ET-1 and TNF-α in HPS: analysis in prehepatic portal hypertension and biliary and nonbiliary cirrhosis in rats

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Luo, Bao, Lichuan Liu, Liping Tang, Junlan Zhang, Yiqun Ling, and Michael B. Fallon. ET-1 and TNF-α in HPS: analysis in prehepatic portal hypertension and biliary and nonbiliary cirrhosis in rats. Am J Physiol Gastrointest Liver Physiol 286: G294–G303, 2004; 10.1152/ajpgi.00298.2003.—Common bile duct ligation (CBDL) triggers a molecular cascade resulting in the hepatopulmonary syndrome (HPS). Both increased hepatic endothelin-1 (ET-1) production and pulmonary vascular ETB receptor expression with stimulation of endothelial nitric oxide synthase and TNF-α mediated inducible nitric oxide synthase and heme oxygenase-1 expression in pulmonary intravascular macrophages occur. Whether biliary cirrhosis is unique in triggering ET-1 and TNF-α alterations and HPS is unknown. We evaluated for HPS in rat prehepatic portal hypertension [partial portal vein ligation (PVL)], biliary (CBDL) and nonbiliary [thioacetamide treatment (TAA)] cirrhosis, and assessed ET-1 infusion in normal and PVL animals. Control, PVL, CBDL, TAA-treated, and ET-1-infused PVL animals had ET-1 and TNF-α levels measured and underwent molecular and physiological evaluation for HPS. HPS developed only in biliary cirrhosis in association with increased plasma ET-1 and TNF-α levels and the development of established molecular changes in the pulmonary microvasculature. In contrast, PVL did not increase ET-1 or TNF-α levels and TAA treatment increased TNF-α levels alone, and neither resulted in the full development of molecular or physiological changes of HPS despite portal pressure increases similar to those after CBDL. Exogenous ET-1 increased TNF-α levels and triggered HPS after PVL. Combination of ET-1 and TNF-α overproduction is unique to biliary cirrhosis and associated with experimental HPS. ET-1 infusion increases TNF-α levels and triggers HPS in prehepatic portal hypertension. ET-1 and TNF-α interact to trigger pulmonary microvascular changes in experimental HPS.

Partial portal vein ligation; common bile duct ligation; thioacetamide; nitric oxide synthase; endothelin B receptor

Hepatopulmonary syndrome (HPS) occurs in a subset of patients with liver disease and/or portal hypertension and results from vasodilatation in the pulmonary microcirculation (22). The syndrome is characterized by arterial hypoxemia and in humans is found across many etiologies of liver disease (9). Nitric oxide (NO) overproduction appears to play a role in the development of intrapulmonary vasodilatation in human HPS (7, 41, 42, 45). However, what triggers the increase and how the alterations relate to the type and severity of liver disease, portal hypertension, and TNF-α overproduction has not been defined.

Chronic common bile duct ligation (CBDL) in the rat is the only recognized model system for the study of HPS (10, 11). In this model, biliary cirrhosis is associated with an early increase in pulmonary endothelial NO synthase (eNOS) levels followed by increased inducible NOS (iNOS) and heme oxygenase-1 (HO-1) expression in accumulated pulmonary intravascular macrophages. These alterations lead to the development and increasing severity of intrapulmonary vasodilatation and gas exchange abnormalities analogous to human HPS (3, 10, 11). Increased hepatic production and plasma levels of endothelin-1 (ET-1) accompanied by pulmonary vascular ETB receptor overexpression are seen early after the onset of CBDL and appear to trigger increases in pulmonary eNOS levels and the onset of HPS (27, 28, 50). Bacterial translocation, leading to TNF-α-mediated increases in pulmonary iNOS and HO-1 expression, is also an important factor in the development of vasodilatation (37). Prehepatic portal hypertension induced by partial portal vein ligation (PVL) is used as a control model, because these animals develop a similar degree of portal hypertension and hyperdynamic circulation to CBDL animals but do not develop HPS (11).

