Adrenergic receptor agonists prevent bile duct injury induced by adrenergic denervation by increased cAMP levels and activation of Akt

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Glaser, Shannon, Domenico Alvaro, Heather Francis, Yoshiyuki Ueno, Luca Marucci, Antonio Benedetti, Sharon De Morrow, Marco Marzioni, Maria Grazia Mancino, Jo Lynne Phinizy, Ramona Reichenbach, Giammarco Fava, Ryun Summers, Julie Venter, and Gianfranco Alpini. Adrenergic receptor agonists prevent bile duct injury induced by adrenergic denervation by increased cAMP levels and activation of Akt. Am J Physiol Gastrointest Liver Physiol 290: G813–G826, 2006. First published December 8, 2005; doi:10.1152/ajpgi.00306.2005.—Loss of parasympathetic innervation after vagotomy impairs cholangiocyte proliferation, which is associated with depressed cAMP levels, impaired ductal secretion, and enhanced apoptosis. Agonists that elevate cAMP levels prevent cholangiocyte apoptosis and restore cholangiocyte proliferation and ductal secretion. No information exists regarding the role of adrenergic innervation in the regulation of cholangiocyte function. In the present studies, we investigated the role of adrenergic innervation on cholangiocyte proliferative and secretory responses to bile duct ligation (BDL). Adrenergic denervation by treatment with 6-hydroxydopamine (6-OHDA) during BDL decreased cholangiocyte proliferation and secretin-stimulated ductal secretion with concomitant increased apoptosis, which was associated with depressed cholangiocyte cAMP levels. Chronic administration of forskolin (an adenyl cyclase activator) or β1- and β2-adrenergic receptor agonists (clenbuterol or dobutamine) prevented the decrease in cholangiocyte cAMP levels, maintained cholangiocyte secretory and proliferative activities, and decreased cholangiocyte apoptosis resulting from adrenergic denervation. This was associated with enhanced phosphorylation of Akt. The protective effects of clenbuterol, dobutamine, and forskolin on 6-OHDA-induced changes in cholangiocyte apoptosis and proliferation were partially blocked by chronic in vivo administration of wortmannin. In conclusion, we propose that adrenergic innervation plays a role in the regulation of biliary mass and cholangiocyte functions during BDL by modulating intracellular cAMP levels. apoptosis; bile ducts; growth; nerves; secretin

CHOLANGIOCYTES ARE THE TARGET cells of cholangiopathies (7), which are characterized by dysregulation of the balance between cholangiocyte proliferation/apoptosis (7). In experimental models, cholangiocyte proliferation is achieved by a number of pathological maneuvers, including bile duct ligation (BDL; see Refs. 6, 7, 20, 31, 34), whereas cholangiocyte damage by apoptosis occurs after acute administration of carbon tetrachloride (CCL4; see Ref. 35) or chronic feeding of the toxin α-naphthylisothiocyanate (33). Although cholangiocyte proliferation is associated with increased basal and secretin-stimulated ductal secretion (6, 20, 31, 34), apoptosis is coupled with decreased basal and secretin-stimulated ductal secretory activity (35).

There is growing information regarding the mechanisms regulating the balance between cholangiocyte proliferation/apoptosis (4, 11, 19, 24). Cholangiocyte proliferation is differentially regulated by a number of hormones and neuropeptides (4, 11, 19, 24). Although somatostatin inhibits cholangiocyte proliferation of BDL rats by a decrease in the synthesis of the intracellular cAMP system (4), gastrin inhibits both hyperplastic and neoplastic cholangiocyte proliferation by an increase in Ca2+/protein kinase C (PKC)-regulated cholangiocyte apoptosis (19, 24). Interruption of the cholinergic innervation by vagotomy impairs cholangiocyte proliferation and enhances apoptosis by a decrease in intracellular cAMP levels, thus leading to a decrease in the number of intrahepatic cholangiocytes in response to BDL (25, 31). Maintenance of cAMP levels, by forskolin administration, prevents the effects of vagotomy on cholangiocyte proliferation and apoptosis (31).

In many different cell types, activation of the cAMP/protein kinase A (PKA)/mitogen/extracellular signal-regulated kinase (MEK)/mitogen-activated protein kinase (MAPK) intracellular pathway is associated with a wide range of biological responses, including differentiation, survival, inhibition of growth, and apoptosis (13, 29, 39, 56). Recent evidence indicates that the cAMP/PKA/MEK/MAPK cascade is involved in the modulation of cholangiocyte functions by different agents (3, 5, 17, 34). We have shown that elevation of cAMP: 1) protects cholangiocytes from vagotomy-induced apoptosis and 2) stimulates the proliferation of normal rat cholangiocytes (17). cAMP has been demonstrated to promote the activation of cell survival factors such as protein kinase B (Akt; see Refs. 12 and 28) by cAMP-dependent phosphorylation of Akt in neurons. cAMP has also been implicated in the activation of phosphatidylinositol 3-kinase (PI3-kinase) to modulate bile acid secretion in WF-B9 cells (23).

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The intrahepatic biliary epithelium displays adrenergic innervation (45, 54); however, no information exists regarding the role and mechanisms of action by which adrenergic nerves regulate intracellular cAMP levels and the balance between cholangiocyte proliferation/apoptosis in rats with cholangiocyte hyperplasia induced by BDL. We addressed the following questions: 1) Do cholangiocytes from normal and BDL rats express \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors? 2) Does administration of 6-hydroxydopamine [6-OHDA, which causes degeneration of adrenergic terminal fibers (14)] alter the expression of \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors in cholangiocytes from BDL rats; 3) Does 6-OHDA induce bile duct damage and cholangiocyte apoptosis with subsequent inhibition of cholangiocyte proliferation and ductal functional activity? 4) Does chronic administration of dobutamine (a specific \( \beta_1 \)-adrenergic receptor agonist; see Ref. 55), clenbuterol (a specific \( \beta_2 \)-adrenergic receptor agonist; see Ref. 15), or forskolin (an adenylyl cyclase activator; see Ref. 27) (which all increase intracellular cAMP levels; see Refs. 1, 17, 41) prevent 6-OHDA activation of cholangiocyte apoptosis, and inhibition of cholangiocyte proliferation and ductal bile secretion? 5) Are 6-OHDA effects on cholangiocyte proliferative and secretory capacity associated

