Endothelin-1 inhibits secretin-stimulated ductal secretion by interacting with ET<sub>A</sub> receptors on large cholangiocytes

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Caligiuri, Alessandra, Shannon Glaser, Rebecca E. Rodgers, Jo Lynne Phinizy, Willie Robertson, Emanuela Papa, Massimo Pinzani, and Gianfranco Alpini. Endothelin-1 inhibits secretin-stimulated ductal secretion by interacting with ET<sub>A</sub> receptors on large cholangiocytes. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G835–G846, 1998.—We studied the expression of endothelin-1 (ET-1) receptors (ET<sub>A</sub> and ET<sub>B</sub>) and the effects of ET-1 on cholangiocyte secretion. The effects of ET-1 on cholangiocyte secretion were assessed in normal and bile duct-ligated (BDL) rats by measuring 1) basal and secretin-induced choleresis in vivo, 2) secretin receptor gene expression and cAMP levels in small and large cholangiocytes, and 3) luminal expansion in response to secretin in intrahepatic bile duct units (IBDU). ET<sub>A</sub> and ET<sub>B</sub> receptors were expressed by small and large cholangiocytes. ET-1 had no effect on basal bile flow or bicarbonate secretion in normal or BDL rats but decreased secretin-induced bicarbonate-rich choleresis in BDL rats. ET-1 decreased secretin receptor gene expression and secretin-stimulated cAMP synthesis in large cholangiocytes and secretin-induced luminal expansion in IBDU from normal or BDL rats. The inhibitory effects of ET-1 on secretin-induced cAMP synthesis and luminal duct expansion were blocked by specific inhibitors of the ET<sub>A</sub> (BQ-610) receptor. ET-1 inhibits secretin-induced ductal secretion by decreasing secretin receptor and cAMP synthesis, two important determinants of ductal secretion.

THE INTRAHEPATIC BILIARY tree consists of a complex, ramified network of interconnecting tubular conduits of different diameters and function lined by intrahepatic bile duct epithelial cells or cholangiocytes (7, 8, 10). Bile is formed at the canalicular domain of hepatocytes, and, before it reaches the duodenum, it is extensively modified by cholangiocyte secretion and reabsorption (5, 6, 19, 30, 50). Ductal bile secretion is regulated by a number of gastrointestinal hormones, including secretin, gastrin, bombesin, and somatostatin (3, 5–10, 12, 15, 19, 28, 30, 50) and neuropeptides (11). Secretin, for example, interacts with specific receptors expressed in rat liver only by cholangiocytes (9), which leads to an increase in intracellular levels of the second messenger cAMP, Cl<sup>-</sup> channel opening and activation of the Cl<sup>-</sup>/bicarbonate exchanger and secretin-induced bicarbonate-rich choleresis (1, 3, 5, 6, 10, 11, 17, 19, 28, 30, 50).

Cholangiocytes are the target cells in a number of animal models of ductal hyperplasia, including bile duct ligation (BDL), α-naphthyl isothiocyanate feeding, or 70% partial hepatectomy (5–7, 9, 10, 19, 30, 50). In BDL rats, the cholangiocyte proliferative response is closely associated with an increase in secretin-stimulated choleresis in vivo and with an augmented secretin receptor gene expression, secretin-induced cAMP synthesis, and Cl<sup>-</sup>/bicarbonate exchanger activity in purified cholangiocytes (5, 6, 9, 19, 50). We have recently shown in distinct subpopulations of cholangiocytes and intrahepatic bile duct units (IBDU) that cholangiocytes are morphologically and functionally heterogeneous along the length of the intrahepatic biliary tree and that secretin-regulated transport of water and electrolytes occurs in large (but not small) cholangiocytes and IBDU (3, 8, 10).

Endothelins (ET-1, ET-2, ET-3) are a family of vasoactive peptides that interact with at least two types of receptors, ET<sub>A</sub> and ET<sub>B</sub>, which are distributed in numerous organs (16). ET-1, a potent vasoconstrictor of 21 amino acids, has a multifunctional role in a variety of tissues and cells (45, 53, 54). In the liver, ET-1 induces cholestasis, which is associated with an increase in portal pressure when administered to the isolated perfused rat liver (IPRL) (14, 25). ET-1 activates glycogenolysis, phosphoinositide synthesis, and intracellular free Ca<sup>2+</sup> oscillations in purified hepatocytes (18, 43). Furthermore, rat sinusoidal endothelial cells in culture synthesizes ET-1 (37). Endothelin receptors have been detected in rat hepatic stellate cells (23). ET-1 has been detected in human gallbladder epithelial cells in culture (22) and in bile ducts in human cirrhotic liver sections (35). However, no data exist regarding the effects of ET-1 on spontaneous and secretin-regulated cholangiocyte secretory processes in different segments of the rat intrahepatic biliary tree. Given the fact that the intrahepatic biliary tree is functionally heterogeneous with regard to ductal secretory processes (1, 3, 8, 10) and that ET-1 plays an important role in the regulation of secretion in a number of organs (21, 29, 33), we evaluated 1) if ET-1 receptors (ET<sub>A</sub> and ET<sub>B</sub>) are expressed (at the gene, protein, and functional levels) in small and large cholangiocytes and 2) if ET-1 regulates basal and secretin-stimulated ductal secre-
ory activity in normal and diseased liver following BDL.

