HIGHLIGHTED TOPIC | Pulmonary Circulation and Hypoxia

Hypoxic pulmonary hypertension is prevented in rats with common bile duct ligation

Masatoshi Imamura,1 Bao Luo,2 Jennifer Limbird,1 Andrea Vitello,1 Masahiko Oka, D. Dunbar Ivy,3 Ivan F. McMurtry,1 Chrystelle V. Garat,1 Michael B. Fallon,2 and Ethan P. Carter1

1Cardiovascular-Pulmonary Research Laboratory, Department of Medicine, University of Colorado Health Sciences Center, Denver; 2Department of Internal Medicine and Liver Center, University of Alabama at Birmingham, Birmingham, Alabama; and 3Pediatric Heart Lung Center and Section of Pediatric Cardiology, Department of Pediatrics, University of Colorado Health Sciences Center and The Children’s Hospital, Denver, Colorado

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Imamura, Masatoshi, Bao Luo, Jennifer Limbird, Andrea Vitello, Masahiko Oka, D. Dunbar Ivy, Ivan F. McMurtry, Chrystelle V. Garat, Michael B. Fallon, and Ethan P. Carter. Hypoxic pulmonary hypertension is prevented in rats with common bile duct ligation. J Appl Physiol 98: 739–747, 2005. First published October 29, 2004; doi:10.1152/japplphysiol.00556.2004.—Biliary cirrhosis in the rat triggers intrapulmonary vasodilatation and gas-exchange abnormalities that characterize the hepatopulmonary syndrome. This vasodilatation correlates with increased levels of pulmonary microcirculatory endothelial NO synthase (eNOS) and hepatic and plasma endothelin-1 (ET-1). Importantly, during cirrhosis, the pulmonary vascular responses to acute hypoxia are blunted. The purpose of this work was to examine the pulmonary vascular responses and adaptations to the combination of liver cirrhosis and chronic hypoxia (CH). In addition to hemodynamic measurements, we investigated whether pulmonary expression changes of eNOS, ET-1 and its receptors (endothelin A and B), or heme oxygenase 1 in experimental cirrhosis affect the development of hypoxic pulmonary hypertension. We induced cirrhosis in male Sprague-Dawley rats using common bile duct ligation (CBDL) and exposed them to CH (inspired PO2 = 76 Torr) or maintained them in Denver (Den; inspired PO2 = 122 Torr) for 3 wk. Our data show 1) CBDL-CH rats had a persistent blunted hypoxic pulmonary vasoconstriction similar to CBDL-Den; 2) the development of hypoxic pulmonary hypertension was completely prevented in the CBDL-CH rats, as indicated by normal pulmonary arterial pressure and lack of right ventricular hypertrophy and pulmonary arteriole remodeling; and 3) selective increases in expression of ET-1, pulmonary endothelin B receptor, eNOS, and heme oxygenase 1 are potential mechanisms of protection against hypoxic pulmonary hypertension in the CBDL-CH rats. These data demonstrate that unique and undefined hepatic-pulmonary interactions occur during liver cirrhosis and chronic hypoxia. Understanding these interactions may provide important information for the prevention and treatment of pulmonary hypertension.

HEPATOPULMONARY SYNDROME is a triad of advanced liver disease, intrapulmonary microvascular dilation, and arterial hypoxemia (6, 11). Blunted hypoxic pulmonary vasoconstriction occurs in many cirrhotic patients and is believed to contribute to ventilation-perfusion mismatching and arterial hypoxemia (Ref. 5, see Ref. 27 for review). Hepatopulmonary syndrome occurs in at least 15% of patients with end-stage liver disease but is thought to be widely underdiagnosed. Mortality within 1 yr of diagnosis is markedly increased in cirrhotic patients with hepatopulmonary syndrome compared with patients without hepatopulmonary syndrome (33).

A well-established experimental model of hepatopulmonary syndrome is biliary cirrhosis in rats induced by common bile duct ligation (CBDL) (8, 13). Our laboratories and others have reported that intrapulmonary vasodilatation and blunted hypoxic vasoconstriction are central to ventilation-perfusion mismatching leading to arterial hypoxemia (5, 13, 28, 35). In addition, expression of vasoactive mediators [e.g., NO/endothelial NO synthase (eNOS)] (5, 12); endothelin (ET-1) (5, 24), and carbon monoxide/heme oxygenase (HO-1) (4, 38)] in the lung and liver is altered. Histological analysis reveals pulmonary artery-to-pulmonary vein anastomoses and dilated alveolar capillaries in this model (13, 34, 35). Importantly, and the focus of this paper, the blunted hypoxic vasoconstriction demonstrates that during cirrhosis the lung’s ability to respond to acute hypoxia is compromised, raising the question of how the lung responds and adapts to chronic hypoxia during cirrhosis.

