Inhibition of $K_{Ca}$ channels restores blunted hypoxic pulmonary vasoconstriction in rats with cirrhosis

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The purpose of this study was to test the hypothesis that increased levels of NO and decreased levels of ET-1 are central to the blunted HPR of the hepatopulmonary syndrome. The expression and activity of both pulmonary eNOS expression and NO production during cirrhosis.

In the pulmonary circulation, a predominant vasoconstrictor opposing the action of NO is ET-1 (3, 12, 21). ET-1 acts through two $G$ protein-coupled receptors, the $\mathrm{ET}_A$ and $\mathrm{ET}_B$ receptors, and is involved in the mechanism of hypoxic pulmonary vasoconstriction via the $\mathrm{ET}_A$ receptor (1, 18). In cirrhosis, hepatic ET-1 is upregulated, consistent with the presence of portal hypertension (13). ET-1 expression in pulmonary vascular tissue during cirrhosis is unknown; however, based on the vasodilation and blunted HPR in the cirrhotic lung, it is possible that ET-1 expression and activity are decreased in response to the elevated NO.

The purpose of this study was to test the hypothesis that increased levels of NO and decreased levels of ET-1 are central to the blunted HPR of the hepatopulmonary syndrome. The expression and activity of both hypoxia, biliary cirrhosis, and intrapulmonary vasodilation (5, 6, 8, 9). Although the systemic vascular changes associated with CBDL have been widely studied, the pulmonary vascular changes are less well documented. Anatomically, the lungs of CBDL rats have larger diameter capillary beds (9, 19) as well as increased alveolar capillary density (19). There are also signs of angiogenesis in several locations in the pulmonary vascular bed, but this has been deemed to be minor relative to the dilation and density changes (19).

There is evidence that nitric oxide (NO) is central to the vascular changes in hepatopulmonary syndrome, namely the intrapulmonary shunting and blunted hypoxic pressor response (HPR). Clinically, cirrhotic patients have elevated levels of exhaled NO (16), and in CBDL rats, there is a strong correlation between intrapulmonary shunting and lung endothelial NO synthase (eNOS) protein expression (8). Pulmonary arterial rings from CBDL rats have a blunted response to phenylephrine that is reversed by NOS inhibition (8). Recent evidence (24) has suggested that circulating endothelin (ET)-1 may be responsible for the increases in pulmonary eNOS expression and NO production during cirrhosis.

In the lung, hypoxic vasoconstriction is mediated by the calcium-activated potassium channel; intrapulmonary shunting; liver cirrhosis; nitric oxide; calcium-activated potassium channel; endothelin-1

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eNOS and ET-1 were investigated in lungs from sham and CBDL cirrhotic rats. Evidence is provided that although CBDL-induced cirrhosis is associated with increased expression of NO and decreased expression of ET-1 in the lung, the blunted HPR is due to factors beyond the acute vasoactive effects of these mediators and appears to involve stimulation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} (K\textsubscript{Ca}) channels.

**METHODS**

**Animal Model of Liver Cirrhosis**

Biliary cirrhosis was induced in rats by ligation of the common bile duct (5, 6, 9). The surgical procedures were approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center (Denver, CO). Male Sprague-Dawley rats (body wt 200–250 g) were allowed to acclimate to Denver’s altitude (1,500 m) for 1 wk before any experimental protocols were performed. Animals had continuous access to food and water. Laparotomy was performed under anesthesia with intramuscular ketamine (100 mg/kg) and xylazine (4 mg/kg). The bile duct was isolated, doubly ligated with 3-0 silk, and resected between the two ligatures. The abdominal wall was closed with 4-0 silk sutures, antibiotic sulfa powder was sprinkled over the closure, and the skin was closed with 4-0 silk sutures. Buprenorphine (0.25 mg/kg) was given subcutaneously twice during the first 24 h after surgery to alleviate postsurgical discomfort. Sham animals underwent laparotomy, bile duct isolation with no ligation and resection, and closure of the surgical opening. In general, experiments were carried out 2–5 wk after surgery. Liver injury was evaluated by measuring serum levels of bilirubin with a colorimetric bilirubin direct assay (Sigma, St. Louis, MO) and by histological analysis (see Histological Analysis).

