Dysregulation of the vascular endothelium plays a fundamental role in a number of cardiovascular diseases (39) and in the altered vascular tone associated with cirrhosis and portal hypertension (37). An imbalance in the local production and/or activity of potent vasoactive agents, including nitric oxide (NO) and endothelin-1 (ET-1), in the vessel wall is thought to underlie endothelial dysfunction and contribute to changes in vascular tone (8, 15, 28, 38). In models of chronic liver disease, splanchnic vasodilatation is accompanied by elevated endothelial nitric oxide synthase (eNOS) levels (28) and enhanced NO activity (30), although the stimuli responsible for increasing nitric oxide synthase (NOS) expression and NO production remain controversial (37). Plasma ET-1 levels are also increased in some forms of experimental and human cirrhosis and are postulated to result from enhanced production in the damaged liver (3, 25, 26, 29, 31). ET-1 is a potent vasoconstrictor when acting through the ET_{A} receptor on the vascular smooth muscle cells, although elevated levels in cirrhosis occur in the setting of systemic vasodilatation and are not associated with measurable vasoconstrictive effects (25). These observations suggest that the effects mediated by ET-1 in chronic liver disease may include stimulation of NO activity through the ET_{A} receptor on endothelial cells (17) or modulation of vasoactive peptide expression (4, 5).

The hepatopulmonary syndrome (HPS) is one well-recognized complication of chronic liver disease that occurs in the setting of cirrhosis with portal hypertension. It is characterized by intrapulmonary vasodilatation, which results in altered arterial oxygenation independent of intrinsic cardiopulmonary disease (23). In previous studies, we have established chronic common bile duct ligation (CBDL) in the rat as a model of HPS in which intrapulmonary vasodilatation occurs in the setting of hepatic injury and portal hypertension (11). In contrast, portal hypertension induced by partial portal vein ligation (PVL) does not cause hepatic injury or HPS, suggesting that portal hypertension alone may be a necessary but insufficient factor in the development of HPS. In CBDL animals, the degree of intrapulmonary vasodilatation and gas exchange abnormalities correlate with a progressive increase in pulmonary vascular eNOS protein levels and enhanced production and activity of NO in intralobar pulmonary rings (10). Recently, we have also observed a progressive increase in hepatic production and plasma levels of ET-1 after CBDL in the absence of altered pulmonary ET-1 levels, which correlate with pulmonary eNOS alterations and gas exchange abnormalities (26). These studies suggest that enhanced ET-1 production during chronic hepatic injury may alter pulmonary eNOS production and contribute to the onset of intrapulmonary vasodilatation in experimental HPS.

In the present study, we test the hypothesis that low levels of circulating ET-1, arising during chronic hepatic injury, induce pulmonary eNOS expression and increased NO production and contribute to the development of intrapulmonary vasodilatation and HPS. Bovine pulmonary artery endothelial cells (BPAECs) are used to assess whether ET-1 administration can trigger eNOS expression and enhanced NO production in vitro and to assess the receptor specificity of the effect. Chronic low-dose intravenous infusion of ET-1 is administered to PVL animals to determine if ET-1 exposure, in the presence of portal hypertension alone, results in increased pulmonary eNOS levels, intrapulmonary va-
sodilatation, and arterial gas exchange abnormalities consistent with the development of HPS.