The pulmonary vascular adaptations to acute and chronic hypoxia and liver cirrhosis are linked through the actions of ET-1 and its receptors ETA and ETB, NO, and HO-1. Chronic hypoxia and cirrhosis independently increase circulating ET-1 levels, but apparently with dramatically different effects on the pulmonary circulation due to alterations in the relative expression of the ETA and ETB receptors (ETA-R and ETB-R) on vascular smooth muscle cells and vascular endothelial cells, respectively. Chronic hypoxia leads to persistent vasoconstriction and pulmonary hypertension that is largely, if not solely, mediated by the elevated circulating and/or lung tissue ET-1 acting on the ETA receptors (3). In contrast, during cirrhosis, elevated ET-1 levels mediate pulmonary vasodilatation via overexpression of ETB receptors located on vascular endothelial cells (24). Pulmonary eNOS expression is also increased during chronic hypoxia (18), presumably to counteract elevated
pulmonary vascular resistance, although the level of NO production might not be higher (26). The experimental overexpression of either eNOS or HO-1 protects against the development of hypoxic pulmonary hypertension (7, 9) and vascular smooth muscle cell proliferation (25), as does ETA receptor blockade (2). Our laboratory and others have demonstrated that pulmonary eNOS and HO-1 are increased during cirrhosis (4, 5, 12), consistent with blunted hypoxic pulmonary vasoconstriction and pulmonary vasodilation.

The purpose of this work was to examine the pulmonary vascular responses to the combination of liver cirrhosis and chronic hypoxia, specifically investigating the role of endothelin receptors in the lung, as well as expression of other critical vasoactive mediators. To study the interaction of chronic hypoxia and liver cirrhosis on the pulmonary circulation, we induced cirrhosis in male Sprague-Dawley rats using CBDL and exposed them and sham-surgery rats (Sham) to chronic hypoxia [CH; inspired PO2 (PIO2) ≈ 76 Torr] or maintained both groups in Denver (Den; PIO2 ≈ 122 Torr) for 3 wk. Our primary findings were that CBDL-CH rats had blunted responses and adaptations to both acute and chronic hypoxia. These rats had blunted hypoxic pulmonary vasoconstriction similar to CBDL-Den rats, indicating that acute hypoxic vasoreactivity was similarly blunted in both CBDL-Den and CBDL-CH rats. Compared with Sham-CH rats, the CBDL-CH rats showed little, if any, signs of pulmonary vascular remodeling and pulmonary hypertension. We propose that selective upregulation of endothelial ETB receptors in the lung after CBDL and elevated hepatic production of ET-1 are the primary mechanisms conferring protection against the development of hypoxic pulmonary hypertension. In addition, the overexpression of eNOS and HO-1 provide potential mechanisms of protection. Some of these data have been previously reported in abstract form (16).

METHODS

Animal model of liver cirrhosis and chronic hypoxia. Biliary cirrhosis in rats was induced by CBDL. Details of the surgery and postsurgical care have been previously reported (5, 8, 13). The surgical procedures are approved by the Institutional Animal Care and Use Committee at the University of Colorado Health Sciences Center. Male Sprague-Dawley rats (200–250 g body wt) were allowed to acclimate to Denver’s altitude (1,609 m) for 1 wk before any experimental protocols. Animals had continuous access to food and water. One day after CBDL or sham operation, rats were either maintained at Denver’s altitude (Den; 1,609 m, barometric pressure 630 mmHg; PIO2 ≈ 122 Torr) or moved to a chronic hypobaric hypoxia chamber (CH; simulated 5,150 m, barometric pressure at Denver’s altitude (Den; PIO2 ≈ 122 Torr) for 3 wk. Our primary findings were that CBDL-CH rats had blunted responses and adaptations to both acute and chronic hypoxia. These rats had blunted hypoxic pulmonary vasoconstriction similar to CBDL-Den rats, indicating that acute hypoxic vasoreactivity was similarly blunted in both CBDL-Den and CBDL-CH rats. Compared with Sham-CH rats, the CBDL-CH rats showed little, if any, signs of pulmonary vascular remodeling and pulmonary hypertension. We propose that selective upregulation of endothelial ETB receptors in the lung after CBDL and elevated hepatic production of ET-1 are the primary mechanisms conferring protection against the development of hypoxic pulmonary hypertension. In addition, the overexpression of eNOS and HO-1 provide potential mechanisms of protection. Some of these data have been previously reported in abstract form (16).

