The Role of Endothelin-1 and the Endothelin B Receptor in the Pathogenesis of Hepatopulmonary Syndrome in the Rat

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Endothelin-1 (ET-1) stimulation of endothelial nitric oxide synthase (eNOS) via pulmonary endothelial endothelin B (ET_B) receptors and pulmonary intravascular macrophage accumulation with expression of inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1) are implicated in experimental hepatopulmonary syndrome (HPS) after common bile duct ligation (CBDL). Our aim was to evaluate the role of ET-1 in the development of experimental HPS. The time course of molecular and physiological changes of HPS and the effects of selective endothelin receptor antagonists *in vivo* were assessed after CBDL. Effects of ET-1 on intralobar pulmonary vascular segment reactivity and on eNOS expression and activity in rat pulmonary microvascular endothelial cells (RPMVECs) were also evaluated. Hepatic and plasma ET-1 levels increased 1 week after CBDL in association with a subsequent increase in pulmonary microvascular eNOS and ET_B receptor levels and the onset of HPS. Selective ET_B receptor inhibition *in vivo* significantly decreased pulmonary eNOS and ET_B receptor levels and ameliorated HPS. CBDL pulmonary artery segments had markedly increased ET_B receptor mediated, nitric oxide dependent vasodilatory responses to ET-1 compared with controls and ET-1 triggered an ET_B receptor dependent stimulation of eNOS in RPMVECs. Pulmonary intravascular macrophages also accumulated after CBDL and expressed HO-1 and iNOS at 3 weeks. Selective ET_B receptor blockade also decreased macrophage accumulation and iNOS production. In conclusion, ET-1 plays a central role in modulating pulmonary micovascular tone in experimental HPS. (HEPATOLOGY 2004;39:1593–1602.)

The endothelium plays a central role in the regulation of vascular tone both under normal circumstances and in cirrhosis by releasing endothelium-derived vasodilators and vasoconstrictors in response to a variety of biochemical and physical stimuli.[1] Nitric oxide (NO) and endothelin-1 (ET-1) are two important endothelial mediators that modulate vascular tone. Endothelial NO production is catalyzed predominately by the endothelial form of nitric oxide synthase (eNOS) and under normal circumstances is constitutively expressed and activated by calcium entry into cells.[2] ET-1 is a 21 amino acid peptide formed from a precursor, big ET-1, through the action of an endothelin-converting enzyme and is produced in a number of cell types in addition to endothelial cells, including hepatic stellate cells and biliary epithelium.[3–6] ET-1 is classically recognized as a potent paracrine vasoconstrictor, and its action is mediated by two G protein coupled receptors.[7,8] The endothelin A (ET_A) receptor mainly exists in vascular smooth muscle cells and mediates contraction and vasoconstriction.[9] Two endothelin B (ET_B) receptor types have been found: one in endothelial cells that upregulates eNOS and NO and the other in smooth muscle cells that functions similar to the ET_A receptor.[10,11] Increased circulating ET-1, in part derived from increased hepatic production and

Abbreviations: ET-1, endothelin-1; eNOS, endothelial nitric oxide synthase; ET_B, endothelin B receptor; iNOS, inducible nitric oxide synthase; HO-1, heme oxygenase-1; HPS, hepatopulmonary syndrome; CBDL, common bile duct ligation; RPMVECs, rat pulmonary microvascular endothelial cells; NO, nitric oxide; ET_A, endothelin A receptor; PVL, partial portal vein ligation; ET, endothelin; MSAP, mean systemic arterial pressure; PVP, portal venous pressure; $AaPO_2$, alveolar-arterial oxygen gradient; NOS, nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; PA, pulmonary artery.

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release, has been found in some forms of experimental and human cirrhosis.[5,6,12,13]

One unique vascular complication of cirrhosis is dilatation in the pulmonary microcirculation leading to systemic hypoxemia; this has been termed hepatopulmonary syndrome (HPS).[14] This syndrome occurs in 10%–15% of patients with cirrhosis and portal hypertension and has no effective medical therapy.[14] Chronic common bile duct ligation (CBDL) in the rat leading to biliary cirrhosis reproduces the physiological abnormalities of human HPS and has been used as an experimental model.[15,16] In prior studies, we have observed a significant increase in hepatic production and plasma levels of ET-1 within 2 weeks after CBDL, in association with increased pulmonary vascular endothelial ET_B receptor and eNOS levels.[17–19] Animals with prehepatic portal hypertension, produced by partial portal vein ligation (PVL), do not have increased ET-1 and do not develop HPS despite elevated pulmonary vascular endothelial ET_B receptor levels unless exogenous ET-1 is given.[20] These findings have led to the concept that increased circulating ET-1 levels in biliary cirrhosis trigger NO-mediated intrapulmonary vasodilatation through activation of endothelial ET_B receptors. However, this hypothesis has not been directly tested *in vivo*. In addition, accumulation of pulmonary intravascular macrophages with expression of inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1) has also been implicated in intrapulmonary vasodilatation, particularly at later time points after CBDL.[21–24] Whether ET-1 influences these events as well is also unknown.

The aim of the present study was to directly evaluate the role of the endothelin (ET) system in the development of experimental HPS. We analyzed the early time course of molecular and physiological changes of HPS in CBDL animals and assessed effects of selective ET receptor antagonists *in vivo*. Direct effects of exogenous ET-1 on intralobar pulmonary vascular segment reactivity from experimental models and on eNOS expression and activity in cultured pulmonary microvascular endothelial cells were also evaluated.