**METHODS**

Animal models. PVL was performed as previously described (7) using male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 200–250 g. At the time of surgery, a miniosmotic pump (ALZET Model 2002, ALZA, Palo Alto, CA) was inserted into the femoral vein. Rats were infused with 3 ng·200 g body wt⁻¹·h⁻¹ of synthetic ET-1 peptide (Peninsula Laboratories, Belmont, CA) or 0.9% saline for 2 wk based on the hepatic venous and plasma ET-1 concentration previously observed in 2-wk CBDL animals (26) and in pilot studies. In pilot studies, the acute effects of ET-1 infusion using 3 ng·200 g body wt⁻¹·h⁻¹ was compared with an infusion of 30 ng·200 g body wt⁻¹·h⁻¹. The lower dose resulted in a mild transient hypotensive effect as previously seen using physiological doses of ET-1, and the higher dose resulted in well-described vasoconstriction (21). ET-1 delivery was monitored by ensuring that the pump was empty and the delivery catheter was positioned in the femoral vein at the termination of the experiment. The stability of ET-1 in miniosmotic pumps over 2 wk was documented by placing a known concentration of ET-1 in four miniosmotic pumps under sterile conditions and incubating these pumps for 2 wk at 37°C. Subsequent RIA for ET-1 revealed a concentration close to the initial concentration in the pumps within 10–15% of initial values. The development of portal hypertension was confirmed by portal pressure and spleen weight measurements. Arterial gas exchange was evaluated as described by arterial blood gas analysis (11), and the alveolar–arterial oxygen gradient was calculated as 150–(P<sub>aco</sub>/0.8)–P<sub>oa</sub>. Lung tissue was collected after perfusion with PBS for histology and eNOS detection by Western blot analysis (10). Mean pulmonary arterial pressure was measured by insertion of an indwelling pulmonary arterial catheter, and mean systemic arterial pressure was measured by insertion of an indwelling femoral arterial catheter both placed 48 h before death as previously described (9). Pressure measurements were made at rest on the pressure catheter both placed 48 h before death as previously described (7) using male Sprague-Dawley rats (Charles River, Waltham, MA) weighing 200–250 g. At the time of surgery, an indwelling femoral arterial and venous catheter was placed with an equal volume of sterile PBS.

Numbers and sizes of microspheres in each sample were assessed using a Leica DMRE microscope (Wetzlar, Germany) with a color video imaging system combined with image analysis software (Pro 3.0 Media Cybernetics, Silver Spring, MD) with area, shape factor, and aspect ratios to distinguish microsphere sizes. Total numbers of microspheres passing through the pulmonary microcirculation were calculated as reference blood sample microspheres per milliliter times estimated blood volume. Blood volume of each animal was derived from the following formula: blood volume (ml) = 0.06 × body wt (g) + 0.77 (see Ref. 24). Intrapulmonary shunting was calculated as an intrapulmonary shunt fraction (%) = (total number of microspheres passing through the pulmonary microcirculation/total beads injected into the venous circulation) × 100.

Cell culture. BPAECs [American Type Culture Collection (ATCC), Manassas, VA] were maintained and passaged in EGM-MV complete media (Clonetics, San Diego, CA) at 37°C with 5% CO₂ on 100-mm Falcon cell culture dishes (Becton Dickinson Labware, Lincoln Park, NJ), and cells of passages 3–10 were used in the study. When cells reached 70% confluence, they were incubated in 4 ml of minimal media containing 0.5% FBS without supplements and stimulated by ET-1 at various concentrations (0.01, 0.1, and 1 µM) for various times (12, 24, and 48 h). In a set of experiments, endothelin receptor antagonists including TBC3214Na for ETA (kind gift from Dr. Y. F. Chen, University of Alabama at Birmingham), BQ-788 for ET<sub>B</sub> (Peptides International, Louis ville, KY), or Bosentan for ET<sub>A</sub> and ET<sub>B</sub> (Roche, Basel, Switzerland) were applied at 10 µM to cells in the presence or absence of ET-1 treatment. To assess cell proliferation, cells were incubated on a 96-well plate (Nalge Nunc, Naperville, IL) at 2000 cells/well in minimal media containing 0.5% FBS in the presence or absence of 0.1 µM ET-1 for 12, 24, and 48 h. A standard curve was constructed by using a defined cell number in the assay. At each time point, 20 µl of freshly made MTS/phenazone methosulfate (PMS) mixture (10:1 vol/vol, Promega, Madison, WI) was added into each well containing 100 µl of media and was incubated at 37°C in a humidified 5% CO₂ atmosphere for 1 h. After the absorbance at 490 nm was recorded using an ELISA microplate reader (Molecular Devices, Sunnyvale, CA), cell number was calculated according to the standard curve.