Isolated perfused lung experiments. Isolated lungs studies were performed on six groups of animals: Sham or CBDL maintained at Denver’s altitude (Sham-Den, CBDL-Den) or exposed to chronic hypoxia (Sham-CH, CBDL-CH) and ETα-deficient transgenic rats (sl/sl) Sham or CBDL (Sham-sl/sl, CBDL-sl/sl). After intraperitoneal administration of 30 mg of pentobarbital sodium and intracardiac injection of 100 IU of heparin, the pulmonary artery and left ventricle were cannulated, with the heart-lung block remaining intact. CH lungs were isolated within 10 min of removing the rats from the hypoxic chamber. After an initial baseline period lasting ~20 min, hypoxic vasoreactivity was assessed by switching the O2 content of inspired air from 21% O2 to 3% O2. Pulmonary arterial pressure (Ppa) was monitored continuously during these maneuvers.

Arterial blood-gas measurement and hemodynamic measurements in vivo. At 3 wk after each condition, in vivo hemodynamics and hypoxic pressor response were measured as previously described (4). Cardiac output (CO) was measured by standard dye-dilution technique and calculated by computer. Hemodynamic measurements were performed on anesthetized rats while all the animals were breathing room air and also subsequently while they breathed hypoxic gas (inspired O2 fraction = 0.03). Pulmonary vascular resistance (PVR, mmHg·ml−1·min−1) was calculated as follows: mean Ppa (mmHg)/CO (ml/min). After hemodynamic measurements, rats were killed with an overdose of pentobarbital (30 mg ip). A thoracotomy verified the position of the catheters. Heats were dissected for measurement of right ventricle (RV) and left ventricle plus septum (LV + S) weights. The RV-to-LV+S weight ratio [RV/(LV+ S)] was calculated to assess RV hypertrophy, a hallmark of hypoxia pulmonary hypertension (31). RV hypertrophy and hematocrit were measured in all rats.

Histology and morphometric analysis. Lung and liver tissue were collected and fixed for histological analysis. After an overdose of pentobarbital, residual blood was washed out of the livers and lungs with phosphate-buffered saline (PBS; pH 7.4, 37°C, 20 cmH2O) followed by 4% paraformaldehyde in PBS from the aorta and pulmonary artery, respectively. During perfusion, the lungs were gently inflated with a 1% agarose-1% paraformaldehyde mixture through the trachea. Both lung and liver were fixed in 4% paraformaldehyde in PBS overnight, dehydrated, embedded in paraffin, cut into 3-μm-thick slices, and stained with hematoxylin and eosin. Under ×200 magnification with Axiovision (Carl Zeiss, Thornwood, NY), pulmonary arteries of 50–100 μm in diameter were chosen. Vessel perimeter was measured by use of NIH image 1.63 software, and the vessel radius was calculated as radius = perimeter/2π.

The wall thickness of vessels was measured at every 90° of circumference, and the ratio of the average of the wall thickness divided by the radius was used as index to quantitate medial wall thickness.

Western blot analyses. Standard techniques were used to collect tissue and prepare tissue homogenates (5). The protein concentration was determined for each sample by using BCA protein assay (Pierce, Rockford, IL). Proteins were separated on SDS-PAGE and electrotransferred to PVDF membranes (Invitrogen, Carlsbad, CA). To confirm that equal amounts of proteins were loaded, membranes were stained with 0.1% Ponceau S in 5% acetic acid (Sigma) for 5 min. For destaining, blots were washed with deionized water for 2 min before blocking and primary antibody incubation. The membranes were probed with a monoclonal antibody against eNOS (BD Biosciences, San Jose, CA) or polyclonal antibodies against HO-1 (StressGen Biotech, Victoria, BC, Canada), and ETα-R and ETβ-R (Calbiochem, San Diego, CA), followed by addition of horseradish peroxidase-conjugated secondary antibodies. Antigenic detection was visualized by enhanced chemiluminescence (Pierce, Rockford, IL) with exposure to X-ray film. Densitometry was performed with a scanner and Kodak 1D Image Analysis software (version 3.5).

