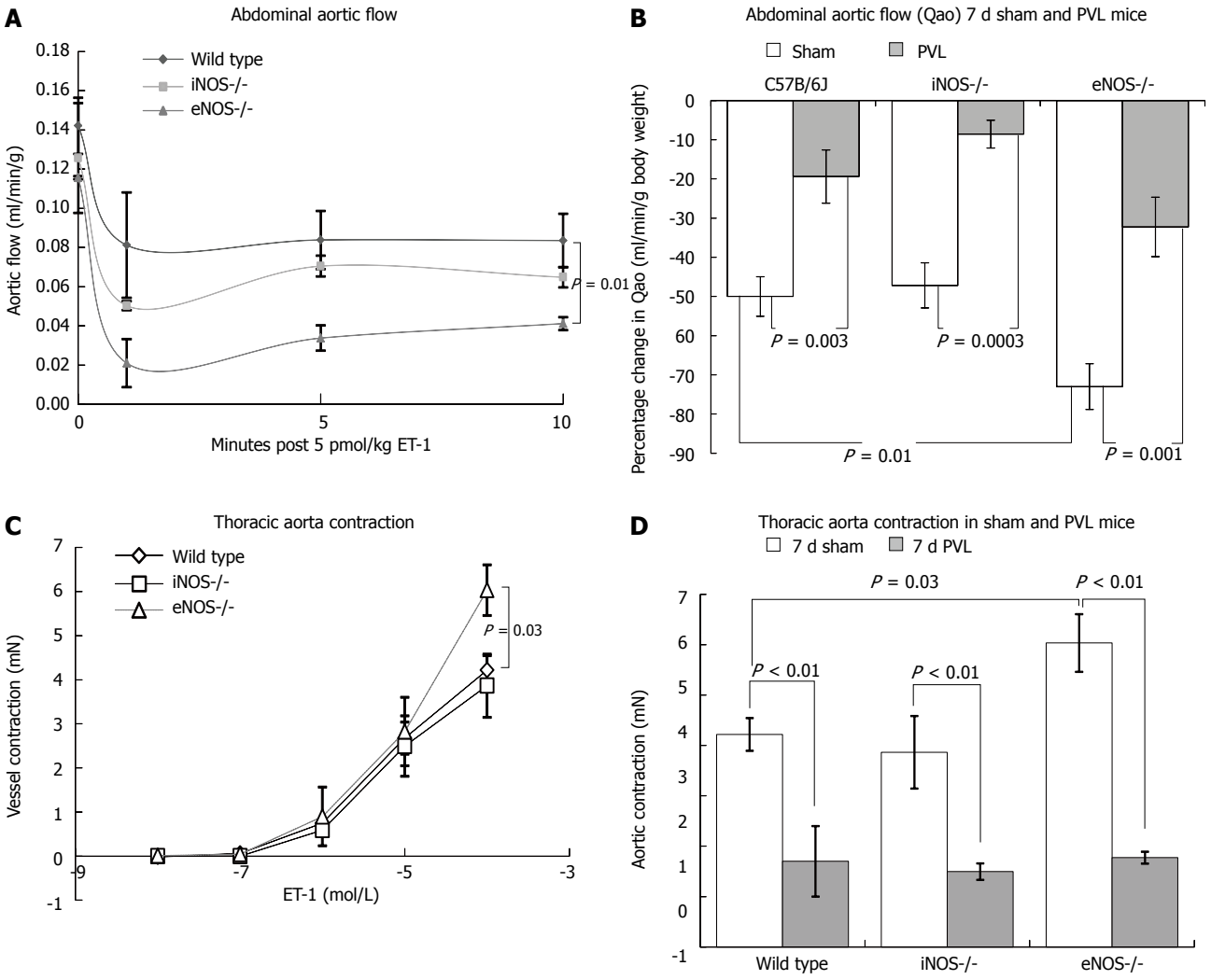

Figure 3 Endothelin-1 hypo-response develops in wild type, iNOS$^{-/-}$ and eNOS$^{-/-}$ mice following portal vein ligation. A: Aortic blood flow was monitored in unadulterated wild type (squares), iNOS$^{-/-}$ (diamonds) and eNOS$^{-/-}$ (triangle) mice prior to and following IV administration of 5 pmol/kg endothelin-1 (ET1). ET1 induced a rapid vessel contraction and subsequent reduction in flow. Response to ET1 was significantly greater in eNOS$^{-/-}$ when compared to wild type controls; B: Wild type, iNOS$^{-/-}$ and eNOS$^{-/-}$ mice were subjected to sham (open bars) or portal vein ligation surgery (PVL) (shaded bars). After 7 d changes in aortic flow was recorded following IV administration of 5 pmol/kg ET1. In all mouse groups the response to ET1 was markedly reduced following PVL (iNOS > wild type > eNOS$^{-/-}$); C: ET1 induced contraction of isolated aortic segments from unadulterated wild type (triangle) iNOS$^{-/-}$ (square) and eNOS$^{-/-}$ (triangle) mice were determined using an ADI 610M small animal myograph. Aortic vessel segments contracted to exogenous ET1. At high ET1 dose (10^{-4} mol/L) aortic vessel segments from eNOS$^{-/-}$ mice contracted significantly greater then segments from wild type controls; D: Wild type, iNOS$^{-/-}$ and eNOS$^{-/-}$ mice were subjected to sham (open bars) or portal vein ligation surgery (shaded bars). After 7 d the aorta was carefully dissected and ET1 contractility was measured. *Ex-vivo* aorta ET1 (10^{-4} mol/L) contractility was significantly decreased in vessels from 7 d wild type, iNOS$^{-/-}$ and eNOS$^{-/-}$ PVL mice when compared to shams (PVL *vs* sham, $P < 0.01$).

rapid engorgement of the portal vein by PVL activates stretch receptors in endothelial cells leading to signaling and phenotypic changes[62]; (3) formation of free radical and oxidative damage. Cell stretching, low blood flow and ET are all linked to the formation of oxygen free radicals[63-65]. Free radicals are linked to up-regulation of NO, which acts as a superoxide radical scavenger, and increases of splanchnic blood flow[66-68]. This would explain why