Western blot analysis. BPAECs or lung tissue was prepared by lysis or Dounce homogenization respectively in radiimmunoprecipitation buffer (10) in the presence of protease inhibitors. Fifteen micrograms of protein from homogenates, quantified by protein assay (Bio-Rad, Hercules, CA), were fractionated on a 7.5% SDS-PAGE gel and then transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Arlington Heights, IL). Incubation with an eNOS monoclonal antibody (Transduction Laboratories, Lexington, KY) was followed by extensive washing and incubation with a horseradish peroxidase-conjugated sheep antimouse secondary antibody (Amersham) and development by enhanced chemiluminescence (Amersham) on Kodak X-ray film (Sigma, St. Louis, MO).

Northern blot analysis. Total RNA from BPAECs was prepared by lysis in TRIzol reagent on the basis of a standard protocol (GIBCO, Grand Island, NY). RNA (20 µg) was subjected to agarose gel electrophoresis and blotted onto a Nytran membrane (Schleicher & Schuell, Keene, NH) as described (26), followed by a quick hybridization procedure (Stratagene, La Jolla, CA) with a 4.0-kb bovine eNOS cDNA probe (kind gift of Dr. William Sessa, Yale University) or a 0.5-kb rat glyceroldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (ATCC) labeled with the Prime-a-Genelabel-
demonstrated a dose-dependent increase in eNOS protein levels.

**RESULTS**

ET-1 increases eNOS protein levels in BPAECs. To determine if ET-1 can induce eNOS protein expression, BPAECs were treated with synthetic ET-1 peptide in a minimal medium containing 0.5% FBS in the absence of other growth factor supplements. A progressive dose- and time-dependent increase in eNOS protein (140 kDa) levels was observed by Western blotting after ET-1 stimulation. The maximal increase occurred at 24 h after the addition of 0.1 µM ET-1 (Fig. 1, A and B) and represented a significant 2.5-fold increase in eNOS protein production (Fig. 1C).

ET-1-mediated effects on eNOS protein are mediated through the ETB receptor in BPAECs. The effects of ET-1 on vascular tone are mediated through two distinct receptors, the ETA receptor and the ETB receptor, which differ in their tissue and cell-type specificity (19). The ETB receptor predominates on endothelial cells. To determine the role of ET receptors in ET-1-mediated alterations in eNOS protein levels, BPAECs were stimulated with 0.1 µM ET-1 for 24 h and eNOS protein levels were assessed in the presence or absence of ET receptor antagonists (Fig. 2). The eNOS protein levels in these cells were compared with control levels in untreated BPAECs, which were arbitrarily set at one. The addition of ET receptor antagonists alone, including TBC3214Na, a selective ETA receptor antagonist (1.0 ± 0.2-fold control), BQ-788, a selective ETB receptor antagonist (1.2 ± 0.06-fold control), and Bosentan, a mixed ETA and ETB receptor antagonist (1.1 ± 0.2-fold control), had no significant effect on eNOS protein levels (n = 4 in each group and in subsequent receptor antagonist studies; differences were not significant for each group relative to untreated controls). The addition of the ETA receptor antagonist did not inhibit the ET-1-mediated increase in eNOS protein (2.3 ± 0.2-fold control; P < 0.05 relative to control untreated cells), which was similar to that seen in control BPAECs treated with ET-1 alone. In contrast, the ET-1-mediated increase in eNOS protein was abolished and was signifi-
cantly different from ET$_A$ receptor antagonist-treated cells when the ET$_B$ receptor antagonist (1.03 ± 0.3-fold control) or Bosentan (1.03 ± 0.04-fold control) was added to the culture medium ($P < 0.05$ relative to ET$_A$ receptor antagonist-treated cells). These findings indicate that ET-1-mediated changes in eNOS protein levels are mediated through the ET$_B$ receptor on BPAECs.