Materials and Methods

Animal Models. Male Sprague-Dawley rats (200–250 g, Charles River Laboratories, Wilmington, MA) underwent sham surgery, CBDL, or PVL as previously described.[16] Specific ET_A (BQ123, 1.0×10^{-7} M/d, Peptides, Louisville, KY) or ET_B (BQ788, 1.0×10^{-7} M/d, from Dr. Y. F. Chen, University of Alabama at Birmingham, Birmingham, AL) receptor antagonists or saline were given intravenously using a miniosmotic pump (ALZET Model 2002, ALZA, Palo Alto, CA) inserted into the femoral vein for 2 weeks beginning 1 week after CBDL. Body weight, mean systemic arterial pressure (MSAP), portal venous pressure (PVP), and spleen weight were measured to evaluate systemic effects and liver abnormalities. The study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and conforms to National Institutes of Health guidelines on the care and use of laboratory animals. Five to eight animals were used in each group.

Western Blot Analysis. ET_B and ET_A receptors, eNOS, ED1 (specific marker for monocytes/macrophages), HO-1, and iNOS protein levels were measured in lungs from animals 1 and 3 weeks after CBDL. Tissue was homogenized in RIPA buffer in the presence of protease inhibitors. Fifteen micrograms of protein were fractionated on Sodium dodecyl sulfate–PAGE gel and transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). Incubation with primary antibodies was followed by addition of horseradish peroxidase–conjugated secondary antibodies and detection with enhanced chemiluminescence.

Immunohistochemistry. Immunohistochemical staining of ED1, HO-1, and iNOS was performed in lung sections as previously described.[20] Five-micrometer sections of 4% paraformaldehyde paraffin–fixed tissue were incubated with primary antibodies and were then washed and incubated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA). After peroxidase-labeled streptavidin (Signet Laboratories, Dedham, MA) and diaminobenzidine (Biogenex, San Ramon, CA) development, sections were photographed using an axiophot microscope (Nikon, Melville, NY). Control sections were incubated with secondary antibody alone.

ET-1 RIA. Plasma and liver ET-1 concentrations were measured with a commercial RIA kit (Phoenix Pharmaceuticals, Mountain View, CA) according to the manufacturer's instructions.[17]

Arterial Blood Gas Analysis. Arterial blood drawn at rest was analyzed on an ABL 520 radiometer (Radiometer America, Westlake, OH) in the clinical laboratory at University of Alabama at Birmingham Hospital.[16] The alveolar-arterial oxygen gradient ($AaPO_2$) was calculated as $150 - (PCO_2/0.8) - PO_2$.

Microsphere Protocol. An established technique was used to evaluate intrapulmonary vasodilatation.[16,25] 2.5×10^6 cross-linked colored polystyrene-divinylbenzene microspheres (range, 5.5–10 μm; Interactive Medical Technologies, Irvine, CA) were injected into animals through the femoral vein, after removing an aliquot of microspheres to verify the numbers and sizes injected. Microspheres passing through the pulmonary microcir-

culation were measured in a blood sample withdrawn from the femoral artery beginning at the time of femoral vein injection. Numbers and sizes of microspheres were assessed using a Leitz microscope (Wetzlar, Germany) with a color video analysis system (Image Pro 3.0, Media Cybernetics, Silver Spring, MD) and counted directly. Total numbers of microspheres passing through the microcirculation were calculated as previously described.[20]

Pulmonary Artery Segment Studies. Intralobar pulmonary arteries (≈1.5 cm) were dissected from normal, 3-week CBDL or PVL lungs using a stereomicroscope, cut into 2- to 3-mm segments, and mounted in jacketed tissue chambers containing Krebs-Henseleit solution at 37°C gassed with 95% O_2/5% CO_2 as previously described.[18] In a set of segments, the endothelium was denuded by gentle mechanical disruption or segments were pretreated with the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 0.3 mM) to define the role of the endothelium in the response to ET-1. Results were similar with mechanical disruption and NOS inhibition; only the later studies are presented. All segments were pretreated with 5 μM of indomethacin. After 40 minutes of equilibration, segments were exposed to maximum depolarizing 70 mM KCL. When contractile responses plateaued, segments were relaxed with accumulated concentrations of acetylcholine, then rinsed with Krebs-Henseleit solution. After equilibration for an additional 40 minutes, submaximal tone was elicited with 0.1–1 μM of phenylephrine and 10 μM of the selective ET_A receptor antagonist BQ123 was added to recapitulate luminal exposure to ET-1. In a set of segments, 10 μM of the selective ET_B receptor antagonist BQ788 was also added. After cumulative concentrations of ET-1 were added (0.3–100 nM), relaxation and vasoconstriction of pulmonary arteries were calculated. Data are expressed as a percentage of decrease or increase in phenylephrine-induced constrictive tone.

Cell Culture. Rat pulmonary microvascular endothelial cells (RPMVECs, VEC Technolgies, Inc, Rensselaer, NY) were maintained and subcultured in MCDB-131 complete medium. After incubating in minimal media without fetal bovine serum for 12 hours, cells were stimulated with ET-1 (10–100 nM) for various times (30 minutes, 12 hours, and 24 hours) in pilot studies. For subsequent stimulation studies, the 10-nM concentration was used. In a set of experiments, cells were pretreated with ET receptor antagonists, including TBC3214Na (20 μM, from Dr. Y. F. Chen, University of Alabama at Birmingham) for ET_A receptor and BQ788 (15 μM, Peptides International, Louisville, KY) for ET_B receptor. Cell culture media was obtained for measurements of NO levels. Cell lysates were used for detecting eNOS messenger RNA and protein expression as well as NOS activity.