Fig. 1.  
(a) Immunohistochemistry for \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors in liver sections from normal and 1-wk bile duct ligation (BDL) rats. A specific reaction appears in bile ducts of normal and 1-wk BDL rats. Original magnification ×250. 
(b) Immunofluorescent staining for \( \beta_1 \) (A and B)- and \( \beta_2 \) (C and D)-adrenergic receptors. Cytospin smears were made from freshly isolated cholangiocytes from normal (A and C) or BDL (B and D) rats. Specific immunoreactivity is shown in red, and the smears were counterstained with DAPI (blue). No staining could be seen when primary antibodies were omitted (E). 
(c) Immunoblotting analysis for \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors in protein (10 \( \mu \)g) from whole cell lysate from BDL rats and BDL rats treated with 6-hydroxydopamine (6-OHDA; c) and apical and basolateral membranes (d) from purified cholangiocytes from BDL rats. c: By immunoblots, we detected the protein for \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors in purified cholangiocytes from BDL rats and BDL rats treated with 6-OHDA. Cholangiocyte protein expression for \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors was not altered in BDL rats compared with BDL + 6-OHDA-treated rats compared with cholangiocytes from BDL rats. AR = adrenergic receptor. Data are means ± SE of 7 experiments. d: These receptor subtypes were found mostly in the basolateral membranes of BDL cholangiocytes.
with changes in the cAMP/PKA and Akt cell survival pathways? 6) Does chronic administration of the cAMP-stimulating agonists (clenbuterol, dobutamine, or forskolin) prevent 6-OHDA-induced alterations in the cAMP/PKA and Akt pathways? and 7) Does administration of wortmannin, a PI3-kinase inhibitor (40), block the protective effects of clenbuterol, dobutamine, and forskolin on 6-OHDA-induced changes in cholangiocyte apoptosis, proliferation, and secretion?

**MATERIALS AND METHODS**

**Animal models.** Male Fischer 344 rats (150 to 175 g) were purchased from Charles River Laboratories (Wilmington, MA), maintained in a temperature-controlled environment (20–22°C) with a 12:12-h light-dark cycle, and fed ad libitum standard rat chow. Animals had free access to drinking water. The studies were performed in: 1) rats with BDL (for preparation of liver blocks and isolated cholangiocytes) or bile duct incannulation (BDI) for bile...
collection (6) for 1 wk and 2 rats that (immediately after BDL or BDI) received a single intraportal injection of 6-OHDA [which induces degeneration of adrenergic terminal fibers (14), 50 mg/kg body wt in 0.9% NaCl plus 0.3% ascorbic acid] followed by daily intraperitoneal injections of 0.9% NaCl, dobutamine [a β2-adrenergic receptor agonist (55), 0.5 μg/g body wt], clenbuterol [a β2-adrenergic receptor agonist (15), 0.5 μg/g body wt], or forskolin [an adenylyl cyclase activator (27), 0.04 mg/100 g body wt, 2 times/day] for 7 days. BDL or BDI were performed as previously described (6). To evaluate if Akt plays a role in the modulation of cholangiocyte proliferation/apoptosis by adrenergic agonists, we evaluated cholangiocyte apoptosis and proliferation in liver sections from rats that (immediately after BDL) received a single intraportal injection of 6-OHDA followed by daily intraperitoneal injections of NaCl, dobutamine, clenbuterol, or forskolin in the presence of daily injections of wortmannin [a PI3-kinase inhibitor (40), 0.7 mg/kg body wt (43)] in DMSO for 7 days. We also evaluated the effects of administration of wortmannin alone on cholangiocyte growth and apoptosis in liver sections from 1-wk BDL rats and rats that (immediately after BDL) received a single intraportal injection of 6-OHDA followed by daily intraperitoneal injections of 0.9% NaCl. Because we have previously shown (31) that chronic intraperitoneal injections of DMSO do not alter cholangiocyte apoptosis and proliferation in BDL rats, we did not include this group in our study. In all animals, body weight, wet liver weight, and liver weight-to-body weight ratio were determined. Study protocols were performed in compliance with institutional guidelines (Institutional Animal Care and Use Committee).

Materials. Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Porcine secretin was purchased from Peninsula (Belmont, CA). The mouse anti-cytokeratin 19 (CK-19) antibody was purchased from Amersham (Arlington Heights, IL). The substrate for γ-glutamyltranspeptidase (γ-GT), N-[γ-L-glutamyl]-4-methoxy-2-naphthylamide was purchased from Polysciences (Warrington, PA). RIA kits for the determination of intracellular cAMP levels were purchased from Amersham. The antibodies for the β1- and β2-adrenergic receptors, proliferating cell nuclear antigen (PCNA), total and phosphorylated PKA, and total and phosphorylated Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Receptor expression in cholangiocytes was confirmed with a β1-adrenergic receptor antibody (PA1–049 from ABR-Affinity BioReagents, Golden, CO) and a β2-adrenergic receptor antibody (M-20 from Santa Cruz). The antibody for β-actin (AC-74) was purchased from Sigma-Aldrich (St. Louis, MO). β2-adrenergic receptor is an affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the COOH-terminus of β2-adrenergic of mouse origin. β2-adrenergic receptor is a rabbit polyclonal antibody raised against amino acids 338–413 mapping at the COOH-terminus of β2-adrenergic of mouse origin. Phosphorylated PKAα (Ser96) is a rabbit polyclonal affinity-purified antibody raised against phosphorylated Ser96 of PKAα of human origin. Phosphorylated Akt1/2/3 (Ser473)-R is a rabbit polyclonal antibody raised against a short amino acid sequence containing phosphorylated Ser473 of Akt1/2/3 of human origin. Total Akt1/2/3 is a goat polyclonal IgG (sc-1618) raised against the COOH-terminus of Akt1, which recognizes Akt1 and to a lesser extent Akt2/3. Water-soluble forskolin [forskolin, 7-deacetyl-7-[O-(N-methylpiperazino)]-γ-butyryl], dibydrochloride; see Refs. 17 and 21], an adenylyl cyclase activator (27), was purchased from Calbiochem-Nova Biochem (San Diego, CA).