ET-1 upregulates eNOS mRNA in BPAECs. To further evaluate the mechanism of the ET-1-mediated increase in eNOS protein, we measured steady-state eNOS mRNA levels by Northern blotting in BPAECs after ET-1 exposure (Fig. 3A). The eNOS mRNA levels normalized to GAPDH levels after ET-1 exposure were compared with control levels in untreated cells and arbitrarily set at 1 ($n = 4$ for each group). There was an increase in the ∼5.0-kb eNOS signal (2) that was statistically significant within 12 h of 0.1 µM ET-1 stimulation (2.3 ± 0.6-fold control) and was maximal by 24 h (3.6 ± 1.2-fold control). The increase was maintained at 48 h (2.9 ± 0.6-fold control). No change in the GAPDH control signal was observed on these blots. The ET-1-mediated increase in eNOS mRNA was confirmed by RT-PCR analysis using eNOS-specific primers. An increase in a ∼380-bp fragment amplified with eNOS primers, but not an α-actin control message, was observed after ET-1 stimulation (Fig. 3B). The ∼380-bp PCR product was confirmed to be eNOS by direct sequencing and comparison with the published eNOS sequence (data not shown) (20). The temporal association between the increase in eNOS mRNA and protein levels after ET-1 exposure is consistent with the hypothesis that ET-1 alters eNOS expression in endothelial cells.

ET-1 stimulates NO production in BPAECs. To confirm that ET-1 stimulation of BPAECs and the subsequent increase in eNOS protein levels result in enhanced NO production, we measured NO levels in BPAEC culture media in the presence and absence of exogenous ET-1. ET-1 administration (0.1 µM) significantly stimulated net NO production (2.8-fold) from cells after 24 h (Fig. 4), as has been previously observed (17). The maximal increase in culture medium NO levels correlated temporally with increased eNOS lev-

Fig. 2. Effects of endothelin receptor antagonists on ET-1-induced eNOS protein levels in BPAECs. Representative Western blot for eNOS obtained from 70% confluent BPAECs treated with 10 µM of endothelin receptor antagonists for 24 h in presence or absence of 0.1 µM ET-1 and analyzed as described in Fig. 1. TBC3214Na is ET$_A$ receptor antagonist, BQ-788 is ET$_B$ receptor antagonist and Bosentan is ET$_A$ and ET$_B$ receptor antagonist. Experiments were repeated ($n = 4$ for each condition) for statistical analysis. Second, fourth, and sixth lanes from left represent cells incubated with ET receptor antagonists alone. No difference in eNOS levels was observed in these cells compared with untreated control cells (first lane from left). Incubation of cells with a selective ET$_A$ receptor antagonist and ET-1 did not block ET-1-mediated increase in eNOS protein levels (third lane from left). In contrast, both ET$_B$-selective and mixed ET$_A$ and ET$_B$ receptor antagonists abolished ET-1-mediated increase in eNOS protein levels (fifth and seventh lanes from left), establishing that ET-1 alters eNOS protein levels in BPAECs through ET$_B$ receptor.

Fig. 3. ET-1 induction of eNOS mRNA in BPAECs. BPAECs (70% confluent) were stimulated with 0.1 µM ET-1 peptide for 0, 12, 24, or 48 h, and total RNA was extracted using TRIzol reagent. A: representative Northern blot of 20 µg of total RNA fractionated on a 0.75% formaldehyde-agarose gel, transferred to nitrocellulose, and detected with a 4.0-kb eNOS probe. A gyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used as an internal control. There was a significant increase in 5.0-kb eNOS mRNA signal within 12 h of ET-1 stimulation (2.3 ± 0.6-fold control) and this increase was maximal by 24 h (3.6 ± 1.2-fold control). No change in the GAPDH control signal was observed on these blots. The increase in eNOS mRNA was confirmed by RT-PCR analysis using specific eNOS primers. An increase in a ∼380-bp fragment amplified with eNOS-specific primers, but not an α-actin control message, was observed after ET-1 stimulation (Fig. 3B). The ∼380-bp PCR product was confirmed to be eNOS by direct sequencing and comparison with the published eNOS sequence (data not shown) (20). The temporal association between the increase in eNOS mRNA and protein levels after ET-1 exposure is consistent with the hypothesis that ET-1 alters eNOS expression in endothelial cells.

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ET-1 induction of nitrite production in BPAECs. After incubation in presence or absence of 0.1 µM ET-1 for 0, 12, or 24 h, NO3 in BPAEC culture media was converted to NO2 by nitrate reductase and then analyzed by Greiss reaction. Absorbance at 550 nm was measured to calculate net NO production according to a standard curve. A significant increase in nitrite was observed at 24 h after ET-1 induction (n = 4 at each time point in each group). *P < 0.05.

