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BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Bile Acid Depletion and Repletion Regulate Cholangiocyte Growth and Secretion by a Phosphatidylinositol 3-Kinase–Dependent Pathway in Rats

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Background & Aims: **We tested the hypothesis that during bile duct obstruction, increased biliary bile acids trigger cholangiocyte proliferation and secretion by a phosphatidylinositol 3-kinase (PI3-K)–dependent pathway.** ***Methods:*** **In bile duct–incannulated (BDI) rats, bile duct obstruction present for 7 days was relieved for 24 hours by external bile drainage. During the 24-hour drainage period, animals received either Krebs Ringer Henseleit (the bile-depleted group), or sodium taurocholate (the bile-depleted, taurocholate-infused group). We evaluated cholangiocyte proliferation and secretin-stimulated ductal secretion. Apical bile acid transporter (ABAT) expression and bile acid transport activity was determined. In pure preparations of cholangiocytes, we examined the effect of taurocholate (in the absence or presence of wortmannin or PI 3,4-bisphosphate the lipid product of PI3-K) on cholangiocyte proliferation and secretin-stimulated cyclic adenosine 3′,5′-monophosphate (cAMP) levels.** ***Results:*** **Bile depletion reduced cholangiocyte proliferation and secretin-stimulated ductal secretion and ABAT expression and bile acid transport activity compared with 1-week BDI control rats. In bile-depleted, taurocholate-infused rats, cholangiocyte proliferation and secretion and ABAT expression and bile acid transport activity were maintained at levels similar to those seen in BDI control rats. In vitro, taurocholate stimulation of DNA replication and secretin-stimulated cAMP levels was blocked by wortmannin. The inhibitory effect of wortmannin on taurocholate stimulation of cholangiocyte proliferation and secretion was prevented by PI 3,4-bisphosphate.** ***Conclusions:*** **Bile acid uptake by ABAT and the PI3-K pathway are important for bile acids to signal cholangiocyte proliferation. In bile duct obstruction, increased biliary bile acid concentration and ABAT expression initiate increased cholangiocyte proliferation and secretion.**

Physiologically, the intrahepatic biliary epithelium is involved in the modification of canalicular bile[1–4] before it reaches the small intestine.[1] Ductal secretion is regulated by gastrointestinal hormones (e.g., secretin, gastrin, bombesin, and somatostatin),[1,2,5–8] nerves,[9] and biliary constituents (e.g., alkaline phosphatase).[10] Secretin stimulates ductal secretion by interacting with receptors expressed only by cholangiocytes in rat liver[11] through an increase in intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) synthesis.[2,3,5–7] The increase in cholangiocyte cAMP levels induces opening of Cl^- channels[6] and activation of the Cl^-/HCO_3^- exchanger,[5,9,12] which leads to bicarbonate secretion in bile.[1–3]

Pathologically, the biliary epithelium is the target for a number of cholestatic liver diseases, including primary biliary cirrhosis, primary sclerosing cholangitis, cystic fibrosis, and idiopathic ductopenic disorders.[4] In normal rat liver, cholangiocytes are mitotically quiescent,[3] but proliferate markedly in response to a number of pathologic injuries/toxins, including bile duct ligation (BDL),[1,4] 70% hepatectomy,[3] acute carbon tetrachloride administration,[13] or chronic bile salt feeding.[14] In these hyperplastic models, cholangiocyte proliferation is associated with in-

Abbreviations used in this paper: ABAT, apical bile acid transporter; BDI, bile duct incannulation; BSA, bovine serum albumin; cAMP, adenosine 3′, 5′-monophosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; γ-GT, gamma-glutamyltranspeptidase; HPLC, high-performance liquid chromatography; KRH, Krebs Ringer Henseleit; PI3-K, phosphatidylinositol 3-kinase.

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creased DNA synthesis, leading to increased intrahepatic ductal mass[1] and increased basal and secretin-stimulated ductal secretion.[1–3,7,11,14,15]

A number of factors (including estrogens and bile acids) have been shown to stimulate the proliferative capacity of cholangiocytes both in vivo and in vitro.[14–17] For example, we have shown that the bile acid sodium taurocholate increases cholangiocyte proliferation and secretin-stimulated ductal secretion both in vitro in purified cholangiocytes and in vivo in the bile acid–fed rat model.[14,15] Because bile acids must be internalized into cholangiocytes to alter their function,[17] it has been proposed that bile acid uptake by the Na^+-dependent apical bile acid transporter (ABAT) initiates the bile acid–dependent changes in cholangiocyte proliferation and secretion.

Although our previous studies[14] showed that increased biliary bile acids induced cholangiocyte hyperplasia, no previous study has tested whether increased cholangiocyte proliferation is triggered by increased biliary bile acids after bile duct obstruction. Our overall strategy was to experimentally reduce biliary bile acids by external bile fistula and to determine whether the effect of bile drainage on cholangiocytes was due specifically to reduced biliary bile acids by infusing taurocholate in a second set of bile-depleted rats. One-week bile duct–incannulated (BDI) rats were depleted of endogenous bile acids by external bile drainage and simultaneously infused with Krebs Ringer Henseleit (KRH) for 24 hours. In another group of animals, 1-week BDI rats were bile depleted by external drainage and simultaneously infused with taurocholate for 24 hours. In BDI control, bile-depleted KRH-infused, and bile-depleted taurocholate-infused rats, indices of cholangiocyte proliferation and secretion, ABAT protein, bile acid transport activity, and biliary bile acid composition were determined.

Finally, through in vitro studies in purified cholangiocytes, we determined the role of phosphatidylinositol 3-kinase (PI3-K), an enzyme that plays an important role in control of cell growth and secretion,[18] in bile acid regulation of cholangiocyte proliferative and secretory activity. We determined whether wortmannin (a specific inhibitor of PI3-K[18]) inhibits taurocholate-stimulated cholangiocyte proliferation and secretion, and whether the lipid products of PI3-K (PI 3,4-bisphosphate, a molecule that has been shown to block the inhibitory effects of wortmannin in hepatocytes[19]) prevents wortmannin inhibition of bile acid–stimulated cholangiocyte proliferation and secretion.