ET-1 mRNA and peptide expression. To determine how the interaction of chronic hypoxia and liver affects ET-1 expression in the
RESULTS

Blunted hypoxic vasoreactivity persists after chronic hypobaric hypoxia. To investigate the impact that CBDL and CH have on hypoxic pulmonary vasoreactivity, we assessed hypoxic vasoreactivity in isolated perfused lungs from Sham and CBDL rats that had been housed either at Denver’s altitude or in chronic hypobaria hypoxia for 3 wk. From representative tracing data (Fig. IA, top) and the summary data from these experiments (Fig. IA, bottom), it is evident that a strong hypoxic pressor response (HPR) was present in Sham-Den rats, which was significantly blunted in the CBDL-Denver rats (Fig. 1A). This blunted hypoxic pressor response was as we expected and have previously reported in CBDL rats (5). Although chronic hypoxia did not alter or possibly even potentiated the hypoxic pressor response in the sham rats (Sham-CH, Fig. IA), hypoxic vasoreactivity was blunted in the CBDL-CH rats similar to that in the CBDL-Den rats (Fig. 1A). Pulmonary artery perfusion pressure was predictably elevated in the Sham-CH lungs, but surprisingly it was not elevated in the CBDL-CH lungs (Fig. 1A, top). This observation prompted us to investigate in vivo systemic (Table 1) and pulmonary hemodynamics (Fig. IB) using systemic and pulmonary vascular catheters to measure systemic arterial pressure, Ppa, CO, and HPR. It is well documented that rats with cirrhosis develop both hyperdynamic circulatory state and hepatopulmonary syn-

lung, real-time reverse transcriptase PCR analysis was used. Total RNA was isolated from frozen lungs by use of the RNeasy RNA extraction kit from Qiagen (Valencia, CA). Reverse transcription was performed on 5 µg of total RNA per sample by established protocols. TaqMan real-time quantitative PCR assay was performed on an ABI Prism 7700 sequence-detection system, according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). The following ET-1 primer sequence was used: ET-1, forward primer: 5'-GCT CCT TGA TGG ACA AGG-3', reverse primer: 5'-AGG GCT TCC TAG TCC ATA CGG-3'. All amplifications were done in triplicate in 96-well plates. All samples were incubated at 50°C for 2 min and at 95°C for 10 min then cycled at 95°C for 15 s and 60°C for 1 min for 40 cycles.

Levels of ET-1 peptide in lung were measured by ELISA analysis as previously described (5).

Statistical analysis. All data are means ± SE. Comparisons between two groups were made with unpaired Student’s t-test. Comparisons between three or more groups were made with ANOVA followed by Tukey-Kramer post hoc analysis. In all cases, P < 0.05 was considered statistically significant.

Table 1. Physical and hematological characteristics

<table>
<thead>
<tr>
<th></th>
<th>Sham-Den</th>
<th>Sham-CH</th>
<th>CBDL-Den</th>
<th>CBDL-CH</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>367.5±11.3</td>
<td>317.8±9.0</td>
<td>318.8±13.0</td>
<td>255.3±5.4†</td>
</tr>
<tr>
<td>Systemic arterial pressure, mmHg</td>
<td>119.8±5.3</td>
<td>122.5±5.6</td>
<td>92.0±9.9‡</td>
<td>89.8±6.8‡</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>46.8±1.2</td>
<td>67.9±2.3§</td>
<td>46.5±1.3</td>
<td>55.8±0.6§</td>
</tr>
<tr>
<td>Biliirubin, mg/dl</td>
<td>0.11±0.02</td>
<td>0.15±0.03</td>
<td>5.28±0.14§</td>
<td>5.28±0.55‡</td>
</tr>
<tr>
<td>Bile acid, µM</td>
<td>22.9±0.8</td>
<td>17.4±4.7</td>
<td>234.6±45.0</td>
<td>248.9±42.2‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–10 rats per group. Den, Denver; CH, chronic hypoxia; Sham, sham operated; CBDL, common bile duct ligated. *Significantly different from Sham-CH, CBDL-Den, and CBDL-CH; P < 0.05. †Significantly different from Sham-Den, Sham-CH, and CBDL-Den; P < 0.05. ‡Significantly different from Sham-Den and Sham-CH; P < 0.05. §Significantly different from Sham-Den, CBDL-Den, and CBDL-CH; P < 0.05.
drome with intrapulmonary vascular dilatation and an increased alveolar-to-arterial $O_2$ tension difference. Systemic arterial pressure was reduced in both CBDL-Den and CBDL-CH rats (Table 1), reflecting the hyperdynamic circulation during cirrhosis. As indicated in the isolated lung data and reinforced by earlier reports (30), chronic hypoxic exposure caused significant pulmonary hypertension (Fig. 1, top). Surprisingly, the CBDL-CH rats did not develop pulmonary hypertension (Fig. 1, bottom). To consider the CO effects, we calculated the total PVR. PVR, computed from Ppa and CO, decreased significantly in cirrhotic rats compared with sham animals. There was no difference between CBDL-Den and CBDL-CH groups (Fig. 1, top). Finally, as observed in isolated lungs (Fig. 1A), the hypoxic pressor response was blunted in both the CBDL-Den and CBDL-CH rats (Fig. 1B, bottom).