To begin to define the role of ET-1 on the modulation of basal and secretin-stimulated ductal bile secretion, we investigated the effect of ET-1 on 1) secretin receptor gene expression and intracellular cAMP synthesis in purified cholangiocytes, 2) basal and secretin-induced lumen expansion in large (>15 µm diameter) IBDU, and 3) spontaneous and secretin-induced bile flow and biliary bicarbonate secretion in vivo. We studied the expression of ET-1 receptors (ETA and ETB) in purified subpopulations of small and large cholangiocytes and subsequently determined the receptor subtype responsible for these effects by using a specific ETA receptor antagonist (BQ-610) (24, 42) and ETB receptor agonists [i.e., ET-3 and sarafotoxin 6c (S6c)] (14, 39, 55). We found that ET-1 receptors (ETA and ETB) are expressed in small and large cholangiocytes from both normal and BDL rats and showed that ET-1 inhibited secretin-stimulated cAMP synthesis and secretin receptor gene expression in large cholangiocytes and the secretin-induced ductal luminal expansion in large, purified IBDU by selectively interacting with ETA receptors. Similarly, ET-1 decreased secretin-induced bicarbonate-rich choleretic and bicarbonate secretion in BDL rats. The inhibitory effects of ET-1 on cholangiocyte secretory processes were completely blocked by BQ-610, a specific inhibitor of the ETA receptor (24, 42).

MATERIALS AND METHODS

Animal Model

Male Fischer 344 rats (150–200 g) were purchased from Charles River (Wilmington, MA) and were maintained at 22°C with a 12:12-h light-dark cycle and fed ad libitum with standard rat chow. Before each experiment, the animals were anesthetized with pentobarbital sodium (50 mg/kg ip). Experiments were performed in normal rats and rats with ductal hyperplasia induced by BDL for 2 wk (5, 6, 19, 50). Experimental protocols were conducted in compliance with institutional guidelines.

Materials

Reagents were purchased from Sigma Chemical (St. Louis, MO), unless otherwise indicated. Endothelins (ET-1 and ET-3) were purchased from Calbiochem (La Jolla, CA). BQ-610, a selective antagonist of the ETA receptor (24, 42), was purchased from Alexis (San Diego, CA). S6c, a specific agonist of the ETB receptor (39, 55), was purchased from American Peptide (Sunnyvale, CA). Dulbecco's PBS was obtained from Celox (Hopkins, MN). Secretin was purchased from Peninsula Laboratories (Belmont, CA). RIA kits for the determination of intracellular cAMP levels in pure preparations of small and large cholangiocytes were purchased from Amersham (Arlington Heights, IL).

Isolation and Morphological and Phenotypic Characterization of Small and Large Cholangiocytes and Large IBDU From Normal and BDL Rats

Pure subpopulations of small and large cholangiocytes were isolated from both normal and BDL rat liver as previously described (2, 8, 10). Briefly, after standard collagenase perfusion was performed, a mixed nonparenchymal cell fraction was obtained from undissociated liver tissue by digestion with a mixture of proteolytic enzymes as described by Ishii et al. (26). The cholangiocyte-enriched fraction (~50% pure by histochemistry for γ-glutamyltransferase (γ-GT) (40), a cholangiocyte-specific marker (5, 7, 26)) was separated into two distinct subpopulations of small and large cholangiocytes by counterflow elutriation using a Beckman J-6-MI centrifuge equipped with a J-E-5.0 rotor (Beckman Instruments, Fullerton, CA). The two subpopulations of small and large cholangiocytes were obtained at the flow rates of 25 and 55 ml/min, respectively, and further purified by immunoaffinity purification (26) using a monoclonal antibody to an antigen ubiquitously expressed on all intrahepatic cholangiocytes (26). Cell number and viability were determined by trypan blue exclusion. Cell purity was assessed by histochemistry for γ-GT (40). Contamination by hepatocytes and mesenchymal cells was determined by glucose-6-phosphatase histochemistry (49) and vimentin immunohistochemistry (2), respectively. Mean diameters of small and large cholangiocytes purified from both normal and BDL rats were measured by computerized image analysis using a technique recently described by us in cholangiocytes (10) and similar to that described in other cells (47).