Materials and Methods

Materials

Reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Porcine secretin was purchased from Peninsula (Belmont, CA); sodium taurocholate was purchased from Calbiochem-Novabiochem (La Jolla, CA). The substrate for γ-glutamyltranspeptidase (γ-GT), N-(γ-L-glutamyl)-4-methoxy-2-naphthylamide, was purchased form Polysciences (Warrington, PA). ^{3}H-taurocholate (3.47 Ci/mmol) was purchased from New England Nuclear (Boston, MA).

Animal Model

Male Fisher 344 rats (175–200 g) were purchased from Charles River (Wilmington, MA), maintained in a temperature-controlled environment (20–22°C) with a 12-hour light/dark cycle and fed ad libitum standard rat chow. Animals had free access to water. In these studies we used 3 experimental groups. The first experimental group comprised rats with extrahepatic bile duct obstruction induced by BDI for 1 week as controls. In the second group, 7 days after BDI, bile duct obstruction was relieved by external bile drainage (the bile-depleted group). During bile depletion, the rats were simultaneously infused for 24 hours with KRH. In the third group, 7 days after BDI, rats were infused with taurocholate (at 1 μmol/h/kg body weight) during the 24 hours of external drainage (the bile-depleted, taurocholate-infused group). After administration of isoforane/oxygen anesthesia, BDI was performed as described previously.[1] Before each experimental procedure (e.g., surgical preparation for bile collection, liver perfusion for cell isolation), the animals were anesthetized with sodium pentobarbital (50 mg/kg). Study protocols were performed in compliance with institutional guidelines.

Assessment of Bile Acid Concentration and Composition in Bile

Total bile salts were measured in bile by the 3 alpha-hydroxysteroid dehydrogenase procedure[20] through absorption spectrophotometry using a commercially available kit (Wako Chemicals USA, Richmond, VA). Aliquots of bile extracted with 4 volumes of isopropanol were analyzed for individual bile salt by reversed-phase high-performance liquid chromatography (HPLC) using an acidic isocratic phosphate buffer.[21]

Purification of Cholangiocytes

Pure cholangiocytes were obtained by immunoaffinity separation[2,3,5,6,11,22] using an antibody to an unidentified antigen expressed by all intrahepatic cholangiocytes.[22] Purity was assessed by histochemistry for γ-GT,[23] a specific marker of cholangiocytes in rat liver.[1,22] Cell number and viability were assessed by standard trypan blue exclusion. Cell viability always exceeded 97%.

Measurement of Cholangiocyte Proliferative Capacity

Cholangiocyte proliferative capacity was evaluated in liver sections ($n = 7$) from normal BDI controls; BDI-bile depleted, KRH infused; and BDI-depleted, taurocholate-infused rats by quantitative localization of proliferating cellular nuclear antigen (PCNA) in bile ducts.[13,24] Sections were counterstained with hematoxylin and examined in a coded fashion with a microscope (BX 40; Olympus Optical, Japan).

The expression of PCNA and H_3 histone mRNAs (2 markers of cell replication[15,24]) in cholangiocytes (5.0×10^6) from the selected group of animals was determined by the lysate RNase protection assay (Direct Protect Kit; Ambion, Austin, TX).[2,3,15] The comparability of the cell lysate used was determined by hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the housekeeping gene.[2,5–7,15] Antisense riboprobes were transcribed from linearized cDNA templates with either T_7 or SP_6 RNA polymerase using [alpha$-^{32}$P UTP] (800 Ci/mmol) (Amersham, Arlington Heights, IL). After exposure for 1–2 days, autoradiograms were quantified by densitometry. We used the following controls: rat spleen (positive) and yeast-transfer RNA (negative) for both PCNA and H_3 histone mRNAs. Rat kidney and yeast-transfer RNA were the positive and negative controls, respectively, for GAPDH mRNA.

The proliferative capacity of cholangiocytes (5×10^6) from the selected group of animals was also evaluated by immunoblots for PCNA as described previously.[24] The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low-light imaging system (Alpha Innotech, San Leandro, CA).

Measurement of Cholangiocyte Secretory Activity

Measurement of secretin receptor gene expression and basal and secretin-stimulated cAMP levels. In pure cholangiocytes from the selected group of animals, secretin receptor gene expression was assessed by lysate ribonuclease protection assay (Direct Protect; Ambion) as previously described.[3,5,11] A 318-bp riboprobe encoding the message for the rat secretin receptor was transcribed from pGEM4Z (a gift from Dr. LaRusso, Mayo Clinic, Rochester, MN). The comparability of the lysates was assessed by hybridization with GAPDH, the housekeeping gene.[5,11] Rat heart was used as the positive control and rat kidney as the negative control.[11]

Basal and secretin-induced intracellular cAMP levels in cholangiocytes from the selected group of animals were measured by radioimmunoassay.[2,3,5–7,15] After purification, cholangiocytes were incubated for 1 hour at 37°C to restore membrane proteins damaged with proteolytic enzyme treatment[25] and stimulated for 5 minutes at 22°C[2,3,5–7,15,25] with 0.2% bovine serum albumin (BSA; control) or secretin (100 nmol/L) in the presence of 0.2% BSA. Intracellular cAMP levels were measured by a commercially available kit (Amersham) in accordance with the vendor's instructions.

In vivo measurement of secretin-induced bicarbonate-rich choleresis. After anesthesia with sodium pentobarbital (50 mg/kg body weight), the selected group of animals was surgically prepared for bile collection.[1–3] When steady-state bile flow was achieved, secretin (100 nmol/L) was infused for 30 minutes, followed by a final infusion of KRH solution for 30 minutes. Bile was collected every 10 minutes in preweighed tubes and immediately stored at −20°C before determination of bicarbonate or total bile salt concentration or before HPLC analysis for evaluating bile salt composition. At the end of each experiment, the animal was killed and the liver removed and weighed. Bicarbonate concentration (measured as total CO_2) in bile was measured with a Natelson microgasometer (Scientific Industries, Bohemia, NY).