Hypoxic pulmonary vascular remodeling and right ventricular hypertrophy are prevented in CBDL rats. On the basis of the observations made during the hemodynamic studies that Ppa was normal in the CBDL-CH rats, we assessed pulmonary arterial vascular remodeling using histological techniques and computed RV hypertrophy from RV/(LV + S) gravimetric measurements. These analyses clearly demonstrated that pulmonary vascular remodeling was completely prevented in the CBDL-CH rats compared with the robust remodeling in the Sham-CH rats (Fig. 2A). This histological data was supported statistically by morphometric analyses of vessel wall thickness and lumen diameter (Fig. 2B). Consistent with the histological, morphometric, and Ppa data, right ventricular mass [RV/(LV + S)] was significantly greater in the Sham-CH rats compared with the Sham-Den ($P < 0.05$), and both CBDL groups were similar to the Sham-Den, as well (Fig. 2C).

Analysis of ET-1, $ET_A$-R, $ET_B$-R, eNOS, and HO-1 expression. To identify potential mechanisms of protection from hypoxic pulmonary hypertension, we investigated the expression of three vasoactive mediators (ET-1, eNOS, and HO-1) because of their demonstrated importance in the vascular pathologies of liver cirrhosis and hypoxic pulmonary hypertension (4, 5, 12).

Expression of ET-1, $ET_A$-R, and $ET_B$-R was evaluated by ELISA, real-time PCR, and Western blotting. Lung ET-1 peptide levels were slightly increased in Sham-CH rats compared with the Sham-Den group, but this did not reach statistical significance (Fig. 3A). In the CBDL groups, peptide levels were significantly lower in the CBDL-Den but significantly higher in the CBDL-CH compared with the Sham-Den (Fig. 3A, $P < 0.05$). These data were repeated using real-time PCR to evaluate levels of ET-1 precursor mRNA, preproET-1 (ppET-1). Notably, pulmonary ET-1 expression was decreased in CBDL-Den lungs compared with both the Sham-Den and CBDL-CH lungs (Fig. 3B). There were no significant differences between CBDL-CH and Sham-Den groups. Pulmonary ET receptor expression was highly subtype dependent. $ET_A$-R expression was lower in both of the CBDL groups compared with Sham (Fig. 4A, $P < 0.05$), whereas $ET_B$-R expression was higher in both of the CBDL groups compared with Sham-Den (Fig. 4B, $P < 0.05$).

To verify the significance of the upregulation of $ET_B$-R in CBDL-Den compared with Sham-Den, and whether the increase in $ET_B$-R expression contributed to the blunted hypoxic pressor response (Fig. 1A), we utilized a special breed of rats that are lacking $ET_B$ receptor in the pulmonary vasculature (slsl rats). This genetic model was produced by rescue of the spotting lethal rat, which is a naturally occurring rat strain that
carries a 301-bp deletion in ETB, rendering the gene nonfunctional (14). These rats underwent bile duct ligation procedure and were studied 3 wk after surgery. Interestingly, we found that the hypoxic pressor response measured in isolated perfused lungs from CBDL-sl/sl was identical compared with those measured in Sham-sl/sl (HPR values were 5.58 ± 1.33 mmHg for CBDL-sl/sl compared with 4.43 ± 0.81 mmHg for Sham-sl/sl). These data clearly demonstrate that ETB is an important mediator of pulmonary vasodilation during cirrhosis.