Purification of cholangiocytes. Purified cholangiocytes [97–98% pure (4, 5, 19, 20, 22, 26) by γ-GT histochemistry (47)] from the selected groups of animals were obtained by immunofluorescence bead separation (22) using a mouse monoclonal OC-2 antibody (IgM, kindly provided by Dr. R. Faris, Brown University, Providence, RI) against an unidentified membrane antigen expressed by all rat intrahepatic cholangiocytes (22). Cell viability assessed by trypan blue exclusion was ~97%.

Expression of β1- and β2-adrenergic receptors in liver sections and purified cholangiocytes. Immunohistochemistry for β1- and β2-adrenergic receptors was performed in paraffin-embedded liver sections (5 μm thick) from normal and 1-wk BDL rats. The endogenous peroxidase activity of the sections was blocked by treatment with 3% hydrogen peroxide for 15 min. Sections were processed by the Signet USA Ultrastrastavidin Detection System-Alkaline Phosphatase kit (Signet Laboratories, Dedham, MA) as follows. Sections were incubated for 30 min with normal serum blocking reagent, washed for 5 min in Tris-HCl (pH 7.4) buffer (Tris buffer), and subsequently incubated for 15 min with avidin and biotin blocking solution. After washes, sections were incubated for 40 min with primary antibody

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inflammation</th>
<th>Necrosis</th>
<th>Lobular Damage</th>
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<tbody>
<tr>
<td>BDL</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BDL + 6-OHDA + NaCl</td>
<td>−/+</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>BDL + 6-OHDA + clenbuterol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BDL + 6-OHDA + dobutamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BDL + 6-OHDA + forskolin</td>
<td>+</td>
<td>+</td>
<td>+</td>
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We evaluated, by hematoxylin and eosin (H&E) staining of paraffin-embedded liver sections (3 slides evaluated/group; 5 μm thick), the degree of portal inflammation, necrosis, and lobular morphology (disarrangement of hepatocytes). At least 10 different portal areas were evaluated for inflammation, apoptosis, and lobular damage. Results were semiquantified into 4 degrees (−, +, ++, ++++) in comparison with the BDL samples (BDL served as internal controls and were judged as +). There were no significant differences in the extent of portal inflammation, necrosis, and lobular damage between the different groups of animals.

Table 1. Liver weight, body weight, and liver-to-body weight ratio in 1-wk BDL rats and BDL rats that (immediately after BDL) received a single intraportal injection of 6-OHDA (50 μg/kg body wt) followed by ip injections of 0.9% NaCl, clenbuterol (0.5 μg/g body wt), or forskolin (0.04 mg/100 g body wt) two times/day for 7 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver Wt, g</th>
<th>Body Wt, g</th>
<th>Liver-to-Body Wt Ratio, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDL</td>
<td>10.01 ± 0.40</td>
<td>164.85 ± 5.61</td>
<td>5.59 ± 0.34</td>
</tr>
<tr>
<td>BDL + 6-OHDA + NaCl</td>
<td>6.85 ± 0.91*</td>
<td>150.91 ± 6.47</td>
<td>4.45 ± 0.42*</td>
</tr>
<tr>
<td>BDL + 6-OHDA + clenbuterol</td>
<td>8.80 ± 0.43</td>
<td>150.93 ± 3.34</td>
<td>5.81 ± 0.22</td>
</tr>
<tr>
<td>BDL + 6-OHDA + dobutamine</td>
<td>8.85 ± 0.50</td>
<td>167.78 ± 5.34</td>
<td>5.31 ± 0.32</td>
</tr>
<tr>
<td>BDL + 6-OHDA + forskolin</td>
<td>8.08 ± 1.00</td>
<td>152.88 ± 13.32</td>
<td>5.22 ± 0.20</td>
</tr>
</tbody>
</table>

Values are means ± SE of 14 values. BDL, bile duct ligation; 6-OHDA, 6-hydroxydopamine. *P < 0.05 vs. corresponding values of 1-wk BDL rats and BDL rats that (immediately after BDL) received a single intraportal injection of 6-OHDA followed by ip injections of clenbuterol, dobutamine, or forskolin two times/day for 7 days.
against the β1- or β2-adrenergic receptor diluted 1:200. After washes with Tris buffer, the sections were incubated for 30 min with biotinylated secondary anti-goat antibody at a dilution of 1:200. After washes with Tris buffer, sections were incubated for 30 min with Ultra Streptavidin-alkaline phosphatase in conjugate buffer. After washes with Tris buffer, the sections were then developed with Histomark RED (KPL Laboratories, Gaithersburg, MD) and counterstained with methyl green. Finally, the sections were dehydrated, mounted with Permount (Fisher Scientific), coverslipped, and examined with a microscope (model BX 40; Olympus Optical, Tokyo, Japan). Sections that were not incubated with a primary antibody served as negative controls.

The expression of β1- and β2-adrenergic receptors was evaluated by immunofluorescence in cytospin smears of purified cholangiocytes from normal and 1-wk BDL rats. The cells were permeabilized in 1× PBS containing 0.2% Triton X-100 (PBST) and blocked in 4% BSA (in PBST) for 1 h at room temperature. Antibodies directed against β1- and β2-adrenergic receptors were diluted (1:100 and 1:10, respectively) in 1% BSA/PBST and were added to the slides for 2 h at room temperature. Cells were then washed 3 × 10 min in PBST, and a 1:50 dilution (in 1% BSA/PBST) of cy3-conjugated anti-rabbit antibody (Jackson Immunochemicals) was added for 1 h at room temperature. Cells were washed again for 3 × 10 min in PBST and mounted on microscope slides with Antifade gold containing DAPI as a counterstain (Molecular Probes). Images were taken on an Olympus IX71 fluorescence microscope with a DP70 digital camera. For the merged pictures, images from each channel were overlaid electronically using Adobe Photoshop software.