Expression of ABAT and Bile Acid Transport Activity in Purified Cholangiocytes

To determine whether the alterations in cholangiocyte proliferative and secretory activity are dependent on changes in the expression of cholangiocyte ABAT, we measured ABAT gene and protein expression and bile acid transport activity in cholangiocytes from the selected group of animals. Quantitative ABAT gene expression was evaluated using the direct lysate ribonuclease protection assay.[2,3,15] The rat ABAT cDNA clone[26] was a gift from B. L. Shneider, Mount Sinai Medical Center, New York. The comparability of the cholangiocyte lysates used was assessed by hybridization with GAPDH.[2,5,6,11,15] Rat ileum (positive) and yeast-transfer RNA (negative) were the controls for ABAT mRNA; rat kidney (positive) and yeast-transfer RNA (negative) were the controls for the GAPDH gene. After exposure for 1–2 days, autoradiograms ($n = 3$) were quantified by densitometry.

Cholangiocyte ABAT protein expression was determined by immunoblots using an anti-ABAT antibody (1:200) (a gift from Dr. P. Dawson, Bowman Gray School of Medicine, Winston-Salem, NC) as a primary antibody and anti-rabbit IgG peroxidase conjugate (ECL Plus Kit, Amersham) diluted 1:50,000 with Tris-buffered saline Tween-20. The intensity of the bands was determined by scanning video densitometry.

ABAT transport activity was determined by Na^+-dependent ^{3}H-taurocholate uptake in purified cholangiocytes as previously described by us.[17] Results were expressed as picomoles of taurocholate uptake per milligram of cholangiocyte protein. All experiments were performed in triplicate. Estimate of K_m and V_{max} were determined by a weighted least squares fit of the sigmoidal curve according to the method of Vaughn et al.[27]

Role of PI3-K in Bile Acid Modulation of Cholangiocyte Proliferation and Secretion

In purified cholangiocytes from 1-week BDI rats, we evaluated whether the PI3-K system plays a role in bile acid modulation of cholangiocyte functions by determining the effects of taurocholate on cholangiocyte proliferative and secretin-stimulated cAMP levels in the presence or absence of wortmannin, a specific PI3-K inhibitor[18,28]; PI 3,4-bisphosphate, an active PI;[19] or PI 4,5-bisphosphate, an inactive PI.[19]

Table 1. Bile Acid Composition in Bile From 1-Week BDI Rats and 1-Week BDI Bile-Depleted Rats Infused With Taurocholate (1 μmol/h/kg body wt) for 24 Hours

Treatment	Tauromuricholic acid (*mEq/L*)	Taurocholic acid (*mEq/L*)	Taurochenocholic acid (*mEq/L*)	Taurodeoxycholic acid (*mEq/L*)
BDI 1 week (control)	7.91 ± 1.21	1.57 ± 0.18	0.38 ± 0.09	0.07 ± 0.05
BDI 1 week (bile depleted + KRH infusion)	0.16 ± 0.007[a]	0.20 ± 0.01[a]	0.14 ± 0.018[a]	Not detectable
BDI 1 week (bile depleted + taurocholate infusion)	4.80 ± 0.79	2.31 ± 0.53	Not detectable	Not detectable

NOTE. Values are mean ± SE for 4 rats and were obtained in the first 10 minutes of bile collection. Bile acid composition in bile samples from the selected group of animals was measured by HPLC. The levels of total bile acids in bile were determined by the 3 alpha-hydroxysteroid dehydrogenase procedure by a commercially available kit.
[a] $P < 0.05$ vs. corresponding value of 1 week BDI control rat.

Cholangiocyte proliferative capacity (PCNA protein expression). Pure cholangiocytes (5×10^6) from 1-week BDI rats were treated at 37°C with (1) 0.2% BSA (basal value) for 60 minutes; (2) taurocholate (20 μmol) with 0.2% BSA for 60 minutes; (3) wortmannin (20 μmol for 20 minutes) + taurocholate (20 μmol for 60 minutes) in the presence of 0.2% BSA; (4) PI 3,4-bisphosphate (1 μmol for 20 minutes), or PI 4,5-bisphosphate (the inactive form of PI3-K,[19] 1 μmol for 20 minutes) + wortmannin (20 μmol for 20 minutes) + taurocholate (20 μmol for 60 minutes) with 0.2% BSA; or (5) wortmannin, PI 3,4-bisphosphate, or PI 4,5-bisphosphate for 20 minutes with 0.2% BSA. Subsequently, we evaluated cholangiocyte DNA replication by Western blot analysis for PCNA as previously described.[24]

Measurement of basal and secretin-stimulated cAMP levels. Ductal secretion was estimated by measurement of basal and secretin-stimulated cAMP levels[2,3,7,11,15] (an indirect index of cholangiocyte secretory capacity[2,5,7]) in purified cholangiocytes from 1-week BDI rats. After purification, pure cholangiocytes (1×10^5) were incubated at room temperature with (1) 0.2% BSA (basal value) for 5 minutes; (2) secretin (100 nmol/L for 5 minutes) with 0.2% BSA; (3) taurocholate (20 μmol for 10 minutes) in the absence or presence of secretin (100 nmol/L for 5 minutes) with 0.2% BSA; (4) wortmannin (20 μmol for 10 minutes) + taurocholate (20 μmol for 10 minutes) in the absence or presence of secretin (100 nmol/L) with 0.2% BSA; or (5) PI 3,4-bisphosphate or PI 4,5-bisphosphate (both at 1 μmol for 10 minutes) + wortmannin (20 μmol for 10 minutes) + taurocholate (20 μmol for 10 minutes) in the absence or presence of secretin (100 nmol/L for 5 minutes) with 0.2% BSA. We also evaluated the effects of wortmannin, PI 3,4-bisphosphate, and PI 4,5-bisphosphate in the presence of 0.2% BSA on basal and secretin-stimulated cholangiocyte cAMP levels. Subsequently, intracellular cAMP levels were evaluated by radioimmunoassay.[2,3,5–7,13,14,25]

Statistical Analysis

All data are expressed as mean ± standard error. The differences between groups were analyzed by the Student unpaired *t* test when 2 groups were analyzed or by analysis of variance if more than 2 groups were analyzed.