Whether biliary cirrhosis is unique in stimulating hepatic production and release of ET-1 and initiating the onset of HPS is undefined. Specifically, no studies have evaluated whether established models of hepatocellular injury leading to cirrhosis may also result in experimental HPS and how plasma TNF-α levels, the degree of portal hypertension, and ET-1 production relate to the molecular and physiological alterations of HPS. The two most widely used rodent models of toxic hepatocellular injury leading to cirrhosis are induced by chronic administration of either carbon tetrachloride (13, 35) or thioacetamide (TAA) (15, 17, 44). Carbon tetrachloride administration has been associated with direct pulmonary toxicity including fibrosis (13, 35), whereas TAA administration has not. Therefore, the present study was undertaken to assess the role of ET-1 in the molecular and physiological changes associated with experimental HPS by evaluating partial PVL, biliary, and TAA-induced cirrhosis.

Materials and methods

Animals. Male Sprague-Dawley rats (200–250 g, Charles River, Wilmington, MA) were used in all experiments. CBDL and PVL were performed as described (6, 8). Normal control animals underwent mobilization of the common bile duct or portal vein without ligation. Some rats were intraperitoneally injected with 200 mg/kg body wt of TAA (Sigma-Aldrich, St. Louis, MO) or saline 3 times each week for 2 or 8 wk as described (17, 44). Some rats were infused with synthetic ET-1 (Peninsula Laboratories, Belmont, CA) at 3 ng kg−1 h−1 or saline by miniosmotic pump (ALZET model 2002; ALZA, Palo Alto, CA) into the femoral vein for 2 wk in normal and PVL animals on the basis of plasma ET-1 concentrations in 2-wk

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CBDL animals as described (27, 50). Five to eight animals from each group (controls, 3-wk PVL, 2- and 5-wk CBDL, 2- and 8-wk TAA treated, and 2-wk ET-1-infused normal and PVL) were used. Plasma, tissue, and physiological measurements were similar in sham PVL, sham CBDL, and saline-treated controls, and measurements were pooled for analysis. All animals had hepatic biochemical and histological analysis, measurements of portal venous pressure, lung wet and dry weights, and pulmonary histological analysis as published (10, 11, 27, 50). Blood, liver, and lung tissues were obtained from each animal. The study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and conforms to National Institutes of Health guidelines on the use of laboratory animals.

Arterial blood gas analysis. Arterial blood was drawn from the femoral artery as described (11) via extension tubing to ensure that values reflected resting arterial gas exchange. Arterial blood gas analysis was performed on an ABL 520 radiometer (Radiometer America, Westlake, OH) in the Clinical Laboratory, University of Alabama at Birmingham Hospital (Birmingham, AL). The alveolar-arterial oxygen gradient was calculated as 150 – (Paco2/0.8) – Paco2.

Microsphere protocol. The pulmonary microcirculation was evaluated by using an established technique (11). Cross-linked (2.5 × 10⁷) colored polystyrene-divinylbenzene microspheres (size range, 5.5–10 μm; International Medical Technological, Irvine, CA) were injected through a femoral vein catheter, after removing an aliquot of microspheres, to verify the numbers and sizes of microspheres injected. A blood sample withdrawn from a femoral arterial catheter beginning at the time of femoral vein injection measured microspheres passing through the lung microcirculation. Numbers and sizes of microspheres were assessed by using a Leitz Laborlux microscope (Wetzlar, Germany) with a color video imaging and digital analysis system (Image Pro 5.0; Media Cybernetics, Silver Spring, MD) and counted directly. Total numbers of microspheres passing through the microcirculation were calculated as arterial blood sample microspheres/milliliter × estimated blood volume (24). Intrapulmonary shunting was calculated as an intrapulmonary shunt fraction (%) = (total numbers of microspheres passing through the pulmonary microcirculation/total microspheres injected into the venous circulation) × 100.

Determination of plasma TNF-α and endotoxin concentrations. Plasma TNF-α levels were measured with a commercially available solid-phase sandwich enzyme-linked immunosorbent assay according to the protocol supplied by the manufacturer (R&D Systems, Minneapolis, MN). Standards and samples were incubated with a TNF-α antibody-coated 96-well microtiter plate. An enzyme-linked polyclonal antibody specific for rat TNF-α was then added after washing. The intensity of the color was measured in a microplate reader (Molecular Devices, Sunnyvale, CA).