Large IBDU from both normal and BDL rat liver were isolated and morphologically characterized as previously described by us (1, 3). After standard collagenase perfusion (3, 38), the intrahepatic biliary tree was exposed by mechanical removal of parenchymal cells until only bile duct structures were present. The mixed bile duct fragments were placed in 50-mm petri dishes, visualized under phase-contrast optics (>10 objective) on a Nikon Diaphot inverted microscope (Tokyo, Japan), picked up by a 10-μl pipette, and subsequently transferred to the stage of a Nikon Diaphot microscope equipped with fluorescence and differential interference contrast optics. To separate large bile ducts from smaller bile ducts, we used a microscope-focused laser (3), an approach previously used in other cells (13). This approach was previously used by us for isolating small and large bile ducts from normal rat liver (3). Briefly, a nitrogen-pulsed dye laser (model LSI-377, LSD Industries, Cambridge, MA, coumarin 481 as a laser dye generating a 120-mJ/pulse, 20-Hz repetition rate energy output) was focused to the diffraction limit through the epilumination port of the fluorescence microscope containing a fluorescein filter cube (Omega Optical, Brattleboro, VT) to produce a very well-localized high-energy light beam (13). Typically, between one and five pulses were delivered to the junction of small and large ducts to cut and separate large ducts from the smaller bile ducts. Isolated large IBDU from normal or BDL rats were then placed in minimum essential medium (GIBCO BRL, Grand Island, NY) containing 10% FCS, allowed to settle on glass coverslips, previously coated with Cell-TAK, and incubated for 24 h at 37°C before effects of ET-1 on both basal and secretin-stimulated ductal lumen expansions were measured (see Measurement of Basal and Secretin-Induced Ductal Lumen Expansion in Large IBDU Purified From Both Normal and BDL Rats).

Molecular, Immunohistochemical, and Immunologic Analyses of ETA and ETB Receptors in Small and Large Cholangiocytes From Normal and BDL Rats

Molecular analyses of ETA and ETB in small and large cholangiocytes from normal and BDL rats were performed. The genetic expression of ETA, ETB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [the housekeeping gene (2, 4, 8–10, 19, 50)] in
pure preparations of small and large cholangiocytes purified from both normal and BDL rats was determined by lysate RNase protection assay (Direct Protect, Ambion, Austin, TX) according to the instructions of the manufacturer. The RNase protection assay was performed with cell lysate samples each containing 4.5 x 10^7 cholangiocytes. This technique has been previously used by us to measure quantitative gene expression in purified cholangiocytes from both normal and proliferating rat liver (2, 19, 30). This procedure has also been used to determine steady-state levels of selected genes in other cell systems (48). Briefly, an antisense 32P-labeled riboprobe was transcribed from the selected linearized DNA template with T7 or SP6 RNA polymerase using [a-32P]UTP (800 Ci/mmole, Amersham). The full-length RNA transcript was purified by excision from a 5% acrylamide-8 M urea denaturing gel with subsequent elution for 3 h at 37°C into a solution of 0.5 M ammonium acetate, 1 mM EDTA, and 0.1% SDS. After hybridization at 45°C for 12 h, the solution was treated with a mixture of RNase A-T1 (150–200 U/ml) to digest unhybridized RNA chains and the protected hybrid was resolved in a 5% acrylamide-8 M urea gel and detected by autoradiography at -70°C for 2 days. After exposure for 2 days, autoradiograms were quantified by scanning video densitometry using the Chemilager 4000 low-light imaging system (Alpha Innotech, San Leandro, CA).

The following RNA probes were used: a 316-bp riboprobe complementary to rat GAPDH mRNA was obtained from cDNA purchased from Ambion. Both ETA and ETB cDNAs were generated in our laboratory by RT-PCR with the Gene-Amp RNA PCR kit (Perkin-Elmer, Branchburg, NJ) using poly(A) + mRNA obtained from pure preparations of cholangiocytes. Primers for ETA mRNA were based on the rat ETA sequence (31) (sense: 5'-GCCCTTGGAGAGACCTTATCTAC-GT-3'; antisense: 5'-GGTGACTACGCAAGCCGATT-3'), with an expected fragment length of 273 bp. Primers for ETB mRNA were based on the rat ETB sequence (41) (sense: 5'-TCTTGATATAACATCGCTG-3'; antisense: 5'-CTG-GACGGGAAGTTGCATTAC-3'), with an expected fragment length of 130 bp. Standard RT-PCR conditions were used with 5 ng of poly(A) + mRNA (35 step cycles: 30 s at 94°C, 30 s at 52°C, and 45 s at 72°C). Poly(A) + mRNA was extracted from cholangiocytes purified by the Microtainer kit (Invitrogen, San Diego, CA) according to the instructions supplied by the vendor. After ligation into the EcoRI site of pCR vector (TA cloning kit, Invitrogen) to confirm their identity, the PCR fragments were sequenced using a Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH).