**Measurement of Plasma Nitrite and Nitrate Levels**

A NO chemiluminescence analyzer (NOA 280, Sievers Research, Boulder, CO) was used to measure levels of plasma nitrite and nitrate levels (NO\textsubscript{x}: NO, NO\textsubscript{2}, NO\textsubscript{3}, nitrosothiols, and peroxynitrite) in plasma (23). Aliquots of plasma (1 \textmu l) were added to 2 ml of 0.1 M vanadium chloride (type II, Aldrich, Milwaukee, WI) dissolved in 1 N HCl and heated to 90°C in the purge vessel of the NO analyzer to reduce all NO\textsubscript{x} to NO. The liberated NO was driven into the chemiluminescence chamber by bubbling the reaction mixture with argon. Calibration curves for NO\textsubscript{x} levels were generated daily by measuring the optical density at 260 nm (= 1 for 40 \mu g/ml of RNA), and the purity was assessed by determining the ratio of the optical density at 260 nm to that at 280 nm (= 2.0 for pure RNA) with a Beckman DU-640 spectrophotometer. Total RNA (20 \mu g) was electrophoresed through a formaldehyde-agarose gel and blotted to a nylon membrane via capillary transfer. RNA was immobilized on the nylon membrane by baking the membrane at 80°C for 2 h. ET-1 mRNA was detected with a 2.0-kb cDNA probe for preproET-1 (ppET-1) labeled with \[^{32}P\]dCTP by random-primer labeling (RTS RadPrime DNA labeling system, Gibco BRL, Life Technologies, Gaithersburg, MD). 18S rRNA levels were measured by hybridization with an oligonucleotide probe (ACGGTAGTGATCGCTCGAA) labeled with \[^{32}P\]dCTP via terminal deoxynucleotidyltransferase. After hybridization, blots were washed at room temperature in 1\times saline-sodium citrate (SSC; 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)-0.1% SDS (low stringency) and then at 65°C in 0.4\times SSC-0.1% SDS (high stringency). Autoradiographs were obtained by exposing the blots to phosphorimaging cassettes, and densitometry was performed with a phosphorimaging system with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Western Blot Analysis of eNOS Expression**

As with the Northern blot analysis, standard techniques were used to evaluate protein expression. The right lung from the blood-free perfusion was homogenized in 250 mM sucrose, 25 mM imidazole, 1 mM EDTA, and a one-tenth volume of a protease solution (25 \mu g/ml of antipain, 1 \mu g/ml of soybean trypsin inhibitor, and 200 \mu M phenylmethylsulfonyl fluoride). After homogenization, samples were sonicated, spun at low speed to clear debris, and frozen at −80°C until assayed for protein content. SDS-PAGE and immunoblotting were performed on 25 \mu g of protein. Samples were electrophoresed through a 7.5% acrylamide gel. eNOS protein expression was detected with a monoclonal antibody (Transduction Laboratories, Lexington, KY). The primary antibody was diluted 1:500 in Tris-buffered saline-Tween 20 containing 5% dry milk. The secondary antibody (sheep antinouse conjugated to horseradish peroxidase) was diluted 1:17,000 in Tris-buffered saline-Tween 20 containing 5% dry milk. Antigenic detection was by enhanced chemiluminescence (Amersham, Arlington Heights, IL) with exposure to X-ray film. Densitometry was performed with a scanner and NIH Image software (version 1.61).

**Measurement of ET-1 Peptide Levels**

For measurement of ET-1 peptide levels, a commercially available kit (Cayman Scientific, Concord, CA) was used, with some slight modifications. Peptide levels were measured in lung homogenate as follows: protein was extracted by adding 10 volumes of 1 M acetic acid-0.1% Triton X-100, boiling for 7 min, and centrifuging for 30 min at 16,000 \texttimes g. The supernatant was sonicated and diluted 1:100 in the acetic acid solution. The diluted homogenate was loaded on methanol-equilibrated C2 columns (Amersham, Arlington Heights, IL), washed with 5 ml of 0.1% trifluoroacetic acid (TFA), and eluted with 2 ml of 80% methanol-0.1% TFA. The eluted samples were dried overnight in a vacuum centrifuge. At this point, the samples were processed per ELISA instructions supplied by Cayman.

**Histological Analysis**

Lungs and livers were perfused free of blood with heparinized PBS. For the lungs, the air space was inflation fixed via
the trachea with 10% buffered Formalin at an inflation pressure of 20 cmH₂O. Livers were perfusion fixed with 10% buffered Formalin. The intact tissues were placed in 10% Formalin for 24 h. Small pieces of lung and liver were paraffin embedded. Paraffin sections 5 μm thick were serially mounted onto Superfrost Plus slides (Fisher Scientific, Fair Lawn, NJ). Hematoxylin and eosin staining was performed on tissue sections from three sham and three CBDL rats to assess histological changes to the lung and liver.