Similar results were recently reported by Luo et al. (24). They reported that pulmonary microvascular ETB receptor levels rose as hepatopulmonary syndrome (HPS) developed, and these levels were correlated with the severity of gas-exchange abnormalities. In contrast, they found unchanged ETA expression documenting that the response was specific for ETB receptor. In their report, selective ETB receptor blockade ameliorated HPS, whereas selective ETA receptor blockade increased intravascular macrophage accumulation and did not improve intrapulmonary shunting. In our study, we reported a decrease in ETA expression levels in both CBDL groups compared with Sham. Further studies need to be done to examine the role of ETA-R regulation in cirrhosis.

As predicted, both CBDL treatment and chronic hypoxia increased pulmonary eNOS expression (Fig. 5A, *P < 0.05). These effects appeared to be additive because the level of expression was greater in lungs of CBDL rats exposed to chronic hypoxia compared with similarly treated Sham rats. Taken together, these data demonstrate that independent mechanisms governing eNOS expression are operating during cirrhosis and hypoxia.

The profile of pulmonary HO-1 expression was different compared with eNOS. HO-1 was potently induced by CBDL (*P < 0.05) with little or no regulation by chronic hypoxia (Fig. 5B). The high level of expression in the CBDL-Den lungs was sustained but not increased by chronic hypoxia (Fig. 5B).

Finally, to make sure that these changes in protein expression were not due to unequal protein loading, each membrane was stained with 0.1% Ponceau S in 5% acetic acid for 5 min and then washed with deionized water before being blocked and primary antibody incubation (data not shown).

Hematological data. The levels of bilirubin, bile salts, and hematocrit were measured in the serum and blood from all four groups of rats. As expected, both bilirubin and bile salts were...
significantly elevated in the CBDL-Den rats ($P < 0.05$). This was not altered by chronic hypoxia, as the CBDL-CH rats had similarly elevated bilirubin and bile salts values (Table 1). Neither bilirubin nor bile salts were directly regulated by hypoxia alone, as the Sham-CH levels were not different from the Sham-Den controls (Table 1).

The hematocrit analysis yielded surprising results. Whereas the Sham-CH rats had the predicted polycythemic response, the CBDL-CH hematocrit values were considerably lower, being intermediate between the Sham-CH values and those of the Sham-Den controls (Table 1).

The purpose of this work was to examine the pulmonary vascular responses to the combination of liver cirrhosis and chronic hypoxia to determine whether the responses and adaptations to chronic hypoxia are blunted during cirrhosis, as the acute responses are. Our results fall into two categories: 1) documenting the pulmonary vascular adaptations to chronic hypoxia in CBDL rats and 2) exploring potential mechanisms for the observed responses to chronic hypoxia. Our most prominent observation was that CBDL rats exposed to chronic hypobaric hypoxia did not develop pulmonary hypertension. This observation is supported by hemodynamic measurements, histological and morphometric analyses, and hematological data. Analyses of protein and gene expression were used to identify potential mechanisms of the blunted pulmonary responses to chronic hypoxia in the CBDL rats. These experiments identified selective increase expression of ET-1 and its ET_{B} receptor, as well as eNOS-NO and HO-1-CO as potential mechanisms of protection.

Previously, we have used both isolated perfused lungs and catheterized rats to evaluate pulmonary vasoreactivity during cirrhosis and hypoxic pulmonary hypertension (4, 5, 17). Isolated lungs have proven very useful in characterizing the perfusion characteristics and vasoreactivity during both CBDL-induced cirrhosis and pulmonary hypertension. In CBDL rats, isolated lungs have been used to characterize the causes and features of blunted hypoxic vasoconstriction that occurs during hepatopulmonary syndrome (5, 8). Similar to the results reported by Chang and Ohara (8), we found that cirrhotic rats exhibited increased CO, normal Ppa, a decreased pulmonary vascular resistance, and a marked depression of HPR compared with Sham-operated rats. Blunted hypoxic vasoreactivity was due to the combined effects of increased ET-1/endothelial ET_{B} receptor expression, NO synthesis, and HO-1 expression, all of which open calcium-activated K^+ (K_{Ca}) channels in the pulmonary artery vascular smooth muscle cells (4, 5, 12, 24). Our perfused lung experiments showed that the CBDL-CH rats had blunted hypoxic pulmonary vasoconstriction, similar to the CBDL-Den rats, demonstrating that the acute responses to hypoxia after bile duct ligation were not impacted by exposure to chronic hypoxia. An important observation in the perfused lung experiments was that, whereas the initial perfusion pressure was predictably elevated in the Sham-CH lungs, it was not elevated in the CBDL-CH lungs, suggesting that bile duct ligation modified or blunted the pulmonary adaptations to chronic hypoxia. It was this observation that led us to examine more closely the responses and adaptations to chronic hypoxia.