Immunohistochemistry for ETA and ETB in small and large cholangiocytes purified from normal and BDL rat liver was performed with the anti-ETA or ETB antibody (1:100 dilution) or ET-1 (10^-7 M) in 0.2% BSA for 30 min at 37°C. The immunohistochemical expression of secretin receptor was subsequently determined by lysate RNase protection assay (Direct Protect, Ambion) according to the instructions from the manufacturer to determine the intracellular levels of cAMP, a functional assay for the secretin receptor (2, 3, 8, 10, 19, 28, 30, 50). Because antibodies against the secretin receptor are not commercially available, the cAMP assay allows us to closely correlate the molecular with the functional expression of the secretin receptor in purified cholangiocytes (8, 10, 19, 30, 50). After isolation, pure preparations of small and large cholangiocytes were incubated for 1 h at 37°C to restore
surface proteins damaged by treatment with proteolytic enzymes (28, 30). Then, small and large cholangiocytes (1 × 10^7) were stimulated for 5 min at 22°C (8, 10, 19, 30, 50) with 0.2% BSA (control), secretin (10^−7 M), ET-1 (10^−10 to 10^−7 M), or secretin (10^−7 M) plus ET-1 (10^−10 to 10^−7 M) in the presence of 0.2% BSA. In separate sets of experiments, we also evaluated the effects of ET-1 (10^−7 M) on basal and secretin-induced cAMP levels in the presence of BQ-610 (10^−7 M), a specific inhibitor for the ET<sub>A</sub> receptor (24, 42). To ensure that BQ-610 does not differentially affect basal cAMP synthesis, cAMP levels of large cholangiocytes from normal or BDL rats were also determined in the absence of BQ-610. Finally, we evaluated the effects of ET-3 and S6c (both at 10^−7 M) on both basal and secretin-stimulated cAMP synthesis. Phosphodiesterase activity was inhibited by 0.5 mM IBMX. After ethanol extraction, spontaneous and agonist-induced intracellular cAMP levels were measured by RIA using commercial kits (Amersham) (2, 3, 8, 10, 19, 28, 30, 50).

Measurement of Basal and Secretin-Induced Ductal Lumen Expansion in Large IBDU Purified From Both Normal and BDL Rats

Large IBDU were incubated for 24 h at 37°C in minimum essential medium containing 10% FCS to allow complete sealing of bile duct lumen (3, 34, 38). With the use of light microscopy to visualize the diameter of bile duct lumen, ductal fluid secretion was estimated from the changes in the volume of duct lumen (3, 34, 38) after stimulation at 37°C with BSA (control, 10 min), secretin (10 min at 10^−7 M), ET-1 (10 min at 10^−7 M), or ET-1 plus secretin (10 min each, both at 10^−7 M) in the absence or presence of the ET<sub>A</sub> inhibitor BQ-610. The methodology for measuring changes in bile duct volume in response to agonists has been described in great detail in previous studies by us and others (3, 34, 38).

In Vivo Biliary Physiology

Normal and BDL rats were surgically prepared for bile collection as previously described (5, 6, 19, 30, 50). One jugular vein was cannulated with a PE-50 cannula (Clay-Adams, New York, NY) to infuse either Krebs-Henseleit bicarbonate solution (KRH), secretin, ET-1, or secretin plus ET-1 (2) dissolved in KRH. Blood was withdrawn every 10 min from one carotid artery (by a PE-60 cannula) to assess the arterial hematocrit, which was constant (41–45%) in all rats during bile collection. The rate of fluid infusion was adjusted to both the rate of bile flow and the value of the arterial hematocrit and ranged from 0.738 to 2.328 ml/h. Body temperature was monitored with a rectal thermometer (Yellow Springs Instrument, Yellow Springs, OH) and maintained at 37°C by using a heating pad. When steady-state spontaneous bile flow was achieved (60–70 min from the beginning of bile collection), we infused secretin (10^−7 M) for 30 min, KRH until new steady state was reached, and then secretin plus ET-1 (both 10^−7 M) for 30 min, followed by a final infusion of KRH. In separate sets of experiments, we also determined 1) the effect of ET-1 at different concentrations (10^−10 to 10^−7 M) and 2) the effect of ET-3 and S6c (both at 10^−7 M) on both basal and secretin-induced cholestasis. The dose of secretin (10^−7 M) used in the present studies is the same as that of in vitro and in vivo studies from us and others (2, 3, 8, 10, 19, 28, 30, 50). In normal rats, blood secretin concentration ranges from 10^−11 to 10^−12 M (44, 52). The doses of ET-1 employed in the present studies (10^−7 to 10^−10 M), slightly above physiological doses are, however, in the range of that used in other cell systems by a number of investigators (27, 32). The plasma concentrations of both ET-1 and ET-3 ranged from 10^−11 to 10^−12 M (20, 36). Throughout the experiment, bile was collected every 10 min and bile flow was determined by weight, assuming a density of 1.0 g/ml. Bile bicarbonate concentration (measured as CO₂) was determined in bile by a Natelson microgasometer apparatus (Scientific Industries, Bohemia, NY).