The expression of β1- and β2-adrenergic receptors was measured by immunoblots (20) in protein (10 μg) from whole lysate from rat heart (positive control) and purified cholangiocytes from 1-wk BDL rats and rats that (immediately after BDL) were treated with a single injection of 6-OHDA. Purified cholangiocytes (3.0 × 10⁶) were resuspended in lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin] and sonicated six times (30-s bursts). Proteins (10 μg/lane) were resolved by SDS-7.5% PAGE and transferred to a nitrocellulose filter. After blocking, the filter was incubated overnight at 4°C with a rabbit anti-β1- or β2-adrenergic receptor antibody (1:200) followed by incubation with a goat anti-rabbit IgG horseradish peroxidase antibody [diluted 1:2,500 with Tris-buffered saline-Tween 20 (TBST)]. After several washes, the filter was visualized using chemiluminescence (ECL Plus kit, Amersham Life Science, Little Chalfont, Buckinghamshire, UK).

To determine the subcellular distribution of the β1- and β2-adrenergic receptors in cholangiocytes, we evaluated by immunoblots (20) the protein expression for these receptor subtypes in protein (10 μg) from membranes from the basolateral or apical domain (18, 32, 52) of purified cholangiocytes from 1-wk BDL rats. The amount of cholangiocyte apical and basolateral membrane protein was determined by 10.220.32.247 on June 25, 2017 http://ajpgi.physiology.org/ Downloaded from

Fig. 2. Measurement of the no. of cholangiocytes undergoing apoptosis in liver sections from 1) 1-wk BDL rats; 2) BDL rats that (immediately after BDL) received a single intraportal injection of 6-OHDA followed by ip injections of NaCl, clenbuterol, dobutamine, or forskolin for 7 days; and 3) rats that (immediately after BDL) received a single intraportal injection of 6-OHDA followed by daily ip injections of NaCl, dobutamine, clenbuterol, or forskolin in the presence of daily injections of wortmannin for 7 days. A single intraportal injection of 6-OHDA increased the no. of apoptotic cholangiocytes compared with BDL rats. Chronic administration of clenbuterol, dobutamine, or forskolin to rats prevented the stimulatory effects of 6-OHDA on the number of apoptotic cholangiocytes. Chronic administration of wortmannin partially blocks the protective effects of clenbuterol, dobutamine, and forskolin on 6-OHDA-induced increases in cholangiocyte apoptosis. *P < 0.05 vs. cholangiocyte apoptosis of BDL rats. Data are means ± SE of 6 experiments. Original magnification ×40.
using a Pierce BSA Protein Assay Kit from Pierce Biotechnology (Rockford, IL). Cholangiocyte apical and basolateral membranes were prepared by isopycnic centrifugation on a three-step sucrose gradient (38, 34, and 31% wt/wt) as described by us and others (18, 33, 52). We and others have characterized the purity of these membranes using specific markers for the basolateral (i.e., Na\(^{+}-\)K\(^{+}\)-ATPase) and apical (i.e., alkaline phosphatase) domain of cholangiocyte membranes as described (33, 52).

**Evaluation of inflammation, necrosis, and lobular damage.** In the selected group of animals, we evaluated, by hematoxylin and eosin (H&E) staining of paraffin-embedded liver sections (3 slides evaluated/animal, 4–5 μm thick), the degree of portal inflammation, necrosis, and lobular morphology (disarrangement of hepatocytes). At least 10 different portal areas were evaluated for inflammation, apoptosis, and lobular damage. Results were semiquantified into four degrees (−++, +++, ++++) in comparison with the BDL samples (BDL served as internal controls and were judged as +). After the selected staining, liver sections were examined in a coded fashion by light microscopy with an Olympus BX-40 microscope equipped with a camera. After being stained, sections were evaluated in blinded fashion with a microscope (Olympus Optical U-PMTVC). One hundred fifty cells per slide were counted in a coded fashion in 10 nonoverlapping fields.

**Evaluation of cholangiocyte apoptosis.** Cholangiocyte apoptosis was evaluated by TUNEL analysis in liver sections from the selected group of animals. TUNEL analysis (the number obtained, n = 6, derives from the analysis of 3 slides/animal) was performed using a commercially available kit (Wako Chemicals, Tokyo, Japan) as described by us (33). After counterstaining with hematoxylin solution, sections were examined by light microscopy with an Olympus BX-40 microscope equipped with a camera. At least 100 cells/slide were counted in a coded fashion in 10 nonoverlapping fields.

**Cholangiocyte proliferative capacity.** Cholangiocyte proliferation was evaluated by measurement of 1) PCNA- and CK-19-positive cholangiocytes (33) in liver sections and 2) PCNA protein expression (20, 33) in purified cholangiocytes from the selected group of animals. Immunohistochemistry for PCNA (in paraffin-embedded liver sections, 5 μm) and CK-19 (in frozen liver sections, 5 μm) was performed as previously described (33, 34). The number obtained, n = 6, derives from the analysis of 3 slides/animal. After the selected staining, sections were counterstained with hematoxylin and examined in a random, blinded fashion with an Olympus BX 40 microscope (Olympus Optical). Data were expressed as number of PCNA- or CK-19-positive cholangiocytes per each 100 cholangiocytes in 7 different fields.

Cholangiocyte proliferation was also assessed by quantitative immunoblotting measurement of protein expression for PCNA (a marker of cell proliferation; see Refs. 19, 20, 24, 25, 33), as previously described (33) in protein (10 μg) from whole lysate samples from purified cholangiocytes from the selected groups of animals. Rat spleen and BSA were the positive and negative controls, respectively. The amount of protein loaded was normalized by immunoblots for β-actin, the internal control (8). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low light imaging system (Alpha Innotech, San Leandro, CA).