Results

Assessment of Bile Acid Concentration and Composition in Bile

Biliary bile acid composition from the selected group of animals is shown in Table 1. We found a significant ($P < 0.001$) decrease in total bile acid concentration in bile from BDI bile-depleted, KRH-infused rats compared with BDI control rats. In BDI bile-depleted, taurocholate-infused rats, bile acid levels were significantly ($P < 0.001$) increased compared with BDI-depleted rats and were comparable to that in BDI control rats.

Measurement of Cholangiocyte Proliferative Capacity

Liver sections from 1-week BDI rats exhibited a marked increase in the number of PCNA-positive cholangiocytes compared with the number of PCNA-positive cholangiocytes observed in normal liver sections (Figure 1). A significant ($P < 0.05$) decrease in the number of PCNA-positive cholangiocytes was observed in the portal areas of BDI bile-depleted, KRH-infused rats compared with BDI control rats. The percentage of PCNA-positive cholangiocytes was similar in 1-week BDI control rats, in BDI bile-depleted rats, and taurocholate-infused rats.

Expression of GAPDH mRNA was similar among purified cholangiocytes from the different experimental groups (Figure 2). The expression of both PCNA and H_3 histone mRNAs was significantly decreased in cholangiocytes from BDI bile-depleted rats compared with cholangiocytes from BDI control rats. In cholangiocytes from BDI bile-depleted, taurocholate-infused rats, the

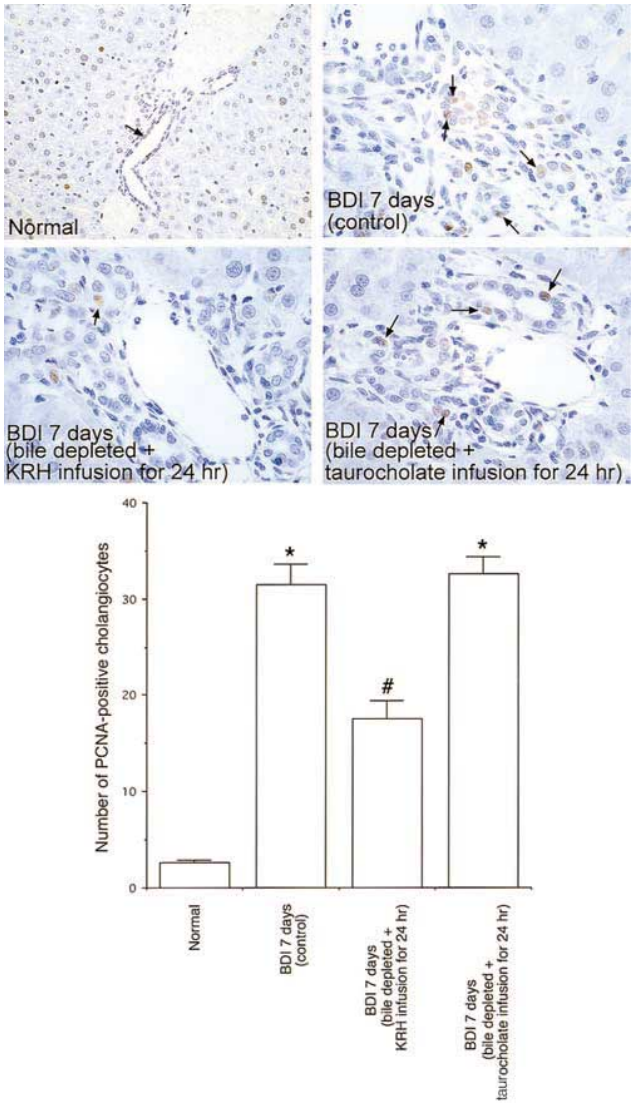

Figure 1. Quantitative measurement of PCNA-positive cholangiocytes in liver sections from normal rats, 1-week BDI control rats, and 1-week BDI bile-depleted rats infused for 24 hours with KRH or taurocholate. The number of PCNA-positive cholangiocytes increased markedly in liver sections from BDI control rats compared with normal liver sections. A significant decrease in the number of PCNA-positive cholangiocytes was observed in liver sections from BDI bile-depleted rats (infused with KRH) compared with those from BDI control rats. The percentage of PCNA-positive cholangiocytes was comparable to that of BDI control rats in BDI bile-depleted, taurocholate-infused rats. $*P \leq 0.05$ vs. normal rat liver section; $^{\#}P \leq 0.05$ vs. the number of PCNA-positive cholangiocytes in liver sections from 1-week BDI rats and 1-week BDI rats infused with KRH for 24 hours. Data are mean ± standard error of 7 experiments. (Original magnification 125× for the normal liver section and 200× for the others.)

expression of both H_3 histone and PCNA mRNA was similar to that of BDI control rats.

We found a marked decrease in PCNA protein expression in cholangiocytes from BDI bile-depleted rats compared with BDI control rats (Figure 3). Expression of PCNA protein was similar to that of BDI control rats in cholangiocytes purified from BDI bile-depleted, taurocholate-infused rats.

Measurement of Cholangiocyte Secretion

Secretin receptor gene expression and intracellular cAMP levels in purified cholangiocytes. There was a significant decrease in secretin receptor gene expression in cholangiocytes from BDI bile-depleted rats compared with BDI control rats (Figure 4*A*). The expression of secretin receptor mRNA was similar to that of BDI control rats in cholangiocytes purified from BDI-depleted, taurocholate-infused rats. Secretin significantly ($P < 0.05$) increased cAMP levels in cholangiocytes from BDI control rats (Figure 4*B*). In contrast, secretin did not increase intracellular cAMP levels of cholangiocytes from bile-depleted BDI rats. In BDI bile-depleted, taurocholate-infused rats, basal and secretin-induced cAMP levels were similar to that of BDI control rats.