The use of arterial and venous catheters in rats from our four groups demonstrated that, although the CBDL-CH rats had persistent blunted hypoxic pulmonary vasoreactivity (similar to CBDL-Den rats), they also failed to develop pulmonary hypertension as the Sham-CH rats did. Specifically, Sham-CH rats had Ppa values of $\sim 44$ mmHg, whereas the CBDL-CH rats had Ppa values of $\sim 22$ mmHg, not different from either of the Denver groups.

In light of our observation of normal Ppa in the CBDL-CH rats, we used histological and morphometric analyses to evaluate pulmonary arterial structure and remodeling. Vessel wall thickness (WT) and radial length (r) were measured in vessels ranging in size from 50 to 100 $\mu$m to compute WT/r. Over 40 vessels from 16 rats (4 from each group) were analyzed. In addition, Ppa was measured in each rat before death and tissue fixation to verify the degree of pulmonary hypertension. Whereas pulmonary vascular remodeling was robust in all of
the Sham-CH rats, the lack of hypoxic remodeling was equally reproducible in all of the CBDL-CH rats. We were not able to discern from our data whether vascular smooth muscle proliferation and remodeling in the CBDL-CH rats was prevented outright or whether hypoxia- and ET-1-mediated proliferation was negated by proapoptotic actions of NO, HO-1, or bilirubin (1, 20, 23).

There are very few interventions cited in the literature that have so completely prevented the development of hypoxic pulmonary hypertension. The results from the analyses of ET-1 and its receptors, eNOS, and HO-1 all indicate potential roles for the prevention of hypoxic pulmonary hypertension. ET-1 is most widely recognized as a potent vasoconstrictor via the ET_A receptor located on vascular smooth muscle. Pulmonary ppET-1 and its protein product ET-1 are upregulated by hypoxia (21), and hepatic ET-1 is upregulated during cirrhosis and bile duct ligation (22). Although we did not measure hepatic ET-1 levels, our results fit with these previous reports. During hypoxic pulmonary hypertension, the upregulation of ET-1 is thought to be central to both the vasoconstriction and increased vascular resistance as well as mediating the mitogenic proliferation of vascular smooth muscle cells. In the present work, these actions of ET-1 appeared to be blunted or negated because the CBDL-CH rats had normal Ppa and vascular structure despite having elevated ppET-1 expression. The basis for this observation is unknown but appears to involve the regulation of endothelin receptors. Recent work by one of the coauthors has shown that, after CBDL, pulmonary endothelial ET_B-R is upregulated, which is responsible for the pulmonary vasodilation that occurs during the experimental hepatopulmonary syndrome (24). Activation of the endothelial ET_B-R mediates vasodilation by increasing NO production by eNOS and prostacyclin synthesis. In our study, we found that ET_B-R was upregulated in CBDL rats. To verify whether this increase contributed to the blunted HPR, we utilized a special breed of rats that are lacking ET_B receptor in their pulmonary vasculature. This genetic model was produced by rescue of the spotting lethal rat, which is a naturally occurring rat strain that carries a 301-bp deletion in ET_B, rendering the gene nonfunctional (14). These rats underwent bile duct ligation procedure and were studied 3 wk after surgery. Interestingly, we found that CBDL transgenic rats did not show a blunted HPR and their response was identical to those measured in Sham transgenic rats. Our data clearly demonstrate that specific upregulation of the ET_B-R is protective against the development of hypoxic pulmonary hypertension and support recent report by Luo et al. (24). Still the role of ET_A-R in the development of HPS remains unclear. To our knowledge, only one source reported some findings on ET_A expression regulation and cirrhosis. Luo et al. reported that lung ET_A-R levels were unchanged after CBDL. Also, in contrast with ET_B-R blockade experiments that improved HPS with a marked reduction of intrapulmonary shunting, these authors reported no improvement of alveolar-arterial PO_2 difference in response to ET_A-R blockade, suggesting a different role for ET_A-receptor during HPS. They suggested a potential role for ET_A-R in modulation of pulmonary intravascular macrophage accumulation and activation. We found that ET_A expression levels decreased in CBDL groups compared with Sham, which was not consistent with these authors’ findings. Further studies need to be done to examine the role of ET_A-R regulation during cirrhosis.