Measurement of Pulmonary Vascular Reactivity

Lungs were isolated from sham and cirrhotic rats after intraperitoneal administration of 30 mg of pentobarbital sodium and an intracardiac injection of 100 IU of heparin. Isolated lungs were ventilated with a humid mixture of 21% O₂-5% CO₂-74% N₂ at 60 breaths/min and at an inspiratory pressure of 9 cmH₂O and an end-expiratory pressure of 2.5 cmH₂O. The lungs were perfused through a cannula in the main pulmonary artery by a peristaltic pump at a constant flow of 0.04 ml·g body wt⁻¹·min⁻¹. The perfusate was a PSS containing (in mM) 116.3 NaCl, 5.4 KCl, 0.83 MgSO₄, 19.0 NaHCO₃, 1.04 NaH₂PO₄, 1.8 CaCl₂·2H₂O, and 5.5 d-glucose. Ficoll (4 g/100 ml, type 70; Sigma) was included as a colloid, and 3.1 × 10⁻⁶ M sodium meclofenamate (Sigma) was added to inhibit synthesis of vasodilator prostaglandins. Lungs were flushed free of blood with 20 ml of PSS and then perfused with a recirculated volume of 30 ml. Effluent perfusate drained from a left ventricular cannula into a perfusate reservoir. Lung and perfusate temperatures were maintained at 38°C, and perfusate pH was kept between 7.3 and 7.4. Mean perfusion pressure was measured continuously with a transducer and pen recorder, and lungs were equilibrated for 20 min before vascular responses were elicited.

Experimental Protocols for Isolated Perfused Lung Experiments

The general experimental paradigm was to first evaluate the HPR under basal conditions by switching the content of the inspired gas from 21% O₂-5% CO₂ to 0% O₂-5% CO₂. The period of hypoxic ventilation was 10 min followed by 10 min of normoxic ventilation. This HPR maneuver was repeated twice for all lungs, and then the specific experimental protocols were initiated. Different animals were used for each of the specified protocols. For all of the pharmacological interventions listed below, HPR was evaluated 10 min after the agent was added to the perfusate, and the timing of each protocol was identical in the sham and CBDL lungs. The number of rats used are listed under each protocol.

Evaluation of NO and cGMP activity on HPR. Five minutes after the second HPR, 100 μM L-arginine (l-Arg; Sigma) was added to the perfusate, and 10 min later, the HPR maneuver was repeated. Then, 50 μM of the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ; Sigma) was added to the perfusate to evaluate the effect of combined NO and cGMP inhibition on HPR. Four sham and four CBDL rats were used.

Evaluation of NO and ET-1 activity on HPR. To determine if the blunted HPR was the result of increased NO and decreased ET-1, l-Arg (100 μM) was added to the perfusate after the initial HPR evaluation. After the HPR was repeated in the presence of l-Arg, 1 nM ET-1 (Sigma) was added to the perfusate, and the HPR was repeated a final time. Four sham and four CBDL rats were used.

Evaluation of hypoxia-independent vasoconstriction. To determine if lungs from CBDL rats were capable of vasoconstriction from stimuli other than hypoxia, the perfusate concentration of K⁺ was increased in a stepwise fashion from 5.4 to 6.4, 10.4, 15.4, and 20.4 mM. Ventilation was maintained at 21% O₂-5% CO₂ throughout these maneuvers. Four sham and four CBDL rats were used.

Evaluation of K⁺ channel activation contribution to blunted HPR. Two protocols were used to study K⁺ channel function. In the first series of experiments, lungs from sham (n = 3) and CBDL (n = 3) rats were first treated with l-Arg (100 μM) and then ODQ (50 μM), and the HPR was assessed after the addition of each. Then, 10 mM tetraethylammonium (TEA; Sigma) was added, and the HPR was repeated a final time. The second series of experiments, in a different set of lungs, was designed to determine if KᵦCa channels were activated after CBDL. For this series, lungs were first treated with the combined application of l-Arg (100 μM) and ODQ (50 μM), and the HPR was evaluated. Then, 50 μM charybdotoxin (ChTX; Sigma) and 50 μM apamin (Sigma) were added together to block the big-conductance and small-conductance KᵦCa channels (7), respectively, and the HPR was repeated. In this protocol, five sham and five CBDL rats were used.