Measurement of secretin-induced bicarbonate-rich choleresis. Basal bile flow of BDI bile-depleted rats was significantly ($P < 0.05$) reduced compared with BDI controls, but was restored by taurocholate infusion (Table 2). Compared with BDI control rats, both biliary

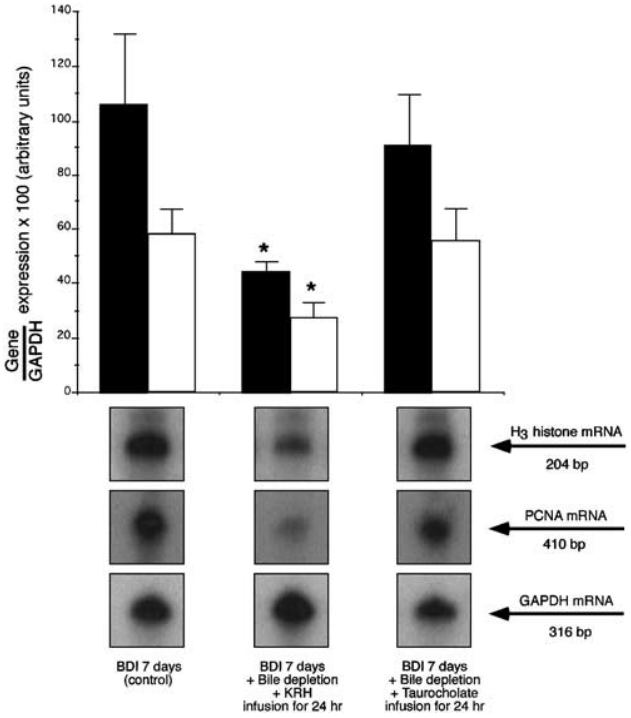

Figure 2. Expression of PCNA (□) and H_3 histone (■) mRNAs in pure cholangiocytes from BDI control rats and BDI bile-depleted rats infused with KRH or taurocholate for 24 hours. The expression of selected messages was determined by direct RNase protection assay. The comparability of the RNA used was assessed by hybridization for GAPDH. $*P < 0.05$ vs. BDI control rats. Autoradiograms ($n = 4$) were quantified by densitometry.

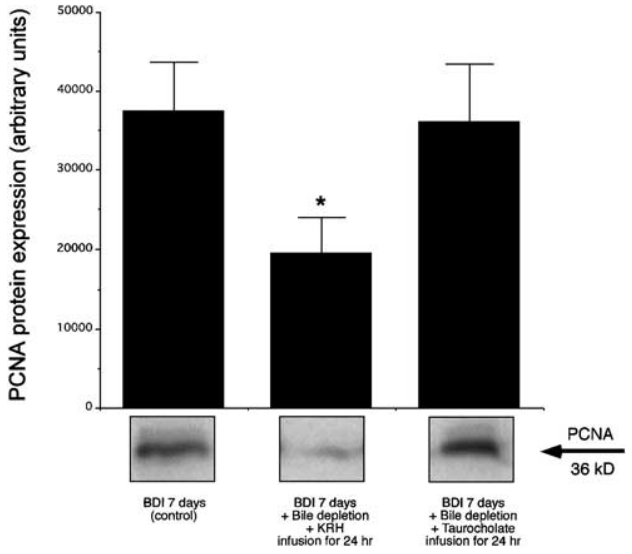

Figure 3. Immunodetection of PCNA in cholangiocytes from BDI control rats and BDI bile-depleted rats infused with KRH or taurocholate for 24 hours. PCNA protein expression decreased in cholangiocytes from BDI bile-depleted, KRH-infused rats compared with BDI control rats. PCNA protein expression in cholangiocytes was similar to that of BDI control rats when BDI-depleted rats were simultaneously reinfused with taurocholate. $*P < 0.05$ vs. BDI control rats. Autoradiograms ($n = 6$) were quantified by densitometry.

bicarbonate concentration and secretion were significantly ($P < 0.05$) reduced in BDI bile-depleted rats. However, when taurocholate was infused in BDI bile-depleted rats, biliary bicarbonate was similar to that of BDI control rats. Secretin significantly ($P < 0.05$) increased bile flow and bicarbonate concentration and secretion in BDI control rats. In contrast, secretin did not induce changes in bile flow or bicarbonate concentration or secretion in BDI bile-depleted rats. In BDI bile-depleted, taurocholate-infused rats, secretin-stimulated increases in bile flow or bicarbonate concentration or secretion were comparable to those in BDI controls.

Expression of ABAT in Cholangiocytes

Reduction of biliary bile acid concentration in bile-depleted rats markedly decreased the genetic expression of ABAT (expressed as a ratio to GAPDH gene expression) in cholangiocytes compared with BDI controls (Figure 5*A*). Taurocholate infusion in BDI bile-depleted rats induced ABAT gene expression similar to that of BDI control rats. Expression of GAPDH mRNA was similar among cholangiocytes purified from the 3 groups of animals.

At the protein level, immunoblots showed significantly decreased ABAT expression in cholangiocytes from BDI bile-depleted rats compared with BDI control rats (Figure 5*B*). ABAT protein expression in cholangiocytes from BDI bile-depleted, taurocholate-infused rats was similar to that of BDI control rats.