Endotoxin concentrations were measured by a Limulus amebocyte lysate test. Plasma samples were serially diluted with sterile endotoxin-free water and heat-treated to destroy inhibitors that can interfere with activation. Endotoxin content was determined as described by the manufacturer (QCL-1000 kit; Bio Whittaker, Walkersville, MD). Endotoxin standards were tested in each run and the concentration of endotoxin in the test samples was calculated by comparison with the standard curve.

ET-1 radioimmunoassay. Plasma and tissue ET-1 concentrations were measured via radioimmunoassay (RIA: Phoenix Pharmaceuticals, Mountain View, CA). Extraction of ET-1 from plasma was accomplished by acidification and elution over Sep-Pak C₁₈ columns (Waters, Milford, MA). Liver and lung tissues were homogenized and prepared as previously described (27). Samples were reconstituted in RIA buffer and subjected to RIA with the use of a rabbit ET-1 antiserum. Recovery from the Sep-Pak C₁₈ columns averaged 90%, and the sensitivity of the assay for ET-1 was 1.5–2.0 pg.

Western blot analysis. eNOS, ET₁ receptor, iNOS, HO-1, or ED1 protein levels were measured in lung as described (10, 27, 28). Equal concentrations of protein from lung were fractionated on Tris/HCl-ready gels (Bio-Rad Laboratories, Hercules, CA) and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Incubation with antibodies to eNOS (Transduction Laboratories and Pharmingen, Lexington, KY), ET₁ receptor (Calbiochem-Novabiochem, San Diego, CA), iNOS (Transduction Laboratories and Pharmingen), HO-1 (StressGen Biotechnologies, Victoria, BC, Canada), or ED1 (Serotec, Raleigh, NC), a general macrophage and monocyte marker, was followed by the addition of horseradish peroxidase-conjugated secondary antibodies and detection with enhanced chemiluminescence (Amersham). Autoradiographic signals were assessed by using an Astra scanner (UMAX, Fremont, CA) and quantitated with ImagePC software (Scion, Frederick, MA). Signal intensity was shown to be a linear function of sample concentration over the range analyzed.

Liver histology and lung immunohistochemistry. Liver samples were fixed in 10% neutral buffered formalin solution, and lung samples were fixed in 4% paraformaldehyde. Paraffin-embedded tissues were sectioned at 5 μm. Liver sections were stained with Masson’s trichrome stain (14). Lung staining was performed as described (32). After preparation and blocking, sections were incubated with ET₁ receptor (Calbiochem-Novabiochem), iNOS (Transduction Laboratories and Pharmingen) or ED1 (Serotec) antibodies; washed; and incubated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA). After peroxidase-labeled streptavidin (Signet Laboratories, Dedham, MA) and diaminobenzidine (Biogenex, San Ramon, CA) development, sections were photographed by using an axiophot microscope (Nikon, Melville, NY). Control sections were incubated with secondary antibody alone.

Statistics. Data were analyzed by using Student’s t-test or ANOVA with Bonferroni correction for multiple comparisons between groups. Measurements are expressed as means ± SE. Statistical significance was designated as P < 0.05.

RESULTS

Physiological assessment for HPS in prehepatic portal hypertension and biliary and nonbiliary cirrhosis. To evaluate the development of HPS in models of cirrhosis and portal hypertension, we measured arterial blood gases and performed in vivo analysis of pulmonary microvascular size using a well-characterized microsphere technique (11, 50) (Table 1). Hepatic histology was assessed in each animal (Fig. 1). PVL animals developed significant portal hypertension with normal hepatic histology. CBDL animals developed progressive hepatic injury culminating in biliary cirrhosis, which resulted in a degree of portal hypertension similar to PVL animals. TAA-treated animals also developed progressive hepatic fibrosis and portal hypertension and had micronodular cirrhosis by 8 wk. Severity of hepatic dysfunction in 2- and 5-wk CBDL animals was greater than PVL and TAA-treated animals on the basis of plasma liver biochemical measurements. Blinded pathological evaluation of lung histological sections and measurement of lung wet-to-dry weight ratios did not reveal the presence of significant lung injury or edema in any model.