Northern Blot Analysis. eNOS messenger RNA levels were measured using standard techniques as described.[26] Twenty-five micrograms of total RNA from RPMVECs, extracted by the Ultraspec-II RNA isolation system (Biotecx, Houston, TX) was electrophoresed through 1% formaldehyde-agarose gel, transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) and hybridized using a [α-^{32}P]dCTP -labeled eNOS complementary DNA probe (from Dr. William Sessa, Yale University, New Haven, CT). 18S ribosomal RNA levels were measured with an oligonucleotide probe (5′-ACGG-TATCTGATCGTCTTCGAACC-3′, from Dr. Y. F. Chen, University of Alabama at Birmingham) labeled with [α-^{32}P]dCTP.

NO Measurement. NO levels in cell culture media were measured using a colorimetric, nonenzymatic assay for determination of total nitrate and nitrite (OXIS, Portland, OR).[19]

NOS Activity Assay. Total NOS activity was measured by determining the biochemical conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline using a commercial NOS assay kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions.[27]

Statistical Analysis. All measurements in this study were expressed as mean ± SEM. Data were analyzed using Student's t test or ANOVA with Bonferroni correction for multiple comparisons between groups, and linear correlation when appropriate. $P < .05$ was considered statistically significant.

Results

Physiological Alterations of HPS After CBDL. To evaluate the onset of HPS in relation to hepatic and systemic alterations after CBDL, we measured MSAP, PVP, spleen weight, arterial blood gases, and shunting of microspheres through the pulmonary microcirculation at serial time points after ligation (Table 1). Relative to sham animals, 1-week CBDL animals had increased PVP and spleen weight, reflecting the development of portal hypertension. HPS was absent at 1 week reflected in normal microsphere shunting and a normal $AaPO_2$. At 2 and 3 weeks after CBDL, time points prior to the development of cirrhosis, a progressive rise in portal pressure and fall in MSAP were noted, indicating the development of a hyperdynamic circulation. The onset of HPS was noted at 2 weeks and did not progress between 2 and 3 weeks after CBDL. Immunohistochemical analysis from each animal revealed no evidence of lung injury or alveolar infiltration of macrophages (Fig. 1).

Table 1. MSAP, PVP, Spleen Weight, Arterial Blood Gases, Intrapulmonary Shunting, Plasma, and Hepatic ET-1 Levels After CBDL

	Control	CBDL (wk) 1	CBDL (wk) 2	CBDL (wk) 3
MSAP (mm Hg)	113.8 ± 7.4	109.9 ± 6.5	99.6 ± 5.0*	97.7 ± 5.9*
PVP (mm Hg)	7.7 ± 0.8	11.5 ± 0.7*	14.6 ± 3.0*	15.8 ± 0.9*
Spleen (g)	0.86 ± 0.07	0.96 ± 0.09	1.69 ± 0.17*	1.71 ± 0.06*
$AaPO_2$	6.7 ± 1.4	7.04 ± 2.37	16.0 ± 1.5*	17.3 ± 2.6*
Intrapulmonary shunt fraction (%)	6.4 ± 1.7	6.7 ± 1.4	13.2 ± 2.6*	15.4 ± 3.4*
Plasma ET-1 (pg/mL)	8.15 ± 0.21	23.63 ± 1.21*	20.53 ± 0.78*	26.68 ± 3.09*
Hepatic ET-1 (ng/g liver)	2.24 ± 0.71	4.84 ± 1.67*	5.29 ± 0.61*	8.02 ± 1.45*

NOTE: Values are mean ± SEM (n = 5–8 for each group).
$^*P < .05$ compared with control animals.

Alterations in Circulating and Pulmonary Mediators and HPS After CBDL. To assess how circulating and pulmonary factors implicated in experimental HPS correlate with the onset of physiological changes, we measured hepatic and plasma ET-1 levels, lung eNOS, ET_B and ET_A receptor, HO-1, iNOS, and ED1 levels after CBDL (Fig. 1; Table 1). Immunohistochemistry was used to localize eNOS, ET_B receptor, ED1, HO-1, and iNOS in the lung (Fig. 2). Hepatic and plasma ET-1 levels increased beginning 1 week after CBDL and rose over the ensuing 2 weeks as HPS developed. eNOS and ET_B receptor protein levels increased beginning at 2 weeks and correlated with the onset of HPS. In addition, ET_B receptor protein levels correlated closely with $AaPO_2$ levels in animals 1, 2, and 3 weeks after CBDL ($r = 0.81$, $P < .005$). Immunohistochemistry localized the increase in eNOS and ET_B receptor to the pulmonary microvascular endothelium as previously observed.[18,19] Lung ET_A receptor levels were unchanged after CBDL, which is consistent with previous findings.[28] Pulmonary intravascular mononuclear cells, quantified by ED1 immunoblotting and localized by immunohistochemistry,[24] began to accumulate within 1 week after CBDL prior to the onset of HPS. However, pulmonary iNOS and HO-1 levels that were derived from production in intravascular macrophages after CBDL[24] were not increased at 2 weeks after CBDL at the onset of HPS. iNOS and HO-1 levels did increase markedly at 3 weeks but were not associated with worsening of HPS.