ABAT transport activity was determined from Na^+-dependent ^3H-taurocholate uptake in cholangiocytes iso-

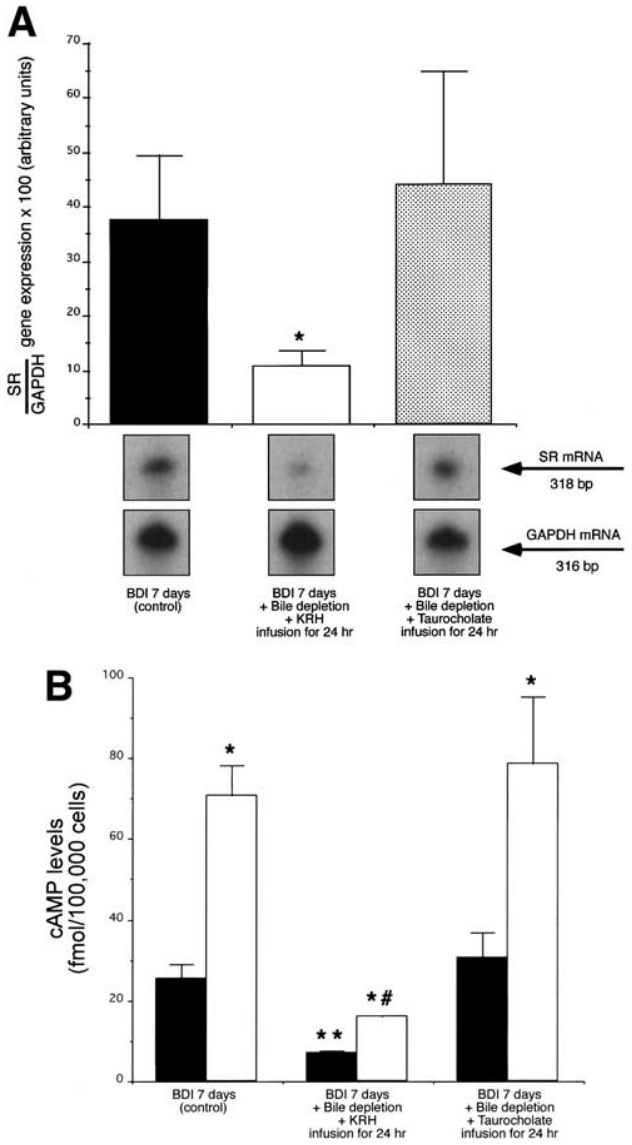

Figure 4. Measurement of secretin receptor gene expression (*A*) and intracellular cAMP levels (*B*) in cholangiocytes from BDI control rats and BDI bile-depleted rats infused with KRH or taurocholate. (*A*) Secretin receptor gene expression was assessed by the lysate ribonuclease protection assay using a 318-bp riboprobe encoding the message for the rat secretin receptor. The comparability of the lysates was assessed by hybridization with GAPDH. $*P \leq 0.05$ vs. basal values. Data are mean ± standard error under the mean of 3 experiments. (*B*) Cholangiocytes were incubated for 1 hour at 37°C and subsequently stimulated at 22°C for 5 minutes with secretin (100 nmol/L). Intracellular cAMP levels were determined by radioimmunoassay. $*P < 0.05$ vs. basal values; $^{\#}P < 0.05$ vs. secretin-induced cAMP levels of BDI control rats and BDI control rats infused with taurocholate. Data are mean ± standard error under the mean of 3–15 experiments. (*B*) ■, Basal; □, secretin.

Table 2. Bile Flow, Bicarbonate Concentration, and Secretion in BDI Control Rats and BDI Bile-Depleted Rats Infused With KRH or Taurocholate (1 μmol/h/kg body wt) for 24 Hours

	Bile flow		Bicarbonate concentration		Bicarbonate secretion	
Treatment	Basal (μL/min/kg body wt)	Secretin (μL/min/kg body wt)	Basal (mEq/L)	Secretion (mEq/L)	Basal (μEq/min/kg body wt)	Secretion (μEq/min/kg body wt)
BDI controls	98.59 ± 6.22	171.90 ± 13.92[a]	38.65 ± 2.75	50.65 ± 2.04[b]	3.87 ± 0.42	7.51 ± 0.52[c]
BDI (bile depleted + KRH infusion)	84.07 ± 7.03[d]	95.42 ± 5.69[d]	27.51 ± 1.41[d]	26.60 ± 1.94[d]	2.28 ± 0.10[d]	2.50 ± 0.12[d]
BDI (bile depleted + taurocholate infusion)	126.57 ± 13.33	183.63 ± 15.91[a]	31.92 ± 0.46	35.86 ± 11.58[b]	3.96 ± 0.41	6.56 ± 0.62[c]

NOTE. Values are mean ± SE of at least 6 values and were obtained at steady-state conditions of bile flow. After an equilibration period of 60 minutes with KRH solution, secretin was infused via a jugular vein for 30 minutes at 100 nmol/L. value of basal bile flow.

[a] $P < 0.05$ vs. its corresponding value of basal bile flow.

[b] $P < 0.05$ vs. its corresponding value of basal bicarbonate concentration.

[c] $P < 0.05$ vs. its corresponding value of basal bicarbonate secretion.

[d] $P < 0.05$ vs. its corresponding value of BDI depleted rats infused with KRH or taurocholate (1 μmol/min/kg body wt) for 24 hours.

lated from BDI control rats, BDI bile-depleted rats, and BDI bile-depleted, taurocholate-infused rats. Na^+-dependent uptake was decreased in cholangiocytes from BDI bile-depleted rats compared with BDI control rats (V_{max} at 109 ± 23 and 221 ± 39 pmol/min/mg protein; $P < 0.05$ compared with control). In BDI bile-depleted, taurocholate-infused rats, ABAT transport activity was similar to that of BDI control rats (V_{max} at 243 ± 48 and 221 ± 39 pmol/min/mg protein, respectively; $P < 0.05$ compared with control). The K_m for ^{3}H-taurocholate uptake was similar in purified cholangiocytes from rats in the 3 study groups (data not shown).

Role of PI3-K in Cholangiocyte Proliferation and Basal and Secretin-Stimulated cAMP Levels

Proliferative capacity. Similar to that shown in normal cholangiocytes,[15] taurocholate significantly ($P < 0.05$) increased PCNA protein expression in purified cholangiocytes from BDI rats compared with cholangiocytes treated with 0.2% BSA (Figure 6). The increase in cholangiocyte DNA synthesis (induced by in vitro taurocholate treatment) was abolished when purified cholangiocytes were preincubated with wortmannin, a PI3-K inhibitor.[18] The inhibitory effect of wortmannin on a taurocholate-induced increase in PCNA protein expression was abolished when purified cholangiocytes were pretreated with the PI 3,4-bisphosphate (an active PI) but not with PI 4,5-bisphosphate (an inactive PI). Wortmannin, PI 3,4-bisphosphate, or PI 4,5-bisphosphate did not change cholangiocyte PCNA protein expression.