**Cholangiocyte secretory activity.** Ductal secretion was evaluated by assessment of basal and secretin-stimulated bile flow and bicarbonate concentration and secretion in vivo from the selected groups of animals. After anesthesia, rats were surgically prepared for bile collection as described (6). One jugular vein was cannulated with a PE-50 cannula (Intramedic, Clay-Adams brand; Becton-Dickinson, Sparks, MD) to infuse either Krebs-Ringer-Henseleit (KRH) or secretin (100 nM). The rate of fluid infusion was adjusted according to both the rate of bile flow and the value of the arterial hematocrit. Body temperature was monitored with a rectal thermometer and maintained at 37°C by using a heating pad (Harvard Homeothermic Blanket Control Unit; Harvard Apparatus, Kent, England). Immediately after the bile duct was cannulated, the biliary fistula tubing was connected to another tube of larger diameter (6) to initiate the collection of bile. When steady-state bile flow was achieved (60–70 min from the beginning of bile collection), secretin (100 nM) was infused for 30 min followed by a final infusion of KRH for 30 min. Bicarbonate concentration (measured as total CO\(_2\)) in bile from the selected group of animals was determined by an ABL 520 Blood Gas System (Radiometer Medical, Copenhagen, Denmark).

**Analysis of intracellular signaling mechanisms.** Intracellular cAMP levels (a functional index of cholangiocyte proliferation/loss; see Refs. 2, 4, 19, 20, 31, 33, 35) were measured as follows. After purification, pure cholangiocytes from the selected group of animals...
were incubated for 1 h at 37°C (to regenerate membrane proteins damaged by proteolytic enzymes during cell isolation; see Ref. 26) and subsequently incubated at room temperature for 5 min (20, 31, 33, 34) with 0.2% BSA (basal) or secretin (100 nM) with 0.2% BSA. Intracellular basal and secretin-stimulated cAMP levels were determined by RIA using a commercially available kit. The protein expression of phosphorylated PKA and Akt (Ser473) was evaluated by immunoblots (20). After stripping of the membrane, the expression of total PKA and Akt (Ser473) was evaluated by immunoblots (20). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low-light imaging system (Alpha Innotech). Statistical analysis. All data are expressed as means ± SE. The differences between groups were analyzed by Student’s t-test when two groups were analyzed or ANOVA if more than two groups were analyzed.

RESULTS

β1- and β2-Adrenergic receptors are expressed by cholangiocytes predominately in the basolateral membrane. Both β1- and β2-adrenergic receptors were found by immunohistochemistry in bile ducts of liver sections (Fig. 1a) and by immunofluorescence in purified cholangiocytes (Fig. 1b) from normal and 1-wk BDL rats. By immunoblots, we detected the protein for β1- and β2-adrenergic receptors (63 and 68 kDa, respectively) in purified cholangiocytes from BDL and BDL + 6-OHDA rats (Fig. 1c). Previous studies have indicated that β1- and β2-adrenergic receptors can form homodimers and heterodimers and that β-adrenergic receptors have complex glycosylation resulting in a smeared band when analyzed by SDS-PAGE (48, 49, 51). The heterogeneity of band sizes present most likely results from different glycosylation states and dimerization. The presence of nonglycosylated monomeric β1- and β2-adrenergic receptors were also detected at 50 and 46 kDa, respectively. Protein expression for β1- and β2-adrenergic receptors was unchanged between cholangiocytes from BDL rats and BDL rats treated with a single dose of 6-OHDA (Fig. 1c). β1- and β2-Adrenergic receptor subtypes were found mostly in the basolateral membranes of BDL cholangiocytes (Fig. 1d).

6-OHDA-induced reduction in the number of CK-19-positive cholangiocytes and liver to body weight ratio was prevented by cAMP activators. No significant differences in body weight were observed between 1-wk BDL rats and BDL rats that (immediately after BDL) received a single intraportal injection of 6-OHDA (50 mg/kg body wt) followed by intraperitoneal injections of NaCl, clenbuterol, dobutamine, or forskolin for 7 days (Table 1). Consistent with the findings that 6-OHDA induces a decrease in the number of bile ducts, a single intraportal injection of 6-OHDA to BDL rats significantly decreased both liver weight and liver-to-body weight ratio (an index of liver growth, including cholangiocytes; see Refs. 6 and 17) compared with BDL control rats (Table 1). Chronic administration of clenbuterol, dobutamine, or forskolin prevented the 6-OHDA-induced decrease in liver weight and the liver-to-body weight ratio, which was similar to that of 1-wk BDL rats (Table 1). Light microscopy of liver sections (stained with H&E) showed that there were no significant differences in the degree of portal inflammation, necrosis, and lobular damage among the selected groups of animals (Table 2).

6-OHDA-induced increases in cholangiocyte apoptosis, which was prevented by chronic administration of clenbuterol, dobutamine, or forskolin. A single intraportal injection of 6-OHDA did not alter cholangiocyte apoptosis in liver sections of normal rats [0.4 ± 0.25 of apoptotic cholangiocytes (normal rats) vs. 0.8 ± 0.38 of apoptotic cholangiocytes (normal rats + 6-OHDA), not significant difference]. 6-OHDA induced an increase in cholangiocyte apoptosis compared with 1-wk BDL rats (Fig. 2). Chronic administration of clenbuterol, dobutamine, or forskolin prevented 6-OHDA-induced increases in cholangiocyte apoptosis, which remained similar to that of 1-wk BDL rats (Fig. 2). Consistent with the concept that Akt plays a role in the modulation of cholangiocyte apoptosis by adrenergic agonists, chronic administration of wortmannin partially blocks the protective effects of clenbuterol, dobutamine, and forskolin on the 6-OHDA-induced increase in cholangiocyte apoptosis of BDL rats (Fig. 2). Administration of wortmannin alone did not change cholangiocyte apoptosis in BDL rats and BDL + 6-OHDA saline-treated rats (not shown).