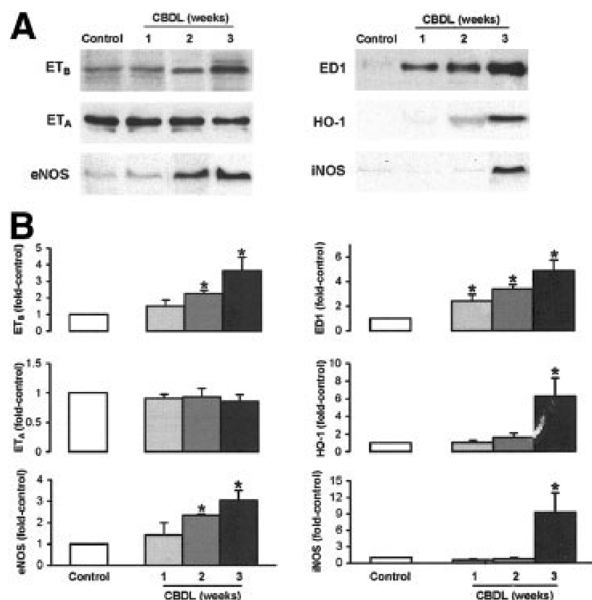

Fig. 1. ET_B receptor, ET_A receptor, eNOS, ED1, HO-1 and iNOS expression in lung from sham and 1 to 3 weeks CBDL animals. (A) Representative Western blots (30 μg lung protein per lane). (B) Protein levels are quantitated by desitometry. All values are expressed as mean ± SEM (n = 5–8 for each group). $^*P < .05$ compared with control. CBDL, common bile duct ligation; ET_B, endothelin B receptor; ET_A, endothelin A receptor; eNOS, endothelial nitric oxide synthase; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase.

Effects of Selective ET Receptor Antagonists on HPS After CBDL. To evaluate if ET-1 influences HPS *in vivo*, selective ET_A or ET_B receptor antagonists were administered intravenously to animals 1 week after CBDL. At 3 weeks, MSAP, PVP, and spleen weights were measured to evaluate systemic effects, and arterial blood gas and microsphere analysis were performed to evaluate HPS (Table 2). Pulmonary eNOS and ET_B and ET_A receptor expression were also measured to assess molecular events (Fig. 3). ET_B receptor blockade significantly increased MSAP but did not affect PVP or spleen weight. A significant improvement in HPS was reflected by a marked reduction in $AaPO_2$ and intrapulmonary shunting accompanied by a significant decrease in pulmonary eNOS and ET_B receptor protein levels. In contrast, ET_A receptor blockade did not alter MSAP or PVP and did not improve $AaPO_2$ or intrapulmonary shunting. No decrease in pulmonary eNOS or ET_B receptor levels were noted in this group. Pulmonary ET_A levels were not altered by either ET receptor antagonist.

Effects of ET-1 on Isolated Pulmonary Artery Segments From CBDL and PVL Animals. To determine if pulmonary vascular ET_B receptor alterations directly

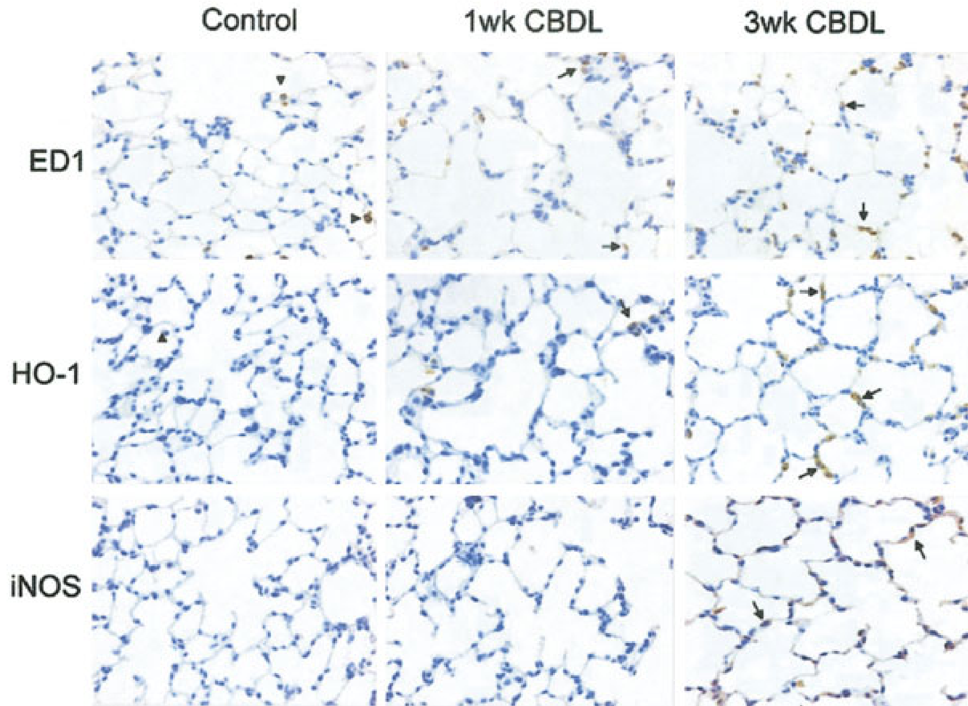

Fig. 2. Immunohistochemical localization of ED1, HO-1, and iNOS in pulmonary alveolar capillary regions from control and 1 and 3 weeks CBDL animals (original magnification ×40). Control lung shows occasional light ED1 (top panel) and HO-1 (middle panel) staining in macrophages in alveolar air spaces (**arrowheads**), whereas no iNOS staining (bottom panel) is found. In the 1-week CBDL lung, there is a small increase in ED1 staining in macrophages in alveolar capillaries (**arrows**) and no change in occasional macrophage staining in alveolar air spaces. No significant HO-1 or iNOS staining is found. At 3 weeks, a marked increase in ED1, HO-1, and iNOS staining is seen in intravascular macrophages (**arrows**). CBDL, common bile duct ligation; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase.