Measurement of basal and secretin-stimulated cAMP levels. Basal cAMP levels of cholangiocytes from 1-week BDI rats were similar to those found in previous studies[2] (Figure 7). Secretin significantly increased cholangiocyte cAMP levels. Similar to its effect in normal cholangiocytes,[15] taurocholate significantly increased both basal and secretin-stimulated cAMP levels, and these increases were ablated when purified cholangiocytes were pretreated with the PI3-K inhibitor wortmannin. PI 3,4-bisphosphate (but not PI 4,5-bisphosphate, an inactive PI) ablated the inhibitory effects of wortmannin on taurocholate-induced increases in basal and secretin-stimulated cAMP levels. Basal cAMP levels were not altered by wortmannin (49.27 ± 9.86 vs. 40.38 ± 4.48 [basal value] fmol/1×10^5 cells), PI 3,4-bisphosphate (43.11 ± 7.89 vs. 40.38 ± 4.48 [basal value] fmol/1×10^5 cells), or PI 4,5-bisphosphate (50.38 ± 14.12 vs. 40.38 ± 4.48 [basal value] fmol/1×10^5 cells). Similarly, secretin-stimulated cAMP levels were not modified by wortmannin (79.20 ± 10.45 vs. 40.38 ± 4.48 [basal value] fmol/1×10^5 cells; $P < 0.05$ vs. basal value), PI 3,4-bisphosphate (70.73 ± 8.77 vs. 40.38 ± 4.48 [basal value] fmol/1×10^5 cells; $P < 0.05$ vs. basal value), or PI 4,5-bisphosphate (59.40 ± 4.01 vs. 40.38 ± 4.48 [basal value] fmol/1×10^5 cells; $P < 0.05$ vs. basal value).

Discussion

The studies showed that bile depletion for 24 hours in BDI rats reduced (1) biliary bile acid concentration; (2) DNA replication and secretin-stimulated cAMP levels in isolated cholangiocytes; (3) the number of PCNA-positive cholangiocytes in liver sections; (4) secretin-stimulated choleresis in vivo; and (5) ABAT gene, protein, and transport activity. In BDI bile-depleted, taurocholate-infused rats, biliary bile acid concentration, cholangiocyte proliferative and secretory capacity, and ABAT expression were similar to that of BDI control rats. Moreover, taurocholate in vitro increased

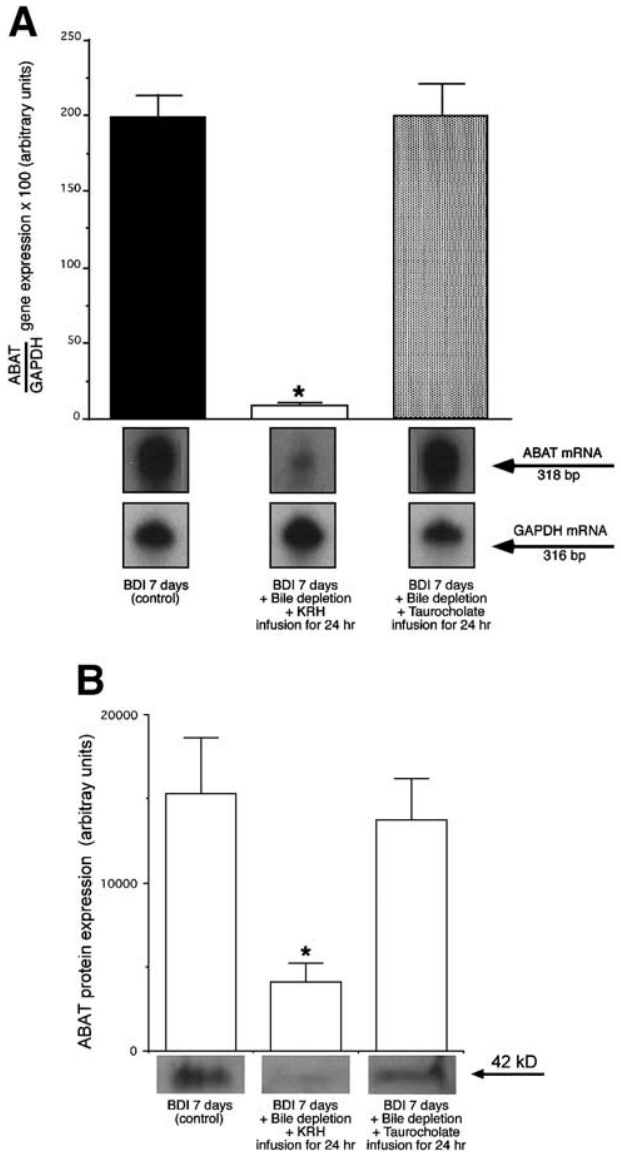

Figure 5. Measurement of ABAT gene (*A*) and protein (*B*) expression in cholangiocytes purified from the selected group of animals. Note that bile depletion markedly reduced both the genetic and protein expression of ABAT in cholangiocytes. When BDI bile-depleted rats were infused with taurocholate, ABAT gene and protein expression was similar to that of BDI control rats. Gene expression of selected messages was determined by lysate ribonuclease protection assay. ABAT protein expression was evaluated by Western blot analysis. Autoradiograms were quantified by densitometry. $*P < 0.05$ vs. the ABAT gene (*A*) or protein (*B*) expression of cholangiocytes from BDI control rats. Data are mean ± standard error under the mean of 3 experiments.