Arterial blood gases and pulmonary microvascular evaluation (Table 1) revealed a progressive increase in the alveolar-arterial oxygen gradient and significant intrapulmonary shunting in CBDL animals, but not PVL animals, indicating the development of HPS after CBDL as observed previously (10, 11). There was a trend toward an increase in the alveolar-arterial oxygen gradient within 2 wk after TAA treatment related to a mild respiratory alkalosis although this did not reach statistical significance. This was not accompanied by a decline in the arterial PO₂ or an increase in the alveolar-arterial arterial-arterial oxygen gradient.
oxygen gradient to >15 mmHg in contrast to findings in CBDL and human HPS (10, 11, 40). Microsphere analysis confirmed that the passage of sized microspheres through the pulmonary microcirculation was unchanged in 2- and 8-wk TAA-treated animals compared with controls, indicating a lack of vasodilatation.

**Evaluation of the endothelin system and plasma TNF-α and endotoxin levels in prehepatic portal hypertension and biliary and nonbiliary cirrhosis.** To evaluate ET-1 production and pulmonary vascular ET_{B} receptor expression and plasma TNF-α and endotoxin levels in relation with development of experimental HPS, we measured these factors in our models (Table 2, Figs. 2 and 3). Hepatic ET-1 production and plasma ET-1 levels rose progressively after CBDL and correlated with the onset and progression of HPS as seen previously (27). In contrast, hepatic ET-1 production and plasma ET-1 levels did not rise after PVL or TAA treatment and lung ET-1 levels were not altered in any model. Pulmonary ET_{B} receptor levels increased significantly in all models as portal hypertension increased, and staining was localized to the pulmonary endothelium (28). There was a progressive increase in plasma TNF-α levels in both CBDL and TAA-treated animals not observed in PVL animals. TNF-α levels were significantly higher in TAA-treated animals than in CBDL animals despite the lack of development of HPS in nonbiliary cirrhosis. Plasma endotoxin levels were measured to assess the contribution of portosystemic shunting and bacterial translocation to TNF-α production. Levels increased significantly after CBDL as TNF-α levels increased but did not rise in either PVL or TAA-treated animals despite the marked increase in TNF-α levels after TAA treatment.

**Pulmonary eNOS, iNOS, HO-1, and ED1 levels in prehepatic portal hypertension and biliary and nonbiliary cirrhosis.** To assess the levels and distribution of proteins that produce vasoactive mediators implicated in the development and progression of experimental HPS in relation with ET-1 and TNF-α alterations, we measured eNOS, iNOS, HO-1, and ED1 levels in lung homogenates and performed immunohistochemical analysis (Figs. 4 and 5). There was a progressive increase in pulmonary eNOS levels after CBDL and a dramatic rise in HO-1 expression at 5 wk associated with the onset and progression of HPS as previously described (3). The eNOS increase was localized to the pulmonary vascular endothelium, and the HO-1 increase occurred in pulmonary intravascular macrophages that accumulated progressively after CBDL, as evidenced by a significant increase in ED1 levels, in line with prior results (3, 10). In contrast, eNOS and HO-1 levels did not increase significantly in PVL or TAA-treated animals. However, there was accumulation of pulmonary intravascular macrophages in TAA lung, to a lesser degree than CBDL, confirmed by both an increase in pulmonary ED1 levels and immunohistochemistry. iNOS levels were increased modestly in these cells in 8-wk TAA-treated animals but were not associated with the development of HPS.