ET1 hypo response was greater in iNOS$^{-/-}$ mice following PVL as iNOS has frequently been linked with NADPH oxidase[69]; (4) alternate NOS isoforms may play a key role. Although, normally associated with neurons NOS1 or neural NOS is increased in the mesenteric artery of PVL rats[70]. Addition studies are required to better understand the etiology of aberrant ET1 response in PHT; and (5) finally, as with all experimental models, especially using gene-modified mice, data should be used with caution. Firstly, murine models are not 100% comparable to human disease. This is especially true of the PVL model, which does not mimic the underlying causation of the majority of patients with PHT. PVL is a model of pre-hepatic PHT, which is usually caused by thrombus, or malignancies encroaching upon the portal vein. However, the PVL is a "clean" model in that it doesn't include hepatic pathology and allows for investigation of PHT vasculopathy in the absence of a pro-inflammatory background. Secondly, alternate mechanisms can compensate for gene deletion. eNOS and iNOS null mice may manifest adaptive effects

such that they may not produce outcomes as a direct consequence of a lack of eNOS or iNOS function. An alternative to using eNOS$^{-/-}$ mice would be to use the reported selective eNOS inhibitor cavtratin, a caveolin-1 derived peptide, developed within the Sessa laboratory[71,72]. Although, cavtratin is reported to inhibit eNOS with little effect on iNOS a question has been raised regarding its solubility and applicability because of its size (3 kDa)[73]. Finally, we measured ET1 response in abdominal aortic vessels rather than in mesenteric vessels used in other studies[30]. This was because of size restrictions. We were unable to isolate responsive mesenteric vessels from either sham of PVL mice. However, are able to demonstrate ET1 hypo-responsive in the abdominal aorta of PVL mice when compared to shams. Demonstrating that in PVL mice the abdominal aorta reaction to ET1 is reduced in a similar manner to mesenteric vessels of PVL rats in contrast to increased reaction to ET1 observed in the thoracic aorta of PVL rats[30,74].

