# Cholangiocyte Endothelin 1 and Transforming Growth Factor β1 Production in Rat Experimental Hepatopulmonary Syndrome

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***Background & Aims:*** **Hepatic production and release of endothelin 1 plays a central role in experimental hepatopulmonary syndrome after common bile duct ligation by stimulating pulmonary endothelial nitric oxide production. In thioacetamide-induced nonbiliary cirrhosis, hepatic endothelin 1 production and release do not occur, and hepatopulmonary syndrome does not develop. However, the source and regulation of hepatic endothelin 1 after common bile duct ligation are not fully characterized. We evaluated the sources of hepatic endothelin 1 production after common bile duct ligation in relation to thioacetamide cirrhosis and assessed whether transforming growth factor β1 regulates endothelin 1 production.** ***Methods:*** **Hepatopulmonary syndrome and hepatic and plasma endothelin 1 levels were evaluated after common bile duct ligation or thioacetamide administration. Cellular sources of endothelin 1 were assessed by immunohistochemistry and laser capture microdissection of cholangiocytes. Transforming growth factor β1 expression and signaling were assessed by using immunohistochemistry and Western blotting and by evaluating normal rat cholangiocytes.** ***Results:*** **Hepatic and plasma endothelin 1 levels increased and hepatopulmonary syndrome developed only after common bile duct ligation. Hepatic endothelin 1 and transforming growth factor β1 levels increased over a similar time frame, and cholangiocytes were a major source of each peptide. Transforming growth factor β1 signaling in cholangiocytes in vivo was evident by increased phosphorylation and nuclear localization of Smad2, and hepatic endothelin 1 levels correlated directly with liver transforming growth factor β1 and phosphorylated Smad2 levels. Transforming growth factor β1 also stimulated endothelin 1 promoter activity, expression, and production in normal rat cholangiocytes.** ***Conclusions:*** **Cholangiocytes are a major source of hepatic endothelin 1 production during the development of hepatopulmonary syndrome after common bile duct ligation, but not in thioacetamide-induced cirrhosis. Transforming growth factor β1 stimulates cholangiocyte endothelin 1 expression and production. Cholangiocyte-derived endothelin 1 may be an important endocrine mediator of experimental hepatopulmonary syndrome.**

The hepatopulmonary syndrome (HPS) causes impaired oxygenation due to vasodilatation in the pulmonary microcirculation in patients with cirrhosis.[1,2] HPS significantly increases mortality in affected patients, and no effective medical treatments are currently available.[3] The pathogenesis of HPS remains an area of active investigation.

Chronic common bile duct ligation (CBDL) in the rat is the only recognized model system for the study of HPS.[4] In this model, biliary cirrhosis is associated with increased pulmonary endothelial nitric oxide synthase (eNOS) levels, intrapulmonary vasodilatation, and gas exchange abnormalities analogous to human HPS.[4,5] Increased hepatic production and plasma levels of endothelin (ET)-1 accompanied by pulmonary microvascular endothelial $ET_B$ receptor overexpression occur at the onset of HPS after CBDL and stimulate eNOS-derived nitric oxide production in the microvascular endothelium.[6,7] Chronic intravenous ET-1 infusion in animals that develop portal hypertension and increased pulmonary microvascular $ET_B$ levels without increased hepatic ET-1 production after partial portal vein ligation also increases

*Abbreviations used in this paper:* bp, base pair; CBDL, common bile duct ligation; CK19, cytokeratin 19; eNOS, endothelial nitric oxide synthase; ET, endothelin; HPS, hepatopulmonary syndrome; LCM, laser capture microdissection; NRC, normal rat cholangiocyte; PCR, polymerase chain reaction; p-Smad2, phospho-Smad2; RT-PCR, reverse-transcription polymerase chain reaction; α-SMA, α-smooth muscle actin; TAA, thioacetamide; TGF, transforming growth factor.

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0016-5085/05/$30.00
doi:10.1053/j.gastro.2005.05.050

pulmonary eNOS levels and triggers HPS.[8] Similarly, exogenous ET-1 directly increases eNOS expression and activity in an $ET_B$ receptor–dependent manner in pulmonary artery endothelial cells.[9] In contrast, hepatic and plasma ET-1 levels do not increase and HPS does not develop in thioacetamide (TAA)-induced nonbiliary cirrhosis despite an increase in pulmonary microvascular $ET_B$ receptor expression.[10] Together, these observations indicate that hepatic ET-1 production and release is a critical event that drives pulmonary vascular eNOS-derived NO production during the onset of experimental HPS.