Fig. 4. ET-1 induction of nitrite production in BPAECs. No ET-1; ET-1 induction.

ET-1 does not induce BPAEC proliferation over 24 h. To exclude the possibility that the mitogenic properties of ET-1 caused BPAEC proliferation and resulted in increased eNOS mRNA and protein levels over the time course studied here, we measured BPAEC proliferation by the MTS/PMS assay in the presence and absence of exogenous ET-1. Cells incubated as above and not exposed to ET-1 were compared with cells exposed to 0.1 µM ET-1 for 12, 24, and 48 h. Cell number was measured at each time point. There was no significant difference in cell number after 24 h between exposed and unexposed cells (Fig. 5). After 48 h, ET-1 treatment resulted in a modest increase in cell number, though the magnitude of the increase relative to untreated cells at 48 h (1.2-fold untreated) was substantially less than the increase in eNOS mRNA and protein levels over the same time frame. These results demonstrate that the increase in eNOS levels in BPAECs after ET-1 stimulation cannot be explained by enhancement of cell proliferation over the time frame studied here.

Western blot analysis of pulmonary eNOS protein levels demonstrated a small but significant 1.5-fold increase in whole lung eNOS levels in ET-1-treated lung relative to saline-treated lung (Fig. 6). This increase is similar but slightly less than that observed in 2-wk CBDL animals with HPS as previously described (9). Western blot for inducible nitric oxide synthase was performed and revealed no detectable signal in lung in either group (data not shown), suggesting that low-dose ET-1 infusion had no measurable effects on the liver. Mean systemic arterial pressures were similar in saline- and ET-1-treated animals and were lower than values we have observed in normal animals (9), reflecting the development of a hyperdynamic state as previously described after PVL (36). Mean pulmonary arterial pressures were also similar in both groups and remained within the range observed in normal animals (9), demonstrating that the doses of exogenous ET-1 administered here had no significant vasoconstrictive effect in the lung.

Chronic intravenous ET-1 administration increases lung eNOS levels, results in intrapulmonary shunting, and impairs gas exchange after PVL. To determine if ET-1 contributes to the development of HPS, we delivered an ET-1 or saline infusion through the femoral vein over 2 wk via a miniosmotic pump in PVL animals. Liver and lung histology, portal vein pressure, mean systemic and pulmonary arterial blood pressure, lung eNOS levels, microsphere assessment of the pulmonary microcirculation, and arterial gas exchange were evaluated. Pressure measurements and arterial blood gas results are summarized in Table 1. All animals developed significant portal hypertension compared with values previously observed in normal rats (11), and the degree of portal hypertension at 2 wk was not different in saline- and ET-1-treated animals. Serum aspartate aminotransferase and total bilirubin levels and hepatic histology were also normal in the saline and ET-1 groups (data not shown), suggesting that low-dose ET-1 infusion had no measurable effects on the liver. Mean systemic arterial pressures were similar in saline- and ET-1-treated animals and were lower than values we have observed in normal animals (9), reflecting the development of a hyperdynamic state as previously described after PVL (36). Mean pulmonary arterial pressures were also similar in both groups and remained within the range observed in normal animals (9), demonstrating that the doses of exogenous ET-1 administered here had no significant vasoconstrictive effect in the lung.

Table 1. Effects of chronic ET-1 infusion on pressure measurements and arterial blood gases in PVL animals