Ultimately, the data presented in this manuscript suggests that targeting eNOS would not abrogate ET1 hypo-response even though previous studies have suggested a link between ET1 and eNOS *via* ETB. This does not refute the positive results seen with ET1 receptor antagonists. Correcting ET1 function has a significant role in the treatment of PHT and prevention of variceal formation and hemorrhage. Selective and non-selective ET-A and ET-B antagonists have significant potential in the treatment of various pathophysiological components of PHT[53]. However questions remain whether such antagonists should be used clinically to treat PHT because of differences between hepatic and extra hepatic outcomes. ET1 response is increased in the liver but is decreased in the systemic vasculature. Moreover, ET-B receptors on vascular smooth muscle can contribute to vasoconstriction in some circumstances and/or locations. Consequently, alternate targets are required that focus on the etiology of ET1 hypo response and receptor downstream changes. However, correcting vascular dysfunction following prolonged inflammatory liver disease might be more complicated than removing the etiological trigger. Wang *et al*[75] (2004) argue that in patients with chronic portal vein hypertension the vascular wall changes, due to the long-term dilation, and recovery will be hard even if the effect of vasodilatation is completely eliminated. More recently, Resch *et al*[76] have described mesenteric arterial remodeling, leading to decreased vessel stiffness, in the CCl_4 model of PHT. In contrast, they found no evidence of vascular remodeling in the rat PVL model of PHT, suggesting that irreversible changes are more likely a response to an inflammatory milieu and not as a consequence of mechanical changes (PVL) or increased NO biosynthesis. Because ET1 hypo response developed in the absence of an inflammatory response and was distant from mechanical/hemodynamic (stretch and low flow in the portal vein) changes it is probably in response to paracrine signaling. Further studies are required to better understand this paracrine signaling.

In conclusion, ET1 dysfunction occurs in the absence of increased NO, chronic liver disease, hyperemia or vascular remodeling and is eNOS and iNOS independent. Moreover, in the PVL model ET1 hypo-response is not sufficient, on its own, to induce a hyper-dynamic circulation or an increase in portal pressure. However, improved ET1 contractility may improve clinical options and thus decrease mortality and morbidity. Additional studies are required to determine the etiology, role and correction of ET1 hypo-response in PHT.

COMMENTS

Background

Portal hypertension is a significant complication of liver disease and can increase morbidity and mortality. Increased hepatic resistance in portal venous flow in combination with elevated portal venous flow raises portal venous pressure and promotes the vascular aberrancies and hemorrhage. Attempts to reduce portal pressure by increasing vascular resistance using vasoconstrictors are hindered by the development of a vascular hypo-response to vasoconstrictors, such as endothelin-1 (ET1). This hypo-response has been linked to increased levels of the vasodilator nitric oxide.

Research frontiers

Current beta-blocker treatment of patients with portal hypertension is problematic. Some patients do not respond and systemic blood pressure is not lowered. Others develop complications and have to terminate treatment. Consequently, alternative approaches are being sort. Amelioration of vascular response to vasoconstrictors in patients with portal hypertension would significantly improve treatment, morbidity and mortality.

Innovations and breakthroughs

By using targeted gene deletion mice this study advances our cognizable knowledge of portal hypertension. Previous studies have suggested that hypo-response to vasoconstrictors is related to an increase in the biosynthesis of the vasodilator nitric oxide. Arguing that inhibition of nitric oxide synthase will ameliorate vascular response to vasoconstrictors. In contrary to this hypothesis we demonstrate that the development of a hypo-response to vasoconstrictors is not due to over production of the vasodilator nitric oxide. Targeted gene deletion of the two main nitric oxide synthase enzymes did not ameliorate vasoconstrictor hypo-response. This information directs future study to look at alternate pathways and mechanisms other than nitric oxide.

Applications

This study guides future investigations aimed towards the development of new treatment options for patients with portal hypertension and are at risk of variceal formation/hemorrhage. By demonstrating that ET1 hypo-response is independent of NOS isoforms alternate approaches can be researched.

Terminology

Vasoconstrictor hypo-response is a condition where vascular tissues have a reduced or absent constrictive response to vasoconstrictors. The use of vasoconstrictors to increase vascular resistance and reduce flow is impaired in patients with portal hypertension. Inter alia, because of ET1 hypo-response.

Peer-review

In this study, the authors examined the importance and etiology of impaired ET1 vasoconstriction in portal hypertension. They used portal vein ligation a prehepatic model of portal hypertension that lacks the milieu of inflammatory and cytokine changes associated with the CCl_4 or bile duct ligation models of intra-hepatic portal hypertension. Although this prehepatic model of portal hypertension somewhat simplifies the complexity of involved pathways, however, it is radically different from the more clinically relevant model of CCl_4 model.

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