6-OHDA-induced inhibition of cholangiocyte proliferation, which was prevented by chronic administration of clenbuterol, dobutamine, or forskolin. A single intraportal injection of 6-OHDA did not alter cholangiocyte proliferation of normal rats [0.2 ± 0.2 PCNA-positive cholangiocytes (normal rats) vs. 0.6 ± 0.25 of PCNA-positive cholangiocytes (normal rats + 6-OHDA), not a significant difference]. Similarly, administration of 6-OHDA to normal rats did not alter the number of CK-19-positive cholangiocytes [32.2 ± 2.35 CK-19-positive cholangiocytes (normal rats) vs. 31.2 ± 2.70 of CK-19-positive cholangiocytes (normal rats + 6-OHDA), not a significant difference]. 6-OHDA induced a decrease in the number of PCNA- and CK-19-positive cholangiocytes compared with 1-wk BDL rats (Fig. 3, a-b). Chronic administration of clenbuterol, dobutamine, or forskolin prevented 6-OHDA-induced decreases in the number of PCNA- and CK-19-positive cholangiocytes, the values of which were similar to that of 1-wk BDL rats (Fig. 3, a and b). Consistent with the concept that Akt plays a role in the modulation of cholangiocyte proliferation by adrenergic agonists, chronic administration of wortmannin partially blocks the protective effects of clenbuterol, dobutamine, and forskolin on the 6-OHDA-induced decrease in the number of PCNA- and CK-19-positive cholangiocytes (normal rats) vs. 32.2 ± 2.35 CK-19-positive cholangiocytes (normal rats) vs. 31.2 ± 2.70 of CK-19-positive cholangiocytes (normal rats + 6-OHDA), not significant difference]. 6-OHDA induced a decrease in the number of PCNA- and CK-19-positive cholangiocytes (Fig. 3). Chronic administration of clenbuterol, dobutamine, or forskolin also prevented the 6-OHDA-induced decrease in the PCNA protein expression in cholangiocytes, the levels of which were similar to that of 1-wk BDL rats (Fig. 3c).

6-OHDA induces inhibition of secretin-stimulated bile and bicarbonate secretion, which was prevented by chronic administration of clenbuterol, dobutamine, or forskolin. Secretin increased bile flow and bicarbonate concentration and secretion compared with the corresponding basal value in 1-wk BDL rats (Table 3). A single intraportal injection of 6-OHDA ablated the increase in secretin-stimulated bile flow and bicarbonate concentration and secretion compared with the corresponding basal value (Table 3). Chronic administration of clenbuterol, dobutamine, or forskolin after intraportal injection of 6-OHDA prevented the 6-OHDA-induced reduction in secretin-stimu-
ADRENERGIC REGULATION OF CHOLANGIOCYTE FUNCTIONS

Table 3. Effect of secretin on bile flow and bicarbonate concentration and secretion in BDL rats that (immediately after BDL) received a single intraportal injection of 6-OHDA (50 mg/kg body wt) followed by daily ip injections of 0.9% NaCl, clenbuterol (0.5 μg/g body wt), or forskolin (0.04 mg/100 g body wt) two times/day for 7 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bile Flow, μl/min⁻¹·kg body wt⁻¹</th>
<th>Bicarbonate Concentration, mEq/l</th>
<th>Bicarbonate Secretion, μg/min⁻¹·kg body wt⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Secretin</td>
<td>Basal</td>
</tr>
<tr>
<td>BDL</td>
<td>88.75 ± 8.59 (12)</td>
<td>133.74 ± 5.76* (12)</td>
<td>44.91 ± 1.20 (12)</td>
</tr>
<tr>
<td>BDL + 6-OHDA + NaCl</td>
<td>86.93 ± 6.38 (13)</td>
<td>96.92 ± 6.67 (13)</td>
<td>34.12 ± 1.87* (13)</td>
</tr>
<tr>
<td>BDL + 6-OHDA + clenbuterol</td>
<td>80.59 ± 4.42 (11)</td>
<td>118.26 ± 4.85* (11)</td>
<td>46.94 ± 4.62 (11)</td>
</tr>
<tr>
<td>BDL + 6-OHDA + dobutamine</td>
<td>84.27 ± 4.77 (19)</td>
<td>118.26 ± 4.85* (19)</td>
<td>37.50 ± 1.20 (19)</td>
</tr>
<tr>
<td>BDL + 6-OHDA + forskolin</td>
<td>113.97 ± 10.11 (8)</td>
<td>154.46 ± 16.67* (8)</td>
<td>67.28 ± 12.69* (8)</td>
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Data are means ± SE; no. of rats in parentheses. BDL bile duct canulation; NS, not significant. *P < 0.05 vs. corresponding basal value of bile flow (a), vs. corresponding basal value of bicarbonate secretion (b), vs. corresponding basal value of bicarbonate secretion (c), vs. basal bile flow of BDL rats (d), vs. basal bicarbonate concentration of BDL rats (e), and vs. basal bicarbonate secretion of BDL rats (f). Statistical analysis was performed by both unpaired Student’s t-test and ANOVA.

6-OHDA inhibits the cAMP/PKA pathway, which was prevented by chronic administration of clenbuterol, dobutamine, or forskolin. We next evaluated if 6-OHDA administration induces a decrease in basal and secretin-stimulated cAMP synthesis (50), a functional index of cholangiocyte proliferation and secretion (20, 26, 31, 33, 34). A single intraportal injection of 6-OHDA significantly decreased basal cAMP levels in purified cholangiocytes compared with basal cAMP levels of cholangiocytes from 1-wk BDL rats (Fig. 4). Secretin increased intracellular cAMP levels in purified cholangiocytes from 1-wk BDL rats compared with the corresponding basal value (Fig. 4). A single intraportal injection of 6-OHDA ablated the increase in secretin-stimulated intracellular cAMP levels compared with the corresponding basal value (Fig. 4). Chronic administration of clenbuterol, dobutamine, or forskolin, after intraportal injection of 6-OHDA, prevented the 6-OHDA-induced decrease in both basal and secretin-stimulated cAMP levels (Fig. 4).

We next determined if 1) 6-OHDA effects on cholangiocyte proliferative and secretory capacity are associated with changes of the cAMP-dependent PKA pathway and 2) chronic administration of clenbuterol, dobutamine, or forskolin prevent 6-OHDA effects on the cAMP-dependent PKA pathway. The data show that a single intraportal injection of 6-OHDA to rats decreased the expression of the phosphorylated PKA protein (Fig. 5). Chronic administration of clenbuterol, dobutamine, or forskolin, following intraportal injection of 6-OHDA, prevented the 6-OHDA-induced changes in the phosphorylation of PKA (Fig. 5).