Statistical Analysis

All data are expressed as means ± SE. The differences between groups were analyzed by Student’s t-test when two groups were compared, by ANOVA when more than two groups were compared, and by the nonparametric Mann-Whitney U-test when two groups were compared.

**Fig. 1. A:** analysis of the genetic expression of endothelin-1 (ET-1) receptors (ET<sub>A</sub> and ET<sub>B</sub>) in small and large cholangiocytes purified from both normal and bile duct-ligated (BDL) rats by counterflow elutriation followed by immunonaffinity separation (see MATERIALS AND METHODS). In both small and large cholangiocytes, expression of the selected messages was determined by direct RNase protection assay using cell lysate samples each containing 4.5 × 10^6 purified cholangiocytes (see MATERIALS AND METHODS). Comparability of the RNA used was assessed by hybridization for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the housekeeping gene. Autoradiograms (n = 2) were quantified by densitometry (B). Note that the messages for ET<sub>A</sub> (273 bp) and ET<sub>B</sub> (130 bp) receptors were present in both small and large cholangiocytes purified from normal or BDL rats.
groups were analyzed or ANOVA if more than two groups were analyzed.

RESULTS

Isolation, Morphological, and Phenotypic Characterizations of Small and Large Cholangiocytes or Large IBDU

We isolated virtually pure (by γ-glutamyltranspeptidase histochemistry) subpopulations of small and large cholangiocytes from both normal and BDL rat liver (results not shown). No hepatocytes or mesenchymal cells were detected in our cholangiocyte preparations (results not shown) (2). Cell viability was >99%. Pure preparations of large IBDU (by γ-GT histochemistry) were obtained from normal and BDL rats (results not shown). The purity and morphological, phenotypic, and functional characteristics of isolated IBDU from normal or BDL rats have been described in great detail in previous studies (1, 3).

Molecular, Immunohistochemical, and Immunologic Analyses of ET$_A$ and ET$_B$ Receptors in Small and Large Cholangiocytes

Molecular analysis. Molecular analysis of purified cholangiocytes shows that the transcripts for ET$_A$ (273 bp) and ET$_B$ (130 bp) receptors were present in both small and large cholangiocytes (Fig. 1A). Densitometric analysis of two experiments showed that no changes in ET$_A$ or ET$_B$ receptor gene expression were observed in small and large cholangiocytes purified from BDL rats compared with small and large cholangiocytes from normal rats (Fig. 1B). The quantitative expression of GAPDH (housekeeping gene) was similar in small and large cholangiocytes purified from normal or BDL rats (Fig. 1).

Immunohistochemistry. At the protein level, parallel with the data shown in the molecular analysis of Fig. 1, immunohistochemistry of purified cholangiocytes (Fig. 2) shows positive staining for ET$_A$ or ET$_B$ receptors in both small and large cholangiocytes from normal or BDL rat liver.

Western blot analysis. Immunoblotting analysis shows that a signal (migrating at 35 kDa) for the ET$_A$ and ET$_B$ receptors was expressed in both small and large cholan-
giocytes purified from normal or BDL rat liver (Fig. 3). Quantitative densitometric analysis of two experiments showed that no changes were observed in the protein expression of ETA or ETB receptors in small and large cholangiocytes purified from BDL rats compared with small and large cholangiocytes from normal rats (results not shown). The data closely parallel with the genetic expression of ETA or ETB receptors in small and large cholangiocytes purified from normal or BDL rats (see Fig. 1).