**Effects of exogenous ET-1 infusion on the development of HPS in PVL animals.** To define how ET-1 influences the development of molecular and physiological changes associated with HPS in vivo, we infused ET-1 intravenously at doses similar to those seen in the plasma of CBDL animals for 2 wk to normal and PVL animals (Table 3, Fig. 6). ET-1 infusion was associated with an increase in pulmonary vascular eNOS levels and the development of HPS in PVL animals in which pulmonary vascular ET_{B} receptor expression is increased but not in normal animals as described previously (28, 50). In addition, ET-1 infusion also increased plasma TNF-α levels in PVL animals without altering portal or systemic pressures or resulting in endotoxemia. These changes were associated with a significant increase in intravascular macrophage accumulation in PVL animals but not with a rise in pulmonary iNOS or HO-1 levels. ET-1 infusion did not significantly alter plasma TNF-α, endotoxin levels, or pulmonary iNOS, HO-1, or ET_{B} receptor levels in normal animals.

**DISCUSSION**

Biliary cirrhosis induced by CBDL is the only identified model that mimics the physiological abnormalities of human HPS. It is distinct from human HPS in that HPS develops in the majority of animals after CBDL, whereas only a minority of humans with cirrhosis develops HPS across a wide range of etiologies of liver disease (9). This observation has led to the hypothesis that CBDL results in a unique confluence of molecular abnormalities culminating in the development of intrapulmonary vasodilatation and HPS. The present study is the first to test this concept by assessing whether toxic hepatocellular injury leading to cirrhosis also results in the molecular alterations in PVL, CBDL, and TAA-treated animals

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>PVL</th>
<th>CBDL, wk</th>
<th>TAA, wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP, mmHg</td>
<td>7.4±0.3</td>
<td>13.8±0.6*</td>
<td>11.8±0.5*</td>
<td>13.8±0.5*</td>
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<tr>
<td>Liver tests</td>
<td></td>
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<tr>
<td>AST, IU/l</td>
<td>84.3±12.8</td>
<td>76.7±4.8</td>
<td>323±84*</td>
<td>758±69*</td>
</tr>
<tr>
<td>T bili, mg/dl</td>
<td>0.10±0.01</td>
<td>0.10±0.01</td>
<td>7.33±0.48*</td>
<td>7.97±1.49*</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>3.0±1.0</td>
<td>2.9±0.2</td>
<td>2.4±0.1*</td>
<td>1.8±0.4*</td>
</tr>
<tr>
<td>Liver histology</td>
<td>normal</td>
<td>normal</td>
<td>bridging fibrosis</td>
<td>biliary cirrhosis</td>
</tr>
<tr>
<td>PaCO₂, mmHg</td>
<td>39.9±0.6</td>
<td>40.0±0.9</td>
<td>38.3±1.0</td>
<td>35.0±1.8*</td>
</tr>
<tr>
<td>AaP O₂, mmHg</td>
<td>7.5±1.5</td>
<td>6.2±1.1</td>
<td>15.2±1.0*</td>
<td>19.0±2.2*</td>
</tr>
<tr>
<td>Intrapulmonary shunt fraction, %</td>
<td>5.8±0.1</td>
<td>5.9±0.2</td>
<td>13.5±0.8*</td>
<td>18.4±1.1*</td>
</tr>
<tr>
<td>Lung, wet/dry wt</td>
<td>5.0±0.1</td>
<td>4.6±0.2</td>
<td>5.0±0.3</td>
<td>4.9±0.1</td>
</tr>
<tr>
<td>Lung histology</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–8 animals per group; PVL, partial portal vein ligation; CBDL, common bile duct ligation; TAA, thioacetamide; PVP, portal venous pressure; AST, aspartate aminotransferase; T bili, total bilirubin; AaP O₂, alveolar-arterial oxygen gradient. *P < 0.05 compared with normal control.
and physiological abnormalities seen in CBDL animals with HPS. Here we find that cirrhosis and portal hypertension induced by TAA does not result in the physiological changes associated with experimental or human HPS. In addition, the cascade of molecular events seen during the onset and progression of HPS in CBDL animals does not fully develop. Specifically, hepatic and plasma ET-1 levels do not increase, and a sequential increase in pulmonary eNOS and HO-1 levels is not observed. However, plasma TNF-α levels do significantly increase after TAA administration, to levels greater than those observed in HPS.