Induction of HO-1 has been shown to prevent the development of hypoxic pulmonary hypertension (9). By use of either NiCl_2 or hemin to induce HO-1, rats exposed to 7 days of normobaric hypoxia (10% O_2) were protected from the development of pulmonary hypertension and vascular remodeling, similar to what we reported here after CBDL. The more than fourfold induction of lung HO-1 by either hemin or NiCl_2 is of a similar magnitude observed here and in our earlier reports after CBDL (4). Taken together, these data indicate that HO-1 may have important protective actions against the development of pulmonary hypertension and vascular remodeling. These protective actions likely involve the vasodilatory and/or antioxidant properties of HO-1 (29) and will be studied further.

To further understand the unique interactions between the injured liver and pulmonary hypertension, we also investigated the expression of eNOS. eNOS is selectively regulated during hypoxia and liver cirrhosis (5, 21). During hypoxia, pulmonary eNOS (and possibly inducible NO synthase) expression and NO production are upregulated according to some (18), but not all reports (32). Upregulation is thought to be due to increased vascular wall shear stress that is the result of global hypoxic vasoconstriction occurring throughout the lung (39). During liver cirrhosis, pulmonary eNOS expression and NO production are upregulated by uncertain mechanisms (5, 12). Controversy still exists about which NO synthase isomers are involved in increased lung NO production in liver disease. For example, some report by Nunes et al. (28) has suggested that the increase in pulmonary NO production was dependent primarily on increases in the expression and activities of inducible NO synthase within pulmonary intravascular macrophages. On the other hand, we and others (38) have shown that intrapulmonary vasodilatation characterizing HPS correlates with increased levels of pulmonary microcirculatory eNOS and hepatic and plasma endothelin-1 (ET-1). Our data clearly show that the mechanisms controlling pulmonary NO during hypoxia and cirrhosis are acting independently and that the response to each is additive. Because the CBDL-CH rats displayed elevated pulmonary eNOS expression above CBDL treatment alone, and this was in the absence of pulmonary hypertension and presumably shear stress, alternate, nonhemodynamic mechanisms such as hypoxia alone or cytokine and hormonal factors must be governing its expression (19).

Serum analysis yielded the predicted increases in bilirubin and bile salts in the CBDL rats. Neither serum bilirubin nor bile salt levels were affected by chronic hypoxia. Although the increase of both bilirubin and bile salts are a direct result of the hepatic injury caused by bile duct ligation (37), these compounds have several known actions that could be important in the analysis and interpretation of our physiological data. Bilirubin has potent antioxidant properties by efficiently scavenging peroxyl radicals, thereby inhibiting lipid peroxidation (36). Although hypoxia has not traditionally been considered to create an oxidant-rich milieu, recent evidence demonstrated that oxidants are generated during hypoxia (15). Antioxidant protection conferred by the elevated bilirubin in the CBDL-CH rats may be a contributing factor in the protection of the pulmonary vasculature to chronic hypoxia. Similarly, bile salts are potent vasodilators that act through the direct activation of K_{Ca} channels in vascular smooth muscle cells (10). We
showed previously that \( \text{K}_{\text{Ca}} \) channel activation was central to the blunted hypoxic pressor response in CBDL rats (5). The activation of this pathway may be protective against the vasoconstriction and remodeling caused by chronic hypoxic. Another finding from the blood and serum analysis was that polycythemia was markedly blunted in the CBDL-CH rats compared with the Sham-CH rats. The Sham-CH hematocrit values were 68\%, compared with 55\% in the CBDL-CH and 46\% and 46\% in the Sham-Den and CBDL-Den, respectively. When considered with the physiological responses of the pulmonary circulation (shown in Fig. 1), these data support the hypothesis that the acute and chronic responses and adaptations to \( \text{O}_2 \) are disrupted during cirrhosis.

In conclusion, we demonstrate that hepatic-pulmonary interactions prevent the development of hypoxic pulmonary hypertension. Our data support the conclusion that, during cirrhosis, increased amounts of ET-1 combined with the selective upregulation of the \( \text{ET}_{\text{A}} \)-R are responsible for the protection from chronic hypoxia observed. Other contributing alterations, including increased expression of eNOS and HO-1, likely play important roles in the adaptations and responses of the pulmonary circulation to both acute and chronic hypoxia.

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