Effect of ET-1 on Secretin Receptor Gene Expression in Purified Cholangiocytes

Because secretin receptor is a specific marker of ductal secretory activity (1, 3, 9, 19, 30), we investigated if ET-1 modulates the expression of this gene. The message for secretin receptor was present in large (but not small) cholangiocytes (8–10), and the expression of the secretin receptor gene was significantly (P < 0.05) increased in large cholangiocytes purified from BDL rats compared with small and large cholangiocytes from normal rats (results not shown). The data closely parallel with the genetic expression of ETA or ETB receptors in small and large cholangiocytes purified from normal or BDL rats (see Fig. 1).

Fig. 4. Effect of ET-1 on the genetic expression of secretin receptor (SR) in small and large cholangiocytes purified from both normal and BDL rats. After purification, virtually pure preparations of small and large cholangiocytes were incubated with ET-1 (10^{-7} M) for 30 min at 37°C, and subsequently SR gene expression was determined by direct RNase protection assays (see MATERIALS AND METHODS). Comparability of the RNA used was assessed by hybridization for GAPDH, the housekeeping gene. Autoradiograms in A (n = 3) were quantified by densitometry (B) using the ChemiImager 4000 low-light imaging system. Densitometric values are means ± SE. *P < 0.05 vs. SR gene expression of large control cholangiocytes obtained from normal rats. Statistical analysis was performed with both ANOVA and unpaired t-test.

Intracellular cAMP Levels

Because antibodies against the secretin receptor are not commercially available, the secretin-stimulated cAMP synthesis is commonly used to measure the functional expression of the secretin receptor in cholangiocytes (1–4, 8, 10, 11, 19, 28, 30, 38, 50). Basal intracellular cAMP levels in small and large cholangiocytes from both normal (Fig. 5A) and BDL (Fig. 5B) rat liver were similar to those reported in previous studies (2, 8, 10). Basal cAMP levels of large cholangiocytes purified from BDL rats were higher than cAMP levels of large cholangiocytes purified from normal rats (see Figs. 5 and 6). Secretin (10^{-7} M) induced a significant increase in intracellular cAMP levels in large (but not small) cholangiocytes purified from both normal (Fig. 5A) and BDL (Fig. 5B) rat liver. ET-1 (at 10^{-10} to 10^{-7} M) did not alter basal intracellular cAMP synthesis in small or large cholangiocytes from both normal and BDL rats (results not shown). In contrast, ET-1 inhib-
ited secretin-stimulated cAMP synthesis in large cholangiocytes from both normal (Fig. 5A) and BDL (Fig. 5B) rats. In the presence of BQ-610, a specific antagonist of the ET\textsubscript{A} receptor (24, 42), ET-1 did not inhibit secretin-induced cAMP synthesis in large cholangiocytes from normal (Fig. 6A) or BDL (Fig. 6B) rats. BQ-610 did not alter basal cAMP levels in large cholangiocytes purified from normal or BDL rats (Fig. 6). No effect on basal or secretin-induced cAMP levels was seen when small or large cholangiocytes were treated with ET-3 or S6c (results not shown), specific agonists for the ET\textsubscript{B} receptor (14, 39, 55), which indicates that ET-1 modulates ductal secretory activity by interacting primarily, if not exclusively, with type A receptors.

**Effect of ET-1 on Basal and Secretin-Stimulated Ductal Lumen Expansion**

To directly assess that the inhibitory effects of ET-1 on secretin-induced ductal bile secretion occurred through a direct action on cholangiocytes rather than by ET-1-induced hemodynamic changes in the liver (51), we isolated large IBDU from both normal and BDL rats and studied their secretory activity in response to 0.2% BSA (control), secretin (10\textsuperscript{-7} M), or ET-1 plus secretin (both at 10\textsuperscript{-7} M). An increase in bile duct lumen volume in large but not small IBDU from normal or BDL rats was observed after stimulation with secretin (10\textsuperscript{-7} M) (Fig. 7). In a fashion similar to that observed for cAMP synthesis, ET-1 alone did not alter IBDU lumen size (Fig. 7). In contrast, when administered in combination with secretin (both at 10\textsuperscript{-7} M), ET-1 inhibited secretin-induced lumen expansion in large IBDU from both normal and BDL rat liver (Fig. 7). The inhibitory effect of ET-1 on secretin-increased ductal lumen expansion was abolished by pretreating purified IBDU with BQ-610 (10\textsuperscript{-7} M) (Fig. 7).