Table 2. ET-1, TNF-α, and endotoxin levels in PVL, CBDL and TAA-treated animals

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PVL</th>
<th>CBDL, wk</th>
<th>TAA, wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>ET-1 Plasma, pg/ml</td>
<td>5.3±0.5</td>
<td>6.8±0.5</td>
<td>13.4±0.4*</td>
<td>22.9±3.1*</td>
</tr>
<tr>
<td>Liver, ng/g tissue</td>
<td>1.8±0.3</td>
<td>1.8±0.2</td>
<td>4.2±0.2*</td>
<td>4.6±0.6*</td>
</tr>
<tr>
<td>Lung, ng/g tissue</td>
<td>8.1±0.9</td>
<td>9.1±0.7</td>
<td>8.1±0.8</td>
<td>8.3±0.7</td>
</tr>
<tr>
<td>TNF-α Plasma, pg/ml</td>
<td>ND</td>
<td>ND</td>
<td>7.7±1.8</td>
<td>34.8±6.9</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>ND</td>
<td>ND</td>
<td>1.3±0.2</td>
<td>2.7±1.1</td>
</tr>
<tr>
<td>Plasma, EU/ml</td>
<td>1.3±0.2</td>
<td>2.7±1.1</td>
<td>1.1±0.2</td>
<td>6.8±1.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–8 animals per group. ET-1, endothelin-1; ND, not detected. *P < 0.05 compared with normal control.
found after CBDL despite less severe hepatic dysfunction and a lack of endotoxemia. These changes are associated with pulmonary intravascular macrophage accumulation to a lesser degree than observed after CBDL and a modest increase in iNOS expression in 8-wk TAA-treated animals that alone do not trigger HPS. Furthermore, the exogenous administration of ET-1 to PVL animals but not normal animals elevates TNF-α levels, increases pulmonary eNOS production and pulmonary intravascular macrophage accumulation, and leads to the development of HPS. Together, these findings support an important role for increased circulating ET-1 in the development of experimental HPS and suggest that ET-1 and TNF-α have synergistic effects in the pulmonary microvasculature in portal hypertension.

Alterations in the endothelin system are well documented in liver disease. Specifically, hepatic production (27, 36, 39) and circulating levels (1, 27, 31) of ET-1 have been observed in some forms of experimental and human cirrhosis. CBDL appears to be unique in that hepatic ET-1 production is increased within 7 days after ligation, before the onset of portal hypertension, whereas ET-1 increases in humans have been detected only in advanced disease (1, 31). In CBDL, the rise in ET-1 is likely related to production in both stellate cell and biliary epithelium (39). In addition, biliary levels of ET-1 are high in rodents both under normal conditions and after bile duct ligation (26) suggesting that retrograde release of ET-1 from bile into blood may be a unique consequence of biliary obstruction. The present study supports that bile duct obstruction is one important contributor to hepatic and plasma ET-1 levels by showing that toxic hepatocellular injury without bile duct obstruction fails to significantly increase hepatic or plasma ET-1 despite the development of cirrhosis and portal hypertension. Our finding that plasma ET-1 levels do not rise in TAA cirrhosis is in agreement with recent work (48).

We have also found that pulmonary vascular endothelial ETB receptor expression is increased in the lung in TAA-treated animals as portal hypertension increases. This finding is consistent with previously documented changes in prehepatic portal hypertension and in biliary cirrhosis (28). Although two vascular ETB receptor types have been identified, one in endothelial cells that upregulates eNOS activity and expression and produces NO and the other in smooth muscle cells functioning similar to the vasoconstrictive ETA receptor (29, 46), our present and prior immunohistochemical results support the finding that the major increase in our models occurs in the endothelium (28). This finding is consistent with the observation that ET-1 causes an ETB receptor-dependent increase in eNOS levels and NO production in short-term cultured pulmonary artery segments from PVL and CBDL animals relative to control (28). Our findings also support the concept that the development of portal hypertension is an important factor in increased lung endothelial ETB receptor expression and in susceptibility to ET-1-mediated vascular alterations. Although the mechanisms have not been directly explored in vivo, ETB
receptor expression can be altered by a number of factors including changes in flow (2, 30, 38) and cytokine production (25, 33) both of which occur in the setting of portal hypertension.