The source and regulation of hepatic ET-1 production in experimental HPS have not been clearly defined. CBDL is unique among cirrhosis models in that marked progressive cholangiocyte proliferation occurs over time and could be an important source of ET-1 production. Both human and rodent cholangiocytes have been found to contain ET-1 in cirrhosis, although detailed analysis after CBDL has not been performed.[6,11,12] Proliferating biliary epithelium has also been recognized to contribute to the development of fibrosis after CBDL by producing a number of profibrogenic cytokines.[13] Among these, transforming growth factor (TGF)-β1 seems to be important, and TGF-β1 can modulate ET-1 production in several hepatic cell types[14,15] and in renal ductular epithelium.[16] TGF-β1 signaling involves binding to a heteromeric complex of 2 cell-surface receptors: type I and type II. Binding triggers phosphorylation of Smad2 and Smad3, followed by complex formation with Smad4 and translocation to the nucleus.[17] Recently, a Smad-binding element has been identified in the ET-1 promoter that is critical for TGF-β1 induction of gene expression.[18] One hypothesis based on these observations is that proliferating biliary epithelial cells may be a major source of hepatic ET-1 production and that TGF-β1 might drive ET-1 expression in these cells after CBDL.

The aim of this study was to define whether cholangiocytes are an important source of hepatic ET-1 production after CBDL and to investigate whether TGF-β1 may influence ET-1 expression in these cells and contribute to the development of HPS. To address this aim, we assessed the cellular localization and expression of hepatic ET-1, TGF-β1, and phospho-Smad2 (p-Smad2) after CBDL in comparison to TAA administration, particularly in biliary epithelial cells. We also directly evaluated whether TGF-β1 modulates ET-1 expression in normal rat cholangiocytes (NRCs). Our findings show that cholangiocytes are a major source of both ET-1 and TGF-β1 production after CBDL and that there is a temporal correlation in their expression in these cells. In addition, we found evidence of TGF-β1 activation in cholangiocytes on the basis of analysis of p-Smad2 levels and localization. Finally, we showed that TGF-β1 directly activates ET-1 expression in NRCs.

## Materials and Methods

### Animals

Male Sprague–Dawley rats (200–250 g; Charles River, Wilmington, MA) were used in all experiments. CBDL was performed as previously described.[19,20] Normal control animals underwent mobilization of the common bile duct without ligation. Some rats were intraperitoneally injected with TAA 200 mg/kg body weight (Sigma-Aldrich, St Louis, MO) or saline 3 times each week for 2 or 8 weeks as previously described.[10] Five to 8 animals from each group (control; 1-, 2-, and 3-week CBDL; and 2- and 8-week TAA administration) were used. Plasma, tissue, and physiological measurements were similar in sham CBDL and saline-treated controls, and measurements were pooled for analysis. All animals had hepatic biochemical and histological analysis and measurements of portal venous pressure and spleen weight.[4,6,8,21] Blood and liver tissues were obtained from each animal. The study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and conformed to National Institutes of Health guidelines on the use of laboratory animals.

### Liver Histology and Immunohistochemistry

Liver samples were fixed in 10% neutral buffered formalin solution. Paraffin-embedded tissues were sectioned at 5 μm. Sections for histology were stained with the Masson trichrome stain.[22] After preparation and blocking, sections were incubated with ET-1 (Peninsula, San Carlos, CA), cytokeratin 19 (CK19; Novocastra, Newcastle Laboratories, UK), a marker for bile epithelial cells, α-smooth muscle actin (α-SMA; Sigma-Aldrich), TGF-β1 (Santa Cruz, Santa Cruz, CA), or p-Smad2 (Ser465/467; Cell Signaling, Beverly, MA) antibodies, washed, and incubated with EnVision-labeled polymer (Dako, Carpinteria, CA). After diaminobenzidine (Biogenex, San Ramon, CA) development, sections were photographed by using an Axiophot microscope (Nikon, Melville, NY). Control sections were incubated with secondary antibody alone.

### Arterial Blood Gas Analysis

Arterial blood was drawn from the femoral artery as previously described,[21] and blood gas analysis was performed on an ABL 520 radiometer (Radiometer America, Westlake, OH) in the clinical laboratory of the University of Alabama at Birmingham Hospital. The alveolar–arterial oxygen gradient was calculated as $150 - (PaCO_2/0.8) - PaO_2$.

### Microsphere Protocol

The pulmonary microcirculation was evaluated with an established technique. Cross-linked ($2.5 \times 10^6$) colored polystyrene-divinylbenzene microspheres (size range, 5.5–10 μm;

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