**In Vivo Studies of Biliary Physiology**

In normal rats, spontaneous bile secretion (73.96 ± 5.36 µl·min\textsuperscript{-1}·kg body wt\textsuperscript{-1}) and biliary bicarbonate secretion (2.05 ± 0.11 µeq·min\textsuperscript{-1}·kg body wt\textsuperscript{-1}) were similar to those reported in previous studies (5, 6, 19, 30, 50). Secretin had no stimulatory effects on bile flow or bicarbonate secretion in normal rats (results not shown). Intravenous infusion of ET-1 (10\textsuperscript{-10} to 10\textsuperscript{-7} M)
did not alter basal bile flow or biliary bicarbonate secretion in normal rats (results not shown).

In agreement with a number of reports (5, 6, 19, 30, 50), following BDL, both basal bile flow (127.86 ± 8.65 µl·min⁻¹·kg body wt⁻¹) and basal bicarbonate secretion (3.90 ± 0.50 meq·min⁻¹·kg body wt⁻¹) were significantly (P < 0.05) increased compared with normal rats. As shown in Fig. 8, secretin increased both bile flow and bicarbonate secretion. Similar to what was observed in normal rats, ET-1 alone (10⁻¹⁰ to 10⁻⁷ M) did not alter basal bile flow and biliary bicarbonate secretion in BDL rats (results not shown). In contrast, simultaneous infusion of ET-1 and secretin (10⁻⁷ M) markedly reduced, in a dose-dependent fashion, both secretin-induced choleresis and secretin-stimulated bicarbonate secretion (Fig. 8). In a fashion similar to that observed for cAMP synthesis, ET-3 and S6c did not affect spontaneous or secretin-induced choleresis or biliary bicarbonate secretion (results not shown).

**DISCUSSION**

The major findings of this study relate to the expression of ET-1 receptors (ETₐ and ETₐ) in small and large cholangiocytes purified from normal and BDL rats and to the inhibitory effect of ET-1 (but not ET-3) on secretin-stimulated ductal bile secretion in both normal and BDL rats. We isolated two pure (by γ-GT histochemistry) and distinct subpopulations of small and large cholangiocytes from normal and BDL rats.
and have shown that the ET_A and ET_B receptors are expressed (at both the message and protein levels) by both small and large cholangiocytes. At the functional level, ET-1 did not alter in vivo spontaneous bile flow or biliary bicarbonate secretion in normal or BDL rats but inhibited the stimulatory effects of secretin on both bile flow and biliary bicarbonate secretion in BDL rats. Parallel with the in vivo effects, ET-1 did not change basal cAMP synthesis but significantly decreased secretin-induced increases in bile flow or biliary bicarbonate secretion. Data are mean ± SE for 6 rats. Statistical analysis was performed with both ANOVA and unpaired t-test. Note that scales on vertical axes are different.

In normal rats, ductal bile flow consists of only 10% of the total bile volume (6). Furthermore, secretin receptor gene expression and secretin-induced cAMP response are very low (8, 9, 28, 30) and secretin does not increase bile flow or biliary bicarbonate secretion in normal rats (5, 6, 19, 30, 50). In the present studies, we used the BDL model in which secretin receptor gene expression and intracellular cAMP levels are markedly increased in purified cholangiocytes (1, 9, 19, 50) and in which secretin markedly increases bile flow and biliary bicarbonate secretion in vivo (5, 6, 19, 50). The secretory and reabsorptive processes of the intrahepatic biliary epithelium are tightly regulated in a coordinated fashion by a number of gastrointestinal hormones and neuropeptides (1–12, 15, 17, 19, 28, 30, 34, 38, 50). The hormone secretin, for example, induces in vivo an increase in bile flow and biliary bicarbonate secretion (5, 6, 19, 30, 50) by interaction with specific receptors on cholangiocytes (9) through an increase in the synthesis of the second messenger system, cAMP (3, 8, 10, 19, 28, 30, 50). In contrast, somatostatin (4, 50) and gastrin (19) regulate ductal bile secretion by countering the in vivo choleretic effects of secretin by decreasing both secretin receptor gene expression and secretin-stimulated intracellular cAMP synthesis. The cholinergic system plays an important role in the regulation of cholangiocyte secretory processes (11). For example, ACh increases the secretin-induced stimulation of cAMP synthesis and Cl-/bicarbonate exchanger activity in both IBDU and purified cholangiocytes by directly interacting with M_3 receptor subtypes on cholangiocytes (11). All these studies demonstrate that the secretin receptor and secretin-induced cAMP response are key factors in the modulation of ductal bile secretion (1–4, 7–12, 19, 28, 30, 50). Our data are consistent and extend the concept that the secretory processes of the intrahepatic biliary epithelium are subjected to a tight regulation with stimulatory effects exerted by secretin (3, 5–8, 10–12, 19, 30, 50), bombesin (15), and ACh (11) and an opposing inhibition by ET-1, somatostatin (4, 50), and gastrin (19). Moreover, the data further support the notion that the secretin receptor and some of the biological events associated with its expression (i.e., cAMP response) tightly regu-
late the secretory processes of cholangiocytes in both normal and proliferating rat liver (1–4, 7–12, 19, 28, 30, 50). Indeed, secretin receptor and secretin-induced cAMP responses are upregulated with all forms of cholangiocyte proliferation studied to date in which we observed a marked increase in ductal secretory activity (2, 9, 10, 19, 30, 50).