Molecular changes associated with the development of experimental HPS include an increase in pulmonary vascular eNOS expression in the lung beginning within 2 wk after CBDL, which has been attributed to circulating ET-1 acting through a selective increase in pulmonary endothelial ETB receptors in the setting of portal hypertension (26–28, 50). In addition, increased iNOS and HO-1 expression in accumulated intravascular macrophages also occurs and appears dependent on TNF-α overproduction related to bacterial translocation (3, 37). Our findings support the importance of ET-1 in triggering eNOS expression in the lung in the setting of portal hypertension by documenting that eNOS changes do not occur in TAA cirrhosis or after PVL in which circulating ET-1 levels are not increased and by confirming that exogenous ET-1 increases pulmonary eNOS levels and the development of HPS in PVL but not normal animals. Increase in plasma ET-1 levels after ET-1 infusion in PVL animals is smaller than that seen in 2-wk CBDL animals but significant relative to saline-infused PVL animals. The finding that small changes in luminal ET-1 concentrations can influence pulmonary vascular tone is not surprising because the release of small quantities of endothelial ET-1 are recognized to stimulate ETB-mediated NO production through an autocrine loop (16, 29). In addition, the lung vasculature is the major site of endothelin clearance, an event that occurs through the high-affinity ETB receptor (12). Therefore, in the setting of increased pulmonary endothelial ETB receptor levels in portal hypertension, small changes in ET-1 levels are likely to have significant effects on eNOS. In addition to effects on pulmonary eNOS, we also find that circulating ET-1 modulates plasma TNF-α levels and intravascular macrophage accumulation in the lung in PVL animals, a finding consistent with the ability of ET-1 to influence TNF-α expression and monocyte adherence in vitro (5, 43). The observation that our low concentration ET-1 infusion did not alter systemic or portal pressures and did not influence circulating endotoxin levels supports that effects were not due to splanchnic vasoconstriction modulating intestinal permeability. Together, these findings suggest that ET-1 influences both pulmonary microvascular eNOS, circulating TNF-α, and intravascular macrophage accumulation in the lung in HPS.

Our results also show that TNF-α overproduction occurs in both biliary and nonbiliary cirrhosis. The finding that both endotoxin and TNF-α levels are elevated in 5-wk CBDL animals is in agreement with the established concept that increased TNF-α levels, driven both by bacterial translocation and decreased hepatic clearance, occur in advanced experimental cirrhosis (49). The increase in circulating TNF-α in these models plays an important role in the maintenance of the hyperdynamic circulatory state by modulating splanchnic and systemic vascular eNOS activity (49) and could regulate pul-

Fig. 4. Lung endothelial nitric oxide (NO) synthase (eNOS), inducible NOS (iNOS), heme oxygenase-1 (HO-1), and ED1 protein levels in PVL, 2- and 5-wk CBDL, and 2-and 8-wk TAA-treated animals. Inserts, representative immunoblots of lung homogenates revealing a single band of ≈140 kDa consistent with eNOS (A), 130 kDa with iNOS (B), 32 kDa with HO-1 (C), or 95 kDa with ED1 (D). Graphs summarize eNOS, iNOS, HO-1, or ED1 protein levels in lung. Values are expressed as means ± SE (n = 5–7 animals for each group). *P < 0.05 compared with normal control.
monary vascular eNOS production in HPS. Our finding that TNF-α levels are more than sixfold higher in both 2- and 8-wk TAA animals than in CBDL animals and are not associated with measurable changes in serum endotoxin levels or biochemical evidence of advanced liver disease supports the concept that decreased hepatic clearance and bacterial translocation are unlikely to be a major source of TNF-α in this model. Increased production in mononuclear cells that have been described to accumulate in the liver after TAA treatment (34) or in mesenteric lymph nodes after intraperitoneal injection of TAA are other potential sources. The dramatic rise in TNF-α levels after TAA treatment is not associated with pulmonary eNOS alterations or with the development of HPS, although it is associated with pulmonary intravascular macrophage accumulation and iNOS overproduction. These findings agree with the recent concept that TNF-α overproduction contributes to pulmonary intravascular macrophage accumulation and HPS after CBDL but support that TNF-α alone is insufficient for the full development of the syndrome (37). These results also suggest that the cell type of presumed NO overproduction in the pulmonary vasculature may influence effects on vascular tone. One mechanism for this effect may relate to an increase in cyclooxygenase-2 activity and vasoactive prostanooid production observed in activated macrophages, an effect mediated in part by interactions between NO and superoxides generated after activation (21). Increased pulmonary levels of the potent vasoconstrictor thromboxane A2 attributed to production in intravascular macrophages have been found in CBDL animals (4). Production of such vasoconstrictors in macrophages could counteract NO-mediated vasodilation in TAA lung, particularly be-
cause endothelial NO production is not increased in this situation. However, understanding why increased iNOS production in intravascular macrophages alone does not cause pulmonary vasodilatation requires further evaluation.