modulate responsiveness to ET-1 in the pulmonary vasculature, we obtained intralobar pulmonary artery (PA) segments from normal, PVL, and CBDL animals. We included both CBDL and PVL PA segments, because ET_B receptor levels increase in the pulmonary vascular endothelium in both models.[19] Acetylcholine administration to segments from all models produced a dose-dependent L-NAME inhibitable relaxation reaching a maximum of above 80%, indicating the presence of a functional endothelium (Fig. 4).[18] ET-1 administration in normal PA segments induced dose-dependent vasoconstriction alone. In contrast, ET-1 at concentrations from 3–30 nM induced a significant initial vasorelaxation in PVL and CBDL PA segments, followed by slowly developing vasoconstriction (Fig. 5). At ET-1 concentrations relevant to those found in CBDL animals with HPS (3–10 nM), vasorelaxation was 5-fold greater in PVL and CBDL animals relative to normal animals, and there was also a trend toward reduced vasoconstriction. ET-1 vasorelaxation in PVL and CBDL PA segments was completely inhibited by pretreatment with either the selective ET_B receptor antagonist BQ788 (10 μM) or with L-NAME, documenting dependence on ET_B receptor mediated NO production. BQ788 did not influence ET-1 mediated vasoconstriction, which is consistent with the minimal role played by ET_B receptors on smooth muscle cells in modulating vascular tone.

Effects of ET-1 on eNOS Expression and Activity in RPMVECs. To assess if ET-1 directly influences eNOS expression and NOS activity in relevant endothelial cell populations, we administered exogenous ET-1 to primary cultures of RPMVECs (Fig. 6). ET-1 exposure for 12 or

Table 2. Effects of Selective ET Receptor Antagonists on MSAP, PVP, and Spleen Weight and the Development of HPS After CBDL

	Control	3 Weeks After CBDL	3 Weeks After CBDL	
			BQ788	BQ123
MSAP (mm Hg)	113.8 ± 7.4	95.4 ± 2.29*	108.1 ± 1.5†	93.7 ± 3.5*
PVP (mm Hg)	7.7 ± 0.8	13.5 ± 0.8*	13.4 ± 0.5*	13.8 ± 0.6*
Spleen (g)	0.86 ± 0.07	1.73 ± 0.05*	1.61 ± 0.06*	1.69 ± 0.09*
$AaPO_2$	6.7 ± 1.4	17.2 ± 0.9*	12.1 ± 1.2*,†	18.4 ± 2.7*
Intrapulmonary shunt fraction (%)	6.4 ± 1.7	16.6 ± 1.8*	10.9 ± 1.4*,†	17.7 ± 1.4*
Carboxyhemoglobin (%)	0.76 ± 0.08	1.26 ± 0.08*	1.05 ± 0.07*	1.36 ± 0.16*

NOTE: Values are mean ± SEM (n = 5–8 for each group).
*$P < .05$ compared with control animals.
†$P < .05$ compared with CBDL animals.

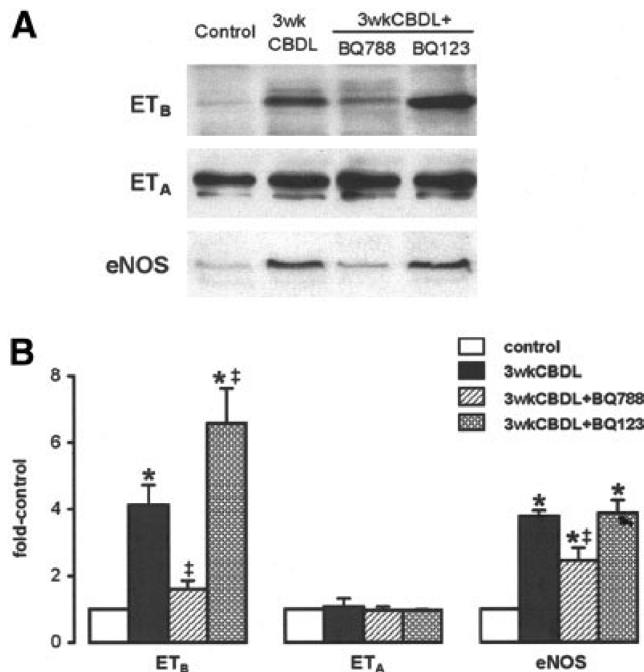

Fig. 3. Effects of chronic ET_B (BQ788) or ET_A (BQ123) receptor antagonist administration on pulmonary ET_B and ET_A and eNOS protein expression. (A) Representative ET_B, ET_A, and eNOS Western blots (30 μg lung protein per lane). (B) Protein levels are quantitated by desitometry. All values are expressed as mean ± SEM (n = 5–8 for each group). $^*P < .05$ compared with control animals; $^‡P < .05$ compared with CBDL animals. CBDL, common bile duct ligation; ET_B, endothelin B receptor; ET_A, endothelin A receptor; eNOS, endothelial nitric oxide synthase.

24 hours induced significant increases in eNOS messenger RNA (2.3-fold) and protein levels (1.7-fold), respectively, which were accompanied by increased NO release into the media. iNOS protein was not detected in control or ET-1 treated RPMVECs (data not shown). Selective ET_B but not ET_A receptor antagonist pretreatment blocked the increases in eNOS expression and NO production. Short-term ET-1 exposure (30 minutes) significantly increased NOS activity in RPMVECs, an effect that is also inhibitable with an ET_B receptor antagonist.