ET-1 has been shown (14, 25) to reduce bile flow in IPRL, an effect that is mediated by an increase in portal pressure. In our studies, ET-1 did not alter basal bile flow, which is due presumably to the different model used to measure bile flow (i.e., bile fistula rats vs. IPRL) during ET-1 infusion. The absence of ET-1 on basal bile flow suggests that ablation of secretin-induced cholestasis by ET-1 is not due to cholestatic changes secondary to altered blood flow. Furthermore, to ensure that the inhibitory effects of ET-1 on secretin-induced ductal bile secretion are due to a direct interaction with cholangiocytes rather than an in vivo indirect effect of ET-1 [e.g., vasoconstriction or increased plasma levels of vasoactive hormones affecting cell function (16, 25, 46)] on cholangiocyte secretion, we examined the effects of ET-1 on ductal bile secretion in pure subpopulations of small and large cholangiocytes and polarized large IBDU that allow us to directly measure cholangiocyte secretory processes (1–3, 7–11, 19, 28, 30, 34, 38, 50).

We found that ETA and ETB (at both the gene and protein levels) were expressed by small and large cholangiocytes purified from normal or BDL rats. Our studies demonstrate a direct interaction of ET-1 with ETA (but not ETB) receptors on cholangiocytes to explain the inhibitory effect that ET-1 has on secretin-induced increases in ductal bile secretion. The inhibitory effect of ET-1 on secretin-induced cAMP synthesis in purified cholangiocytes and secretin-induced bicarbonate-rich choleresis supports the concept of specific, physiologically active receptors for ET-1 on cholangiocytes. Surprisingly, we found that in normal rats secretin-stimulated cAMP synthesis in large cholangiocytes is inhibited by even low concentrations of ET-1, whereas in cholangiocytes from BDL rats the inhibitory effect of ET-1 on secretin-induced cAMP synthesis is only observed at 10^-8 M. Perhaps after BDL there is loss of ET-1 control over secretin-induced cholangiocyte secretory function. For example, the loss of negative feedback on cholangiocyte secretion by ET-1 may lead to higher ductal secretion in BDL rats. This would be a potential mechanism for increased ductal secretion in BDL that is independent of increased secretin receptor expression. In support of the view that ET-1 interacts with cholangiocytes through the ETA receptor, we have demonstrated that, in the presence of BQ-610, a specific inhibitor of ETA (BQ-610) receptor (24, 42), ET-1 does not inhibit secretin-stimulated cAMP synthesis in large cholangiocytes and secretin-induced lumen duct expansion in large IBDU. Moreover, in different sets of experiments, we have demonstrated the presence of endothelin receptors (ETA and ETB) at both the mRNA and protein levels in rat cholangiocytes. Finally, consistent with the concept that ET-1 inhibition of secretin-induced cholangiocyte secretory processes occurs by interaction with ETA, but not ETB, receptors on cholangiocytes, we have shown that ET-3 and S6c [specific agonists of the ETB receptor (14, 39, 55)] do not alter basal or secretin-induced cAMP synthesis and secretin-stimulated bicarbonate-rich choleresis. Because we found that cholangiocytes also contain ETB receptors, we suggest that ET-3 and S6c [specific agonists of the ETB receptor (14, 39, 55)] do not affect secretin-stimulated ductal secretory activity because they are not involved in the cAMP signaling pathway of cholangiocytes.

We believe that our findings have important physiopathological implications, since little is known about the cellular and molecular regulatory mechanisms of ductal bile secretion. This may lead to a better understanding of the mechanisms of enhanced cholangiocyte secretory processes in a number of cholestatic liver diseases, including extrahepatic biliary obstruction, primary biliary cirrhosis, and primary sclerosing cholangitis. Because endothelin receptor antagonists may be therapeutically employed in the future to promote collagen matrix degradation in patients with chronic liver diseases (39), it is important that we understand the effect of ET-1 on biliary physiology. In summary, the results of the present study provide further insight into the understanding of the regulatory mechanisms of ductal bile secretion and the potential involvement of ET-1 in clinical cholestatic conditions characterized by increased bile duct proliferation and ET-1 overexpression.

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