One hypothesis, on the basis of our observation that both ET-1 and TNF-α levels are increased in experimental HPS, is that these mediators interact to influence the development of pulmonary vascular abnormalities. Our finding that exogenous administration of ET-1 to PVL animals influences both circulating TNF-α levels and pulmonary intravascular macrophage accumulation during the development of HPS is consistent with this concept and with a growing body of evidence. ET-1 has been shown to modify monocyte adhesion by both direct effects on monocytes as well as by modulation of cell adhesion molecule and macrophage chemokine expression and NO production in endothelial cells (5, 18, 20, 23). In addition, ET-1 can increase TNF-α production by macrophages (43). TNF-α also modulates monocyte adhesion directly and influences cell adhesion molecule expression and can influence ET B receptor expression and NO production in endothelial cells (19, 47, 49). These findings support that ET-1 and TNF-α interactions, possibly occurring both locally in the lung vasculature and in other vascular beds and organs, contribute to the development of experimental HPS. Defining the cellular sites and mecha-

Table 3. Hepatic and pulmonary alterations and plasma ET-1, TNF-α, and endotoxin levels in ET-1-infused normal and PVL animals

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>2-wk PVL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>saline  ET-1</td>
<td>saline ET-1</td>
</tr>
<tr>
<td>PVP, mmHg</td>
<td>7.4±0.3 7.6±0.6</td>
<td>12.6±0.4* 12.5±0.7*</td>
</tr>
<tr>
<td>AaPO2, mmHg</td>
<td>7.5±1.5 9.8±2.4</td>
<td>6.7±1.4 22.3±2.0**</td>
</tr>
<tr>
<td>Intrapulmonary shunt</td>
<td>5.7±0.1 5.8±0.2</td>
<td>5.9±0.1 14.2±0.5**</td>
</tr>
<tr>
<td></td>
<td>fraction, %</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1, pg/ml</td>
<td>6.9±0.2 11.1±0.4†</td>
<td>7.1±0.6 9.5±0.9**</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>ND 1.8±0.4</td>
<td>ND 37.9±13.1</td>
</tr>
<tr>
<td>Endotoxin, EU/ml</td>
<td>1.3±0.2 1.0±0.1</td>
<td>2.6±1.1 3.0±0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–8 animals per group. PVP, portal venous pressure; *P < 0.05 compared with saline-infused normal control. †P < 0.05 compared with saline-infused 2-wk PVL.

![Figure 6](https://example.com/figure6.png)

Fig. 6. Lung eNOS, ET B receptor, iNOS, HO-1, and ED1 protein levels in 2-wk saline or ET-1-infused normal and PVL animals. Top, left: representative immunoblots in lung. Graphs summarize eNOS (A), ET B receptor (B), iNOS (C), HO-1 (D), or ED1 (E) protein levels in lung. Values are expressed as means ± SE-fold saline-infused normal as control values (n = 5–7 animals for each group). *P < 0.05 compared with saline-infused normal control. †P < 0.05 compared with saline-infused 2-wk PVL.
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nisms of these interactions in experimental HPS may provide insight into the pathogenesis of human disease and allow targeting of new therapies.

GRANTS
This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-02030 and a Veterans Affairs Merit Review Grant (to M. B. Fallon).

REFERENCES