Effects of Selective ET Receptor Antagonists on Pulmonary Intravascular Macrophage Accumulation, iNOS and HO-1 Levels after CBDL. To determine if ET receptor antagonists also influence other mediators implicated in the development of experimental HPS, we measured pulmonary intravascular macrophage accumulation and pulmonary iNOS and HO-1 levels after ET receptor antagonist treatment of CBDL animals (Fig. 7). In untreated CBDL animals, a significant increase in pul-

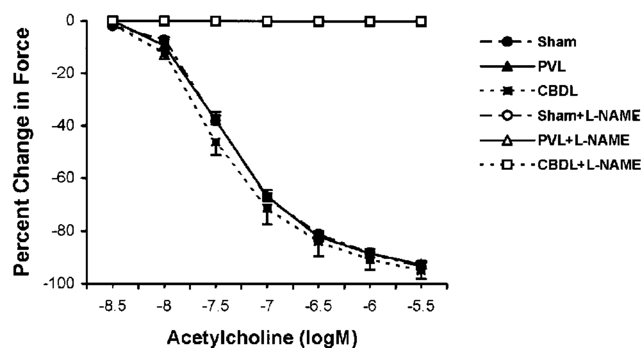

Fig. 4. Comparison of acetylcholine-induced endothelial NO-dependent relaxation in PA segments from sham, PVL, and CBDL animals. Acetylcholine induced a marked vasorelaxation in all segments; this effect was completely inhibited by L-NAME pretreatment. Six to eight segments per group were used. Data are expressed as mean ± SEM. PVL, partial portal vein ligation; CBDL, common bile duct ligation; L-NAME, N^G-nitro-L-arginine methyl ester.

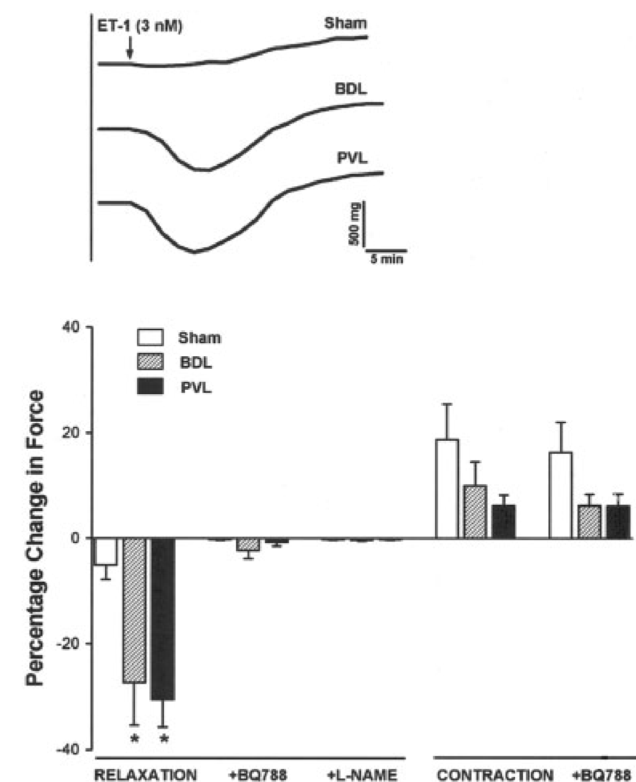

Fig. 5. Effects of exogenous ET-1 on vascular tone in PA segments. (A) Representative tracings of ET-1–induced changes in vascular tone in indomethacin and ET_A receptor antagonist (BQ123, 30 nM) pretreated segments from sham, PVL, and 3-week CBDL animals. ET-1 administration induced minimal relaxation in sham control PA segments. In contrast, a marked relaxation in response to ET-1 was detected in all CBDL and PVL segments followed by slowly developing contraction. (B) Summary of relaxation and constriction responses measured relative to baseline tone in ET-1–treated (3 nM) PA segments. There was a significant fivefold increase in ET-1–induced relaxation in CBDL and PVL segments compared with sham. This effect was completely inhibited by the addition of either the selective ET_B receptor antagonist BQ788 (10 μM) or by the NOS inhibitor L-NAME (0.3 mM). Constrictive responses were not different between the groups, although there was a trend towards decreased constriction in CBDL and PVL animals. The addition of BQ788 did not influence constrictive responses. Data are expressed as mean ± SEM. ET-1, endothelin-1; BDL, bile duct ligation; PVL, partial portal vein ligation; L-NAME, N^G-nitro-L-arginine methyl ester.

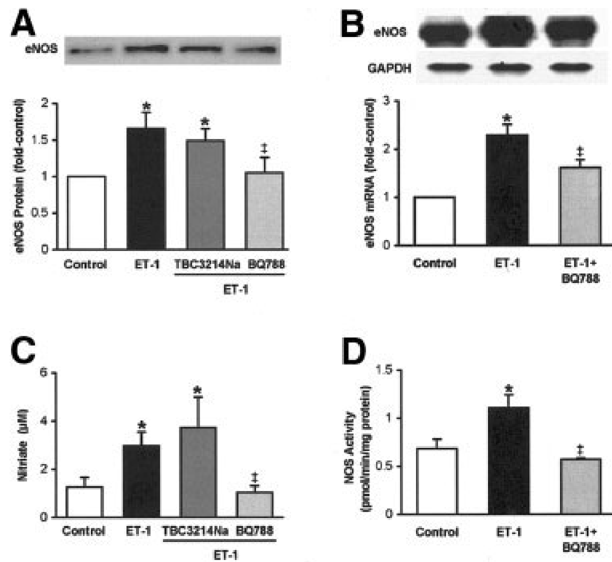

Fig. 6. Effects of ET receptor antagonists on ET-1–induced eNOS expression and activity in RPMVECs. (A) Representative Western blots and densitometric quantitation from RPMVECs stimulated for 24 hours. (B) Representative Northern blots and densitometric quantitation for eNOS obtained from RPMVECs stimulated for 12 hours. (C) Nitrite production in media from RPMVECs treated as in panel A. (D) Total NOS activity in cell lysates from RPMVECs stimulated for 30 minutes. Data are expressed as mean ± SEM (n = 4–5 for each group). $*P < .05$ compared with control; $‡P < .05$ compared with ET-1 treatment. eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; ET-1, endothelin-1; NOS, nitric oxide synthase.