<table>
<thead>
<tr>
<th></th>
<th>Normal Range (12,17)</th>
<th>PVL Control (17)</th>
<th>Saline Pump</th>
<th>ET-1 Pump</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pressure, mmHg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVP</td>
<td>7.3 ± 0.3</td>
<td>13.5 ± 0.7*</td>
<td>12.4 ± 0.5*</td>
<td>12.6 ± 1.1*</td>
</tr>
<tr>
<td>MPAP</td>
<td>18.0 ± 1.3</td>
<td>16.4 ± 1.6</td>
<td>15.0 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>MSAP</td>
<td>113.0 ± 6.0</td>
<td>96.8 ± 3.0*</td>
<td>96.6 ± 5.2*</td>
<td></td>
</tr>
<tr>
<td><strong>Arterial blood gases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.43 ± 0.01</td>
<td>7.43 ± 0.01</td>
<td>7.49 ± 0.02†</td>
<td></td>
</tr>
<tr>
<td>PCO2, mmHg</td>
<td>40.0 ± 0.5</td>
<td>40.1 ± 0.9</td>
<td>32.1 ± 1.5†</td>
<td></td>
</tr>
<tr>
<td>PO2, mmHg</td>
<td>93.8 ± 2.2</td>
<td>93.8 ± 2.2</td>
<td>88.4 ± 2.9†</td>
<td></td>
</tr>
<tr>
<td>AaPO2, mmHg</td>
<td>7.2 ± 0.2</td>
<td>6.0 ± 1.4</td>
<td>21.5 ± 2.6†</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4–6 for each group. ET-1, endothelin-1; PVP, portal venous pressure; MPAP, mean pulmonary arterial pressure; MSAP, mean systemic arterial pressure; AaPO2, alveolar-arterial oxygen gradient. Numbers in parentheses are reference numbers. *P < 0.05 relative to normal range. †P < 0.05 relative to portal vein ligation (PVL) control and normal range.
pulmonary eNOS levels in ET-1-treated animals was accompanied by increased shunting of microspheres across the pulmonary microcirculation and significant alterations in arterial gas exchange. Figure 7A demonstrates that similar numbers and sizes of microspheres were injected into the venous system in saline- and ET-1-treated animals. Figure 7B shows the results of arterial counts of microspheres after venous injection, reflecting passage through the pulmonary microcirculation. Saline-treated PVL animals demonstrated a similar pattern of microsphere passage through the lungs as previously observed in normal and PVL animals (11). ET-1-treated animals demonstrated a significant increase in the size and number of microspheres passing through the lungs relative to saline-treated animals, as evidenced by a rightward shift in the microspheres recovered from the femoral artery in these animals. Saline-treated PVL animals had a shunt fraction of 2.6 ± 0.8%, similar to shunt values found for 6.5 × 10^6 µm microspheres in normal and PVL animals in previous studies (11). In contrast, ET-1-treated animals had a significantly increased shunt fraction of 14.7 ± 2% (P < 0.05) similar to that previously observed in CBDL animals with HPS (11), reflecting intrapulmonary vasodilatation and enhanced passage of microspheres through the lung.

Evidence of dilatation of the pulmonary microcirculation was also accompanied by abnormalities in arterial oxygenation in ET-1-treated animals (Table 1). Compared with animals without liver disease and to untreated PVL animals (11), each ET-1-treated PVL animal developed a respiratory alkalosis, relative hypoxemia, and a widened alveolar-arterial oxygen gradient, similar to changes observed in animals and humans with HPS (10). An arterial blood gas was available from one saline-treated PVL animal, and values from this animal were within the normal range that we have previously observed (11). These observations demonstrate that chronic low-dose ET-1 infusion in PVL animals results in the development of molecular alterations in lung eNOS, dilatation of the pulmonary microcirculation, and gas exchange abnormalities similar to changes observed in animals with HPS.

DISCUSSION

Reciprocal paracrine and autocrine interactions between ET-1 and NO are important in mediating endothelium-dependent regulation of vascular tone (8, 15, 38, 39). An imbalance in these mediators has been implicated in the pathogenesis of several vascular pulmonary eNOS levels in ET-1-treated animals was accompanied by increased shunting of microspheres across the pulmonary microcirculation and significant alterations in arterial gas exchange. Figure 7A demonstrates that similar numbers and sizes of microspheres were injected into the venous system in saline- and ET-1-treated animals. Figure 7B shows the results of arterial counts of microspheres after venous injection, reflecting passage through the pulmonary microcirculation. Saline-treated PVL animals demonstrated a similar pattern of microsphere passage through the lungs as previously observed in normal and PVL animals (11). ET-1-treated animals demonstrated a significant increase in the size and number of microspheres passing through the lungs relative to saline-treated animals, as evidenced by a rightward shift in the microspheres recovered from the femoral artery in these animals. Saline-treated PVL animals had a shunt fraction of 2.6 ± 0.8%, similar to shunt values found for 6.5 × 10^6 µm microspheres in normal and PVL animals in previous studies (11). In contrast, ET-1-treated animals had a significantly increased shunt fraction of 14.7 ± 2% (P < 0.05) similar to that previously observed in CBDL animals with HPS (11), reflecting intrapulmonary vasodilatation and enhanced passage of microspheres through the lung.