6-OHDA inhibition of Akt phosphorylation was prevented by chronic administration of clenbuterol, dobutamine, or forskolin. Treatment with 6-OHDA in BDL rats induced a decrease in Akt1/2/3 (Ser473) phosphorylation (Fig. 6) in comparison with control BDL rats. However, chronic administration of forskolin, clenbuterol, or dobutamine prevented the impaired Akt phosphorylation induced by 6-OHDA (Fig. 6).

DISCUSSION

The data demonstrate that normal and BDL cholangiocytes express β1- and β2-adrenergic receptors. 1) The expression of β1- and β2-adrenergic receptors was similar between cholangiocytes from BDL rats and BDL rats treated with 6-OHDA; 2) chemical sympathetic denervation of the liver by 6-OHDA administration increased cholangiocyte apoptosis and decreased cholangiocyte proliferation and secretin-stimulated ductal secretion in BDL rats; 3) the effects of 6-OHDA were associated with decreased cAMP levels and decreased phos-
phorylation of PKA and Akt; 4) 6-OHDA-induced changes in cholangiocyte proliferative and secretory capacity were prevented by the β1-adrenergic receptor agonist dobutamine and the β2-adrenergic receptor agonist clenbuterol (adrenergic receptors that both couple to Gs-proteins to activate adenyl cyclase; see Ref. 57) and by forskolin (adenylyl cyclase agonist; see Ref. 27) administration; and 5) consistent with the concept that Akt plays a role in the modulation of cholangiocyte apoptosis by adrenergic agonists, chronic administration of wortmannin to rats partially blocks the protective effects of clenbuterol, dobutamine, and forskolin on 6-OHDA effects on cholangiocyte apoptosis and proliferation.

Recent studies have demonstrated how the nervous system plays a key role in the modulation of apoptotic, proliferative, and secretory activities of the intrahepatic biliary epithelium (9, 25, 31, 42). The cholinergic system positively modulates the proliferative and secretory activities of cholangiocytes by regulating adenylate cyclase activity by an intracellular pathway involving Ca2+/calcineurin but not PKC (9, 31). The serotoninergic system exerts an antiproliferative effect by inhibiting the cAMP/PKA/Src/MAPK pathway via inositol trisphosphate/Ca2+/PKC signaling (37). The dopaminergic system inhibits secretin-stimulated ductal secretion of BDL rats through activation of PKC-β2 and downregulation of cAMP-dependent PKA activity (18). No information exists on the role of the adrenergic system in the modulation of cholangiocyte pathophysiology, although the adrenergic innervation of the intrahepatic biliary epithelium has been described (45). In the present study, we evaluated the effect of chemical sympathectic denervation of the liver on the enhanced proliferative and secretory activities of the intrahepatic biliary epithelium of the BDL rat model of experimental cholestasis (2, 4, 6, 7, 19, 20). Ligation of the common bile duct induces ductal hyperplasia (4, 6, 7, 20), which is associated with enhanced secretory activity and responsiveness to the choleretic stimulus of secretin (6). Immediately after BDL, rats were submitted to one intraportal single injection of 6-OHDA, a procedure described to induce a selective chemical adrenergic denervation of the liver (14). After BDL + 6-OHDA (1 wk), we evaluated intrahepatic bile duct mass and the proliferative and secretory activities of cholangiocytes in comparison with control BDL rats treated with 6-OHDA. We next evaluated whether the effects of chemical adrenergic denervation of the liver could be reversed by adrenergic agonists or by the adenylate cyclase stimulator forskolin (27). For this purpose, BDL + 6-OHDA rats were treated by daily intraperitoneal injections of dobutamine (a β1-adrenergic receptor agonist; see Ref. 55), clenbuterol (a β2-adrenergic receptor agonist; see Ref. 15), forskolin (adenylyl cyclase stimulator; see Ref. 27), or NaCl. Subsequently, the proliferative and secretory activities of the intrahepatic biliary epithelium were evaluated compared with BDL rats treated with 6-OHDA. The first finding of our study is the expression of both the β1- and β2-adrenergic receptors in normal and BDL cholangiocytes with preferential basolateral localization. This has been demonstrated by immunoblotting of apical and basolateral membranes in purified cholangiocytes from 1-wk BDL rats.
(Fig. 1d). 6-OHDA causes degeneration of adrenergic terminal fibers (14) but does not destroy the β1- and β2-adrenergic receptors, since the protein for these two receptors was present at similar levels in cholangiocytes from BDL rats and BDL rats treated with a single intraportal injection of 6-OHDA. Thus the β1- and β2-adrenergic receptor agonists are able to interact with their own receptor, preventing 6-OHDA-induced duct damage. The finding that β-adrenergic receptors are involved in the modulation of the proliferative response of cholangiocytes to BDL is also supported by the fact that the chemical destruction of hepatic adrenergic fibers by 6-OHDA is followed by decreased bile duct mass, which in turn is caused by depression of cholangiocyte proliferation (evaluated by PCNA immunoblots) and by the activation of apoptosis (evaluated by TUNEL analysis in liver sections).

The specificity of 6-OHDA effects was demonstrated by the capability of β-adrenergic agonists (clenbuterol and dobutamine) to completely reverse the effects of 6-OHDA on bile duct mass, cholangiocyte proliferation, and apoptosis. In addition, this is consistent with decreased liver weight and liver-to-body weight ratio after 6-OHDA and with the prevention of these effects by administration of dobutamine or clenbuterol. We next evaluated the intracellular transduction pathways by which β-adrenergic receptors modulate cholangiocyte proliferation.