monary intravascular macrophage accumulation and iNOS and HO-1 expression occurred at 3 weeks after CBDL and were accompanied by increased arterial carboxyhemoglobin levels, as previously reported.[24] After ET_B antagonist treatment, there was a modest reduction in intravascular macrophage accumulation and normalization of iNOS levels. In contrast, ET_A antagonist treatment resulted in a dramatic increase in intravascular macrophage accumulation without altering HO-1 or iNOS levels. Arterial carboxyhemoglobin levels were not altered by either ET receptor antagonist.

Discussion

In this study, we addressed the role of ET-1 in experimental HPS. We found that hepatic and plasma ET-1 levels increased within 1 week after CBDL and remained elevated over 3 weeks. At 2 weeks, pulmonary microvascular eNOS and ET_B receptor levels rose as HPS developed, and ET_B receptor levels correlated with the severity of gas exchange abnormalities. Selective ET_B receptor inhibition *in vivo* decreased pulmonary endothelial eNOS and ET_B receptor levels, improved mean arterial pressure, and ameliorated HPS. In isolated PA segments from CBDL animals, ET-1 triggered a markedly enhanced ET_B receptor mediated, NO-dependent vasodilatory response, and in RPMVECs, ET-1 triggered an ET_B receptor dependent increase in eNOS expression and activity. Intravascular macrophages began to accumulate in the lung at 1 week after CBDL and increased steadily over 3 weeks. These cells produced iNOS and HO-1 at 3 weeks, but this event was not accompanied by progression of HPS. Selective ET_B receptor blockade also decreased pulmonary intravascular macrophage accumulation and iNOS production, while selective ET_A receptor blockade increased intravascular macrophage accumulation. These findings demonstrate a central role for ET-1 in modulating pulmonary micovascular tone during the onset of experimental HPS. The pulmonary vasodilatory effect of ET-1 appears to result from activation of the ET_B receptor in the pulmonary microvascular endothelium and may be contributed to by ET_A receptor and ET_B receptor mediated modulation of pulmonary intravascular macrophage accumulation and activation.

One fundamental question regarding HPS is how its development relates to impaired splanchnic and systemic vascular contractility in cirrhosis, and whether or not similar mechanisms are involved in both situations. Overpro-

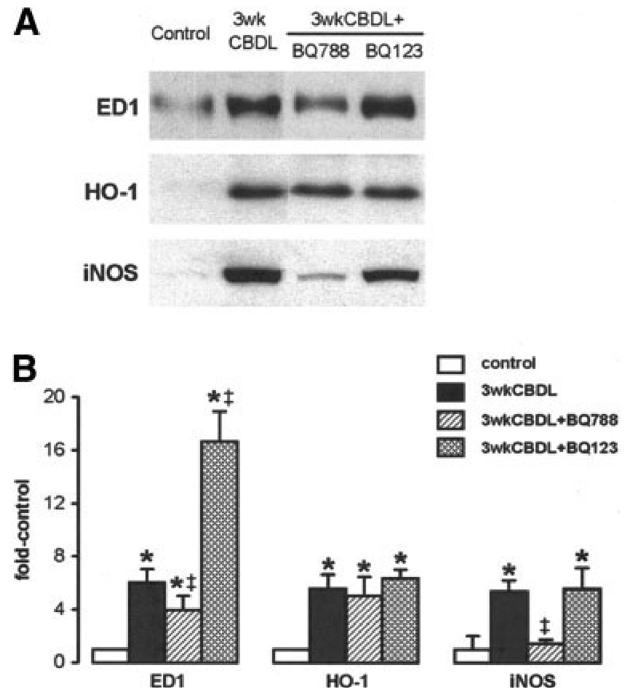

Fig. 7. Effects of chronic ET_B (BQ788) or ET_A (BQ123) receptor antagonist administration on pulmonary ED1, HO-1, and iNOS protein expression. (A) Representative ED1, HO-1, and iNOS Western blots (30 μg lung protein per lane). (B) Protein levels are quantitated by densitometry. All values are expressed as mean ± SEM (n = 5–8 for each group). $*P < .05$ compared with control animals; $‡P < .05$ compared with CBDL animals. CBDL, common bile duct ligation; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase.

duction of endothelial-derived vasodilators, most notably NO from eNOS, appears to play an important role in the hyperdynamic state.[29,30] Here, we find that the onset of HPS does temporally correlate with the development of systemic hypotension and with increased pulmonary eNOS levels at 2 weeks after CBDL. However, we also find that circulating ET-1 and pulmonary endothelial ET_B receptor levels are significantly elevated at the onset of HPS and that ET_B receptor inhibition markedly improves systemic as well as intrapulmonary vasodilatation in CBDL animals. These results suggest that ET-1 may play a role in decreased vascular tone in both the pulmonary and systemic vasculature in experimental biliary cirrhosis. Whether endothelial ET_B receptor expression is altered in the splanchnic and systemic circulation in cirrhosis has not yet been determined.