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DISCUSSION

Reciprocal paracrine and autocrine interactions between ET-1 and NO are important in mediating endothelium-dependent regulation of vascular tone (8, 15, 38, 39). An imbalance in these mediators has been implicated in the pathogenesis of several vascular
disorders (13, 18, 39). The present study extends this concept on the basis of our previous observations, which suggest a pathogenetic association between elevated circulating ET-1 levels, increased pulmonary vascular eNOS levels, and functional alterations in the pulmonary vasculature in an animal model of HPS (26).

In the present study, we document that administration of ET-1 stimulates increased eNOS protein and mRNA levels in BPAECs through an ET α receptor-mediated effect, which correlates with increased NO production in these cells. In addition, chronic low-dose intravenous infusion of ET-1 in PVL animals triggers an increase in lung eNOS levels, alterations in the pulmonary microcirculation, and an impairment in arterial gas exchange similar to that observed in animals with HPS, without increasing pulmonary arterial pressure. These studies provide direct evidence that circulating ET-1 may increase eNOS production in endothelial cells and contribute to the pathogenesis of HPS.

We and others have demonstrated that hepatic production of ET-1 increases after CBDL (26, 34) and likely results in release into the venous system with circulation to the pulmonary vasculature. The increase in circulating ET-1 correlates with increased eNOS levels, enhanced NO activity, and gas exchange abnormalities in the lung in these animals that develop HPS (26) and implies that ET-1 may contribute to the development of pulmonary abnormalities. Our in vitro studies demonstrate the novel observation that exposure to ET-1 does increase eNOS mRNA and protein levels in BPAECs in a dose- and time-dependent fashion, supporting a role for ET-1 in altering eNOS expression. This effect occurs through the ET α receptor. Although the molecular mechanism through which ET-1 alters eNOS production remains under study, the hypothesis that ET-1 alters eNOS expression in the pulmonary vasculature is consistent with the observation that ET-1 can alter vasoactive mediator expression in other cell types (4, 5) and with the finding that a variety of agents are now known to influence the expression of the eNOS gene (6, 16, 22, 32). The potential physiological significance of this effect is highlighted by our in vivo observation that chronic low-dose intravenous ET-1 infusion also increases pulmonary eNOS levels and is associated with vascular and gas exchange abnormalities in the lung.

ET-1 has potent vasoconstrictive properties when released from vascular endothelial cells and targeted to the ET α receptor on vascular smooth muscle cells or when administered intravenously in pharmacological doses (35). However, this effect is not observed when doses closer to levels found in vivo are administered (21). The ET-1 peptide also exerts an autocrine vasoactive effect by increasing eNOS activity through the ET β receptor on vascular endothelial cells (17) and alters the gene expression of other vasoactive peptides in smooth muscle cells (4, 5). Liver injury with portal hypertension appears to represent a unique pathological situation where elevated circulating ET-1 levels (3, 26, 29), derived from increased hepatic production (26, 31, 34), occur in the setting of systemic vasodilatation. In this situation, systemic vascular tone is not altered by the addition of ET receptor antagonists (25), suggesting that ET-1 production does not simply reflect a response to vasodilatation. Our in vivo findings demonstrate that low-dose intravenous ET-1 infusion in the setting of portal hypertension does not significantly alter systemic or pulmonary arterial pressure, confirming that vasoconstrictive effects of ET-1 are not detectable. The attenuated vasoconstrictive effect of circulating ET-1 in portal hypertension is unexplained but may be contributed to by the relatively low circulating levels observed, which are generally below levels associated with significant vasoconstriction (27). Alternatively, flow-mediated changes in ET receptor expression in the vasculature (33) or alterations in the levels of other vasoactive substances observed during the development of the hyperdynamic circulation of portal hypertension could influence the effects of ET-1 on the vasculature.