Statistical Analysis

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

RESULTS

Evaluation of Cirrhosis

Liver function was assessed in all sham and CBDL rats. Serum bilirubin was significantly elevated after CBDL (Table 1). CBDL animals also were hypoxemic and generally had ascites. Normal liver structure was significantly disrupted 5 wk after CBDL surgery (data not shown). In contrast, no major structural changes were evident in the lungs (data not shown) as previously reported (8, 24). Baseline pulmonary arterial pressure of isolated perfused lungs was not different between sham (5.7 ± 0.2 mmHg) and CBDL (5.5 ± 0.3 mmHg) rats (n = 25/group).

Molecular Correlates to Explain Blunted HPR

The blunting or complete absence of hypoxic pulmonary vasoconstriction during CBDL-induced cirrhosis has been documented (7). This observation was confirmed in our initial set of perfused lung experiments.

Table 1. Characterization of cirrhosis and hepatopulmonary syndrome after common bile duct ligation

<table>
<thead>
<tr>
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<th>Sham</th>
<th>CBDL</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>232 ± 12</td>
<td>239 ± 9</td>
</tr>
<tr>
<td>Arterial Po₂, mmHg</td>
<td>88 ± 2</td>
<td>66 ± 1*</td>
</tr>
<tr>
<td>Plasma bilirubin, mg/dl</td>
<td>0.11 ± 0.02</td>
<td>12.21 ± 2.07*</td>
</tr>
<tr>
<td>Ascites, ml</td>
<td>ND</td>
<td>&gt;4.2*</td>
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Values are means ± SE; n = 25 rats/group except for arterial Po₂, n = 5 rats/group. CBDL, common bile duct ligation; ND, not detected. *Significantly different from sham, P < 0.05.
The next set of experiments was designed to find molecular and biochemical correlates to explain the blunted HPR, and we examined the gene expression of the two most prominent vasoactive mediators in the lung, the vasodilator NO and the vasoconstrictor ET-1. By 2 wk postsurgery, eNOS protein expression in CBDL lung homogenates was 1.9-fold higher than paired sham values (Fig. 2A). This upregulation persisted for at least 5 wk postsurgery when the CBDL lung eNOS protein expression was 1.5-fold higher than paired sham values (Fig. 2A). Similarly, plasma levels of NOx tended to be elevated in CBDL rats at 2 wk and were significantly elevated at the 5-wk time point (Fig. 2B).

By 2 wk post-CBDL, ppET-1 mRNA expression was decreased by 66% (Fig. 3A). This decrease persisted at least until 5 wk postsurgery. Analysis of ET-1 peptide content in lung homogenates at 5 wk postsurgery revealed a 38% decrease in the lungs from CBDL rats compared with that in sham lungs (Fig. 3B).

Investigation of the Physiological Basis for Blunted HPR

In view of the increased expression of eNOS and the decreased level of ET-1 in the CBDL lungs, we tested to see if increased NO and decreased ET-1 accounted for the blunted HPR. In both sham and CBDL lungs, HPR was potentiated by L-NNA and further augmented by the sequential addition of ODQ (Fig. 4). However, the acute inhibition of NO and cGMP production did not fully reverse the blunted HPR in cirrhotic lungs. Baseline pulmonary arterial pressure was not altered by L-NNA and ODQ treatment in either the sham or the CBDL lungs (P > 0.05; data not shown).

A similar experimental approach was used to evaluate the vasoconstrictor actions of ET-1 in the cirrhotic lungs. After the initial HPR maneuver, NO production was inhibited by L-NNA. As described above, this re-
sulted in a marked potentiation of the HPR in the sham lungs compared with that in the CBDL lungs (Fig. 5). Next, the addition of exogenous ET-1 (1 nM) tended to increase HPR in sham lungs but failed to reverse the blunted HPR in CBDL lungs. This protocol did not alter baseline pulmonary arterial pressure in either group of lungs ($P > 0.05$; data not shown).