The β1- and β2-adrenergic receptors (rhodopsin/β-adrenergic receptors) are prototypes of class I G protein-coupled receptors, since they induce cAMP increase via adenylyl cyclase activation and then PKA phosphorylation in a number of cells (46, 53). Intracellular cAMP plays an important role in the regulation of cholangiocyte proliferation (4, 20, 31, 33–35). In support of this concept, experimental maneuvers causing inhibition of duct proliferation are associated with decreased cAMP levels in cholangiocytes (31, 35), whereas induction of proliferation is associated with enhanced cholangiocyte cAMP levels (4, 20, 31, 34). Maintenance of cAMP levels, by forskolin administration, prevents the activation of cholangiocyte apoptosis and inhibition of duct proliferation/secreation induced by vagotomy (31). Furthermore, in vivo and in vitro activation of cholangiocyte cAMP levels alone is sufficient to increase duct proliferation and secretion through changes in the PKA/Src/MAPK pathway (17). A number of hormones (4, 10, 19, 20), neuropeptides (31, 37, 38), and bile salts (2, 8, 36, 38) modulate cholangiocyte proliferation by acting on cAMP and PKA activity, which, in turn, induce changes in the Ras/Raf/Shc/Src/extracellular/signal-regulated kinase cascade. In agreement, we found that reduction of cholangiocyte proliferation by 6-OHDA administration is associated with decreased cAMP levels and PKA phosphorylation and that prevention of 6-OHDA effects by clenbuterol or dobutamine is linked with maintenance of these intracellular mediators. Most importantly, the inhibitory effects of 6-OHDA on cholangiocyte proliferation were maintained by forskolin, and this is consistent with a central role of cAMP in mediating the β-adrenergic...
modulation of cholangiocyte proliferation. Several hypotheses can explain our findings. The first possibility is that proliferating cholangiocytes respond directly to manipulation of adrenergic nerves, and to this regard a sparse adrenergic innervation of the rat intrahepatic biliary epithelium has been documented (45, 54). A second, most likely possibility is that cholangiocytes respond to extracellular catecholamines. The expression of β-adrenergic receptors in cholangiocytes and changes of the signaling pathways associated with these adrenergic receptors during treatment with 6-OHDA or β-adrenergic agonists are all consistent with this explanation. Although, we have shown that α1 (phenylephrine; see Ref. 32) and α2 (UK14,304; see Ref. 16)-adrenergic receptor agonists regulate biliary functions by increases in cholangiocyte intracellular Ca2+ concentration and decreases in cholangiocyte cAMP levels, respectively, we cannot exclude that changes in α-adrenergic vascular tone after 6-OHDA treatment might have influenced our findings. Indeed, a number of effects of the α-adrenergic system have been documented in the normal and injured rat liver, including fibrogenesis, regulation of vascular tone, proliferation of the oval cell compartment, and release of somatostatin and prostaglandins (30, 44). However, the fact that all the effects of the chemical sympathectomy on cholangiocytes and prostaglandins (30, 44) were prevented by the administration of clenbuterol, dobutamine, and forskolin indicates that changes in both vascular tone and the β-adrenergic system may play a role in the effects of cholangiocyte proliferation/loss.

We also demonstrated that 6-OHDA administration resulted in a decrease in the phosphorylation of Akt (Ser473) and that the chronic administration of the cAMP-stimulating agonists clenbuterol, dobutamine, and forskolin activated the phosphorylation of Akt. Previous reports have demonstrated that cAMP can promote the activation of cell survival factors such as Akt (12, 28) via cAMP-dependent phosphorylation of Akt in neurons. Also, cAMP can activate PI3-kinase-dependent bisle acid secretion in WIF-B9 (23). Our data suggest a link between cAMP and activation of cell survival pathways via an Akt-dependent mechanism in cholangiocytes. Our studies show that clenbuterol and dobutamine have very similar effects on cAMP but that dobutamine may influence Akt phosphorylation to a lesser degree compared with clenbuterol (Fig. 6). This may be because of differences in the cholangiocyte β1- and β2-receptor signaling that influence cholangiocytes or the doses of the β1- and β2-adrenergic receptor agonists used. Because wortmannin (a PI3-kinase inhibitor; see Ref. 40) only partially blocks the protective effects of clenbuterol, dobutamine, and forskolin on 6-OHDA-induced changes in cholangiocyte apoptosis, proliferation, and secretion, further studies are warranted to evaluate the other possible intracellular mechanisms that (in addition to cAMP and PI3-kinase) may regulate adrenergic modulation of cholangiocyte function. Further evaluation of the link between cAMP and Akt-dependent cell survival mechanisms is being conducted.

Regarding ductal secretion, we found that 6-OHDA administration abolished secretin-induced choleresis typical of BDL rats (6) and that the administration of clenbuterol, dobutamine, and forskolin prevented 6-OHDA inhibition of ductal secretion. The enhanced response to the choleretic effect of secretin is a typical feature of BDL rats (6), which is associated with amplification of the cAMP/PKA response to this hormone (4, 18, 20). A number of previous studies have shown that changes in proliferation are associated with parallel changes in basal and secretin-stimulated cholangiocyte secretory activities (2, 6, 8, 19, 20, 31, 33–35, 38). In fact, maneuvers inducing inhibition of cholangiocyte proliferation in BDL rats (i.e., by vagotomy, acute CCl4 treatment, or chronic administration of gastrin or the bile salts ursodeoxycholate or tauroursodeoxycholate) are associated with impairment of secretin-stimulated choleresis (2, 19, 20, 31, 35), whereas, on the contrary, stimulation of cholangiocyte proliferation (i.e., after partial hepatectomy, by feeding of the bile acids taurocholate and taurolithocholate; see Refs. 5 and 34) induces amplification of secretin-stimulated cAMP levels and secretin-induced choleresis. The findings of the present study further confirm this general concept that 1) inhibition of proliferation by 6-OHDA is associated with abolishment of secretin-stimulated choleresis and secretin-induced cAMP levels and 2) 6-OHDA effects on cholangiocyte functions are prevented by maintaining proliferation through the administration of β-adrenergic agonists or forskolin.

In conclusion, the role of the β-adrenergic system in the modulation of cholangiocyte apoptotic, proliferative, and secretory activities, shown in this study, adds a new piece in the complex puzzle of the regulation of cholangiocyte pathophysiology by the nervous system. Understanding the mechanisms by which nerves regulate the balance between cholangiocyte proliferation/loss may be important in patients with denervated livers after liver transplantation.

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acid transporter expression in both small and large rat cholangiocytes. 