We and others do not support that circulating ET-1 contributes to the development or maintenance of systemic vasodilatation in portal hypertension, as saline- and ET-1-treated PVL animals develop a similar degree of systemic hypotension. Thus ET-1 appears to exert a selective vasoactive effect in the pulmonary vasculature in our system.

The observation that low levels of circulating ET-1 may have a localized effect in the pulmonary vasculature that alters eNOS levels and influences the pulmonary microcirculation is perhaps not unexpected. ET-1 released into the circulation during hepatic injury or infused intravenously will first encounter the pulmonary vasculature. In addition, significant clearance of ET-1 from the circulation occurs in the lung, in large part through an ET β receptor-mediated effect (12). Thus ET-1 may influence pulmonary vascular NO production by increasing eNOS levels and by increasing eNOS enzyme activity, effects that are each mediated through the ET β receptor. Our in vitro and in vivo studies support this concept. However, we cannot be certain that eNOS is not also produced in cell types other than endothelial cells in the vascular wall or may increase in cell types outside the vasculature in the lung. In addition, we have not completely excluded that other forms of nitric oxide synthase may be contributing to changes in pulmonary vascular tone, although we have not found detectable levels of iNOS in the lung in the present studies. Finally, the increase in lung eNOS levels observed after ET-1 infusion is less than that observed in CBDL animals despite the development of a similar degree of vasodilatation and gas exchange abnormalities. This observation suggests that ET-1 may also contribute to intrapulmonary vasodilatation through effects distinct from nitric oxide production in the pulmonary vasculature.

In humans and animals the presence of portal hypertension appears to be required for the development of HPS. Thus we designed our in vivo studies to administer low-dose intravenous ET-1 to animals that normally develop portal hypertension in the absence of pulmonary vascular or hepatic changes to provide a direct means of analyzing whether ET-1 triggers changes in the pulmonary microcirculation in the setting of portal hypertension.
hypertension. We have evaluated the effects of chronic intravenous ET-1 infusion on the liver as well as the lung in PVL animals and have observed no effect on portal venous pressure, liver tests, or hepatic histology. These results confirm a lack of measurable effects of exogenous low-level ET-1 infusion in the liver in PVL animals. Our findings in the lung are consistent with the concept that chronic ET-1 infusion, in the setting of portal hypertension, causes intrapulmonary vasodilatation. Studies evaluating ET-1 infusion in normal animals will define whether portal hypertension is required for ET-1 to alter pulmonary microvascular tone.

Our microsphere assessment of the pulmonary microcirculation provides a direct assessment of small vessel tone and intrapulmonary shunting in unselected animals. The technique is an adaptation of methods used to evaluate intrapulmonary shunting in humans with HPS (1) and portosystemic shunting in anesthetized rats (7) and has been previously employed in PVL animals (11). Results in our saline-treated PVL animals in the present study are similar to those observed in PVL animals previously (11), confirming the reproducibility of this technique. Our ET-1-treated animals shunted significantly more and larger microspheres across pulmonary microcirculation, similar to changes previously observed in CBDL animals with HPS (11). One limitation of this technique is the inability to discriminate between intrapulmonary and intracardiac shunting of microspheres. Thus if ET-1 infusion resulted in a significant selective increase in pulmonary arterial pressures, a gradient for right-to-left intracardiac shunting of microspheres through potential intracardiac septal defects could be created. We did not observe a significant rise in pulmonary arterial pressures in ET-1-treated PVL animals and could find no gross anatomical evidence for the presence of intracardiac septal defects in previous studies (11) or in our present animals.

Our current findings, in conjunction with previous observations, suggest that increased ET-1 production found during certain forms of hepatic injury may stimulate pulmonary vascular eNOS expression and activity and contribute to the pathogenesis of HPS. In vitro studies demonstrate that this effect may be mediated through the ET-B receptor on pulmonary vascular endothelial cells. Together, these observations provide a novel pathogenetic mechanism for the development of HPS. In addition, they provide a direction for future investigation in humans and imply that unique therapeutic approaches focused on inhibiting the pulmonary vascular ET-B receptor or on modulating hepatic ET-1 production may provide useful medical therapies for this disorder.

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