Because neither increased NO and cGMP nor decreased ET-1 appeared to be solely responsible for the blunted HPR in CBDL rat lungs, alternate mechanisms were explored. To determine if lungs from CBDL rats had a generalized blunting of vasoconstrictor function, we measured KCl-evoked vasoconstriction in the absence of any hypoxic stimuli. After the initial perfusion at the standard KCl concentration of 5.4 mM, the concentration of KCl was increased stepwise to 20.4 mM while the pulmonary arterial pressure was monitored continuously (Fig. 6). The content of inspired gas was kept constant at 21% O$_2$-5% CO$_2$ throughout these maneuvers. This experiment yielded two important observations. First, lungs from CBDL rats were capa-
ble of vasoconstriction to the same degree as the sham lungs, indicating that the cirrhosis-induced pulmonary vascular changes were not irreversible structural changes but rather alterations to mediators that regulate vascular tone and/or direct alterations in ion channels and intracellular ion homeostasis. Second, after the final increase in perfusate KCl, the HPR was significantly potentiated in both groups and the groups did not differ from each other (Fig. 6, inset).

The normalization of the blunted HPR by high extracellular K\(^{+}\) suggested the involvement of K\(^{+}\) channel activation, and to test if there were specific differences between sham and CBDL lungs, a series of perfused lung experiments was carried out with the nonspecific K\(^{+}\) channel blocker TEA and the K\(_{ca}\) channel blockers apamin and ChTX. The experiments shown in Fig. 7 were done to test if NO, cGMP, and K\(^{+}\) channel activity were acting in concert to blunt HPR. NOS and guanylate cyclase were inhibited first with L-NNA and ODQ, respectively. Then, TEA was added, and the HPR was repeated a final time. As in the previous experiments, this maneuver potentiated HPR in lungs from both sham and CBDL rats, but the blunted HPR was not fully corrected in the CBDL lungs nor was the baseline pulmonary arterial pressure altered. The second set of experiments, shown in Fig. 8, was done to test more selectively if activation of K\(_{ca}\) channels was responsible for the blunted HPR. As demonstrated above, the inhibition of NOS and guanylate cyclase with L-NNA and ODQ, respectively, did not fully correct the blunted HPR in CBDL rats. However, when apamin and ChTX were added after L-NNA and ODQ treatment, HPR was identical between the sham and CBDL rats. K\(_{ca}\) channel inhibition did not alter baseline pulmonary arterial pressure in the sham and CBDL lungs (P > 0.05; data not shown). This normalization of HPR between sham and CBDL lungs by the combined K\(_{ca}\) channel blockers was similar to that observed after high-perfusate KCl.

**DISCUSSION**

Several lines of evidence implicate NO in the development of intrapulmonary vasodilation in hepatopulmonary syndrome (8, 16). Cirrhotic patients have impaired arterial oxygenation ranging from an increased alveolar-arterial oxygen gradient to hypoxemia, which correlates with elevated NO in exhaled air (16). Experimentally, rats with hepatopulmonary syndrome induced by CBDL have elevated expression of eNOS in the lung as well as increased NO levels (8). It has been proposed that the increased NO, through its vasodilatory actions, mediates intrapulmonary vasodilation and blunted HPR, leading to arterial hypoxemia and hepatopulmonary syndrome. Typically, a balance between NO and the vasoconstrictor ET-1 contributes to regulation of pulmonary vascular tone, and ET-1-induced stimulation of the ETA receptor is an important component of the mechanism of hypoxic pulmonary vasoconstriction (1, 18). Pulmonary ET-1 expression during cirrhosis has not been investigated. Thus we investigated the roles of NO and ET-1 in the blunted HPR of the hepatopulmonary syndrome.

The major findings of this study were that in addition to elevated lung eNOS expression, biliary cirrhosis led to decreased ET-1 expression in the lung. Although these changes in eNOS and ET-1 expression are consistent with the pulmonary vascular abnormalities of the hepatopulmonary syndrome, they do not fully explain our physiological findings in isolated perfused lungs. The acute inhibition of both NO and cGMP production and the addition of exogenous ET-1 did not restore normal pulmonary vasoreactivity to airway hypoxia in perfused lungs from CBDL rats. However, inhibition of NO and guanylate cyclase and blockade of K\(_{ca}\) channels did normalize the HPR between the
sham and CBDL rats. This suggests that the blunted HPR in CBDL rats is not solely a result of the acute actions of increased NO or decreased ET-1 but rather appears to also involve the activation of $K_{Ca}$ channels in pulmonary vascular smooth muscle. This finding differs from that of Fallon et al. (8), who reported that in isolated pulmonary arterial rings from CBDL rats, NOS inhibition alone restored normal phenylephrine vasoconstriction. Thus the mechanisms of the blunted vasoconstriction may be different in conduit versus resistance pulmonary arteries and/or for hypoxia versus phenylephrine stimulation.