The increase in ET-1 levels after CBDL appears to be unique in that the rise is nearly 3-fold within 1 week after ligation and does not correlate with the progression of liver injury or the degree of portal hypertension in contrast to human cirrhosis.[5,31] The early increase in circulating ET-1 after CBDL appears to derive from increased hepatic production in both stellate cells and biliary epithelium.[13,17] In addition, biliary levels of ET-1 are high in rodents, both under normal conditions and after CBDL,[26] suggesting that retrograde release of ET-1 from bile into blood may be a unique consequence of biliary obstruction Our observation that chronic selective ET_A receptor inhibition does not decrease systemic arterial pressure is in agreement with previous work and suggests that ET-1 does not play a major role in vasoconstriction in the systemic or pulmonary vasculature in cirrhosis.[32–34] However, these observations suggest that ET-1 may have an important and unique vasodilatory effect on systemic and pulmonary vascular tone after CBDL.

Our *in vivo* and *in vitro* findings demonstrate that a component of the ET-1 induced pulmonary microvascular vasodilatation after CBDL results from direct effects via endothelial ET_B receptor coupling to eNOS. ET_B receptor antagonist infusion studies and results in PA segments demonstrate that the functional effect of ET_B receptor activation in the lung vasculature is vasodilatation. Our PA segment studies were performed in the presence of indomethacin, so we do not know if endothelial eicosanoids contribute. Increased ET_A and ET_B receptor levels are found in cirrhotic liver,[35,36] and elevated ET receptor messenger RNA levels ($ET_B > ET_A$) have been detected in hepatic arteries from patients undergoing liver transplantation.[37] However, no detailed evaluation of splanchnic or systemic vascular ET receptor expression and localization has been undertaken.

The mechanism for the increase in pulmonary vascular ET_B receptor expression in portal hypertension has not been fully explored. ET_B receptor expression can be modulated by pressure,[38] shear stress,[39,40] and cytokines, including tumor necrosis factor α,[41] all of which may contribute in CBDL. Our *in vivo* ET receptor antagonist data suggest that ET-1 itself may influence ET_B receptor levels either directly or through secondary effects. Another novel observation is that pulmonary endothelial ET_B receptor stimulation increases eNOS levels *in vivo*, an effect supported by direct ET_B receptor dependent modulation of eNOS expression in RPMVECs. This result is consistent with our previous findings in PA segments[19] and supports that ET-1 activation of the ET_B receptor in the pulmonary endothelium increases eNOS expression.

We also evaluated if ET-1 influenced other pathways implicated in experimental HPS. Specifically, we assessed effects on intravascular macrophage accumulation and iNOS and HO-1 production in lung. Pulmonary intravascular macrophage accumulation began at 1 week after CBDL,[42] but iNOS and HO-1 expression did not occur until after HPS was established,[24] suggesting that the initiation of experimental HPS is not dependent on macrophage iNOS and HO-1 production. However, ET_B receptor inhibition did not completely reverse HPS, and previous work has found that intestinal decontamination after CBDL reduces macrophage accumulation and HPS severity at late time points.[23] These results are in agreement with our recent finding that pulmonary intravascular macrophage HO-1 mediated carbon monoxide production contributes to vasodilatation in the later stages of experimental HPS.[24] Selective ET_B receptor inhibition decreased macrophage accumulation and reduced iNOS levels. These alterations may also decrease overall NO production in 3 weeks. However, the lung iNOS increase after CBDL is found only at 3 weeks,[23] suggesting that non-iNOS mediated vasodilatation is central to the onset and progression of experimental HPS. Whether or not carbon monoxide production accounts for the persistent intrapulmonary vasodilatation after ET_B receptor inhibition remains unknown.

Although ET-1 can inhibit macrophage adhesion[43,44] and iNOS expression,[45] these events are not generally mediated through the ET_B receptor. Therefore, the mechanisms for the ET_B receptor mediated effects on macrophages seen here remain undefined. Recently, eNOS mediated NO production has been recognized to stimulate macrophage activation and iNOS expression,[46] and ET-1 has been shown to modulate ET_B receptor mediated NO production by eNOS in these cells.[44] Thus ET_B receptor inhibition might directly lower iNOS expression.

In contrast, ET_A receptor inhibition significantly increased macrophage accumulation but was not paralleled by increases in iNOS or HO-1 expression. One explanation for the differential effects of ET receptor inhibition on macrophage accumulation relative to iNOS and HO-1 levels is that other sources of iNOS and HO-1 are present in the lung. However, our present and previous work has not found other sources to support such an explanation.[23] Alternatively, indirect effects of ET receptor inhibition—such as changes in systemic arterial pressure and/or shear stress, modulation of translocation of gut-derived endotoxins, or alterations in cytokine production—could influence macrophages. However, these mechanisms remain speculative. Together, our findings support that ET-1 effects in the pulmonary microvasculature include effects on intravascular macrophages as well as endothelial cells and provide the first evidence that the two pathways may influence one another.

Whether the mechanisms involved in experimental HPS are unique to CBDL and whether they play a role in human HPS are important unresolved questions. CBDL is unique in that hepatic production and plasma levels of ET-1 increase early after CBDL, do not correlate with progression of liver dysfunction, and are generally higher than plasma levels found in advanced human cirrhosis. However, plasma ET-1 levels have not been reported in human cirrhosis with HPS. Assessing if other models of cirrhosis are associated with the development of HPS and whether similar mechanisms are involved will determine if alterations in the endothelin system are a requirement for the initiation of experimental HPS. In addition, evaluating if intravascular macrophages accumulate in human HPS and defining the mechanisms that regulate adhesion in the pulmonary vasculature is also important. These studies are likely to provide important pathophysiological insights and lead to novel and effective medical therapies.

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