The additional potentiation of HPR by ODQ after NOS inhibition with L-NNa indicates that some factor in addition to NO was also stimulating cGMP production. One such candidate is carbon monoxide (CO). CO is liberated from the enzymatic action of heme oxygenase-1 (HO-1) and can mediate vasodilation via both cGMP-dependent and independent pathways (10, 17). HO-1 is potently induced under a variety of conditions, particularly as a stress-response gene (20). We have preliminary evidence that HO-1 is markedly upregulated in both the liver and the lung after CBDL (4). Although this suggests increased CO-mediated vasodilation in CBDL lungs, the physiological significance of HO-1 upregulation remains untested.

Our observation that ET-1 mRNA and peptide were decreased in the lungs of CBDL rats prompted us to try restoring normal hypoxic vasoconstriction by adding exogenous ET-1 to the perfusate of isolated lungs. When combined with NOS inhibition, exogenous ET-1 partially restored the blunted HPR but not to the levels observed in the lungs from sham animals. There are several possibilities to explain why exogenous ET-1 combined with NOS inhibition did not fully reverse the blunted HPR. A decrease in the expression or function of ETA and ETB receptors is one possibility. A second possibility is that increased NO and decreased ET-1 are not the sole causes of the blunted HPR. Finally, adding exogenous ET-1 may not mimic the effects of endogenous ET-1. In retrospect, our negative finding with respect to adding exogenous ET-1 to isolated lungs is consistent with evidence of blunted HPR in intact rats despite increased circulating levels of ET-1 (24).

A generalized inability to vasoconstrict under normoxic or hypoxic conditions could be the cause of the blunted HPR in hepatopulmonary syndrome. We tested this possibility and observed that vasoconstriction in response to increasing amounts of perfusate KCl was identical between the sham and CBDL lungs. It is noteworthy that after the KCl-evoked vasoconstriction, the HPR in both groups of lungs was markedly potentiated and was not different between them. There are several ways to interpret these observations. One is that pulmonary arterial smooth muscle cells (PASMCs) from CBDL lungs are hyperpolarized. The high extracellular KCl depolarizes the PASMCs, eliminating differences in resting membrane potential between the sham and CBDL lungs. Another possibility is that the vascular remodeling (e.g., increased diameter and density of capillary beds) reported to occur after CBDL (19) is associated with pulmonary vessels that are unresponsive to hypoxia but are still capable of KCl-evoked vasoconstriction. However, if this is the case, then it is unclear how the increase in perfusate KCl would have normalized the HPR.

In normal lungs, a critical event in the mechanism of hypoxic pulmonary vasoconstriction appears to be K+ channel inhibition and membrane depolarization (2, 14, 15, 22). There are several families of K+ channels in PASMCs that modulate the hypoxic response, including $K_{Ca}$ channels, ATP-sensitive K+ channels, and delayed rectifier K+ channels. Activation of any or all of these K+ channels could cause the blunted HPR of cirrhotic lungs. Although the combined application of L-NNa, ODQ, and the nonselective K+ channel blockers TEA did not restore the blunted HPR in CBDL lungs, the combination of L-NNa, ODQ, and the $K_{Ca}$ channel blockers apamin plus ChTX did. This observation combined with the restoration of normal HPR by high extracellular KCl in the absence of L-NNa and ODQ is evidence that the blunted hypoxic response is largely a result of $K_{Ca}$ channel activation and membrane hyperpolarization. Because it has been observed in some isolated artery studies that the combination of apamin and ChTX is more effective than TEA in inhibiting endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation (7), our findings raise the possibility that increased EDHF activity is involved in the blunting of HPR in CBDL lungs. Further experiments will be required to more directly test this idea and to determine the relative roles of NO, EDHF, and EDHF-independent $K_{Ca}$ channel activity in the blunted HPR.

In conclusion, we have observed that lungs from cirrhotic rats have increased eNOS and decreased ET-1 expression. However, evidence is provided that the blunted HPR of the hepatopulmonary syndrome is not caused solely by the acute vasoregulatory actions of NO and ET-1 but may also involve activation of $K_{Ca}$ channels. We speculate that the blunted HPR is multifactorial, involving the acute and chronic actions of vasoactive agents such as NO and ET-1 on both other vasoactive agents and vascular smooth muscle ion channels.

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