DNA replication and secretin-stimulated cAMP levels, and this increase was blocked by wortmannin. The inhibitory effect of wortmannin on taurocholate-induced increases in cholangiocyte DNA replication and secretin-stimulated cAMP levels was prevented by PI 3,4-bisphosphate but not by PI 4,5-bisphosphate, the inactive form of PI3-K.[19]

Recent studies have demonstrated that conjugated bile acids are transported by cholangiocytes through apical and basolateral transporters identified as Na^+-dependent ABAT[17,29] and an alternatively spliced Na^+-independent form of ABAT,[30] respectively. The direction of bile acid transport is from the apical to the basolateral pole of cholangiocytes,[17,30,31] suggesting that a portion of conjugated bile acids secreted in bile by the hepatocytes is reabsorbed into the intrahepatic biliary epithelium to undergo cholehepatic shunting. The functional and biological significance of the reabsorption of conjugated bile acids by the biliary epithelium is undefined, although recent studies indicate that bile acids may act in cholangiocytes as signaling molecules, regulating the functions of these cells.[15,17] Our previous studies[14,15] have shown that taurocholate increases cholangiocyte proliferation and secretion in vitro in isolated cholangiocytes and in vivo in the bile acid–fed rats. Furthermore, regulation of

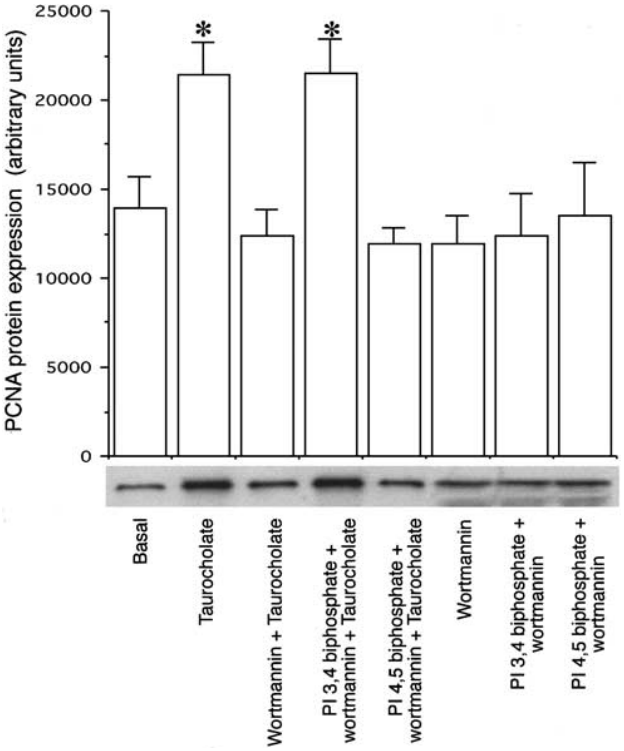

Figure 6. Measurement of PCNA protein expression (by Western blot analysis) in pure cholangiocytes from 1-week BDI rats stimulated at 37°C with 0.2% BSA (basal value) for 60 minutes; taurocholate (20 μmol) with 0.2% BSA for 60 minutes; wortmannin (20 μmol for 20 minutes) + taurocholate (20 μmol for 60 minutes) in the presence of 0.2% BSA; PI 3,4-bisphosphate or PI 4,5-bisphosphate (both at 1 μmol for 20 minutes) + wortmannin (20 μmol for 20 minutes) + taurocholate (20 μmol for 60 minutes) with 0.2% BSA; or wortmannin (20 μmol for 20 minutes), PI 3,4-bisphosphate or PI 4,5-bisphosphate (both at 1 μmol for 20 minutes) in the presence of 0.2% BSA. $*P < 0.05$ vs. BDI control rats. Autoradiograms ($n = 10–15$) were quantified by densitometry.

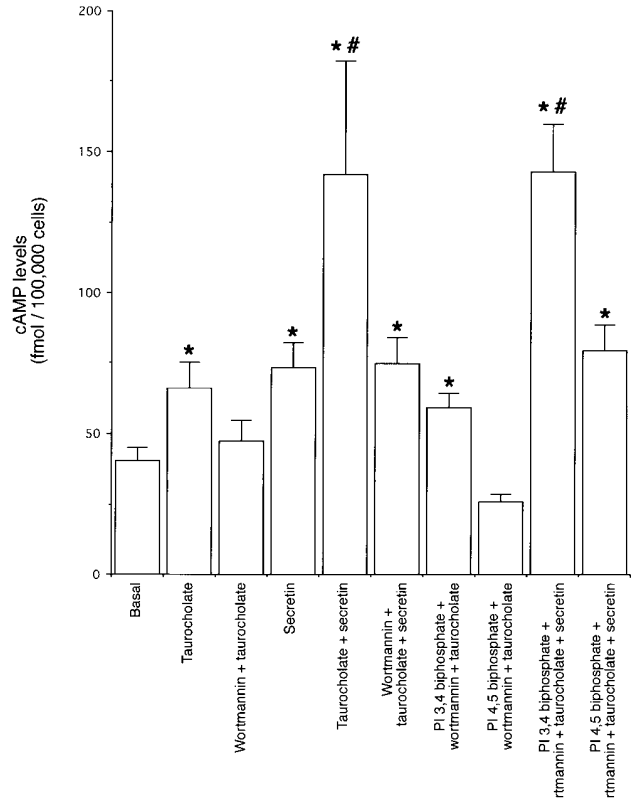

Figure 7. Intracellular cAMP levels in cholangiocytes from 1-week BDI rats stimulated at 37°C with 0.2% BSA (basal value) for 5 minutes; secretin (100 nmol/L for 5 minutes); taurocholate (20 μmol/L) with 0.2% BSA for 10 minutes in the absence or presence of secretin (100 nmol/L for 5 minutes); wortmannin (20 μmol/L for 10 minutes) + taurocholate (20 μmol/L for 10 minutes) in the absence or presence of secretin (100 nmol/L) with 0.2% BSA; or PI 3,4-bisphosphate or PI 4,5-bisphosphate (control) (both at 1 μmol/L for 10 minutes) + wortmannin (20 μmol/L for 10 minutes) + taurocholate (20 μmol/L for 10 minutes) in the absence or presence of secretin (100 nmol/L for 5 minutes). Cholangiocyte cAMP levels were measured by radioimmunoassay using commercially available kits. $^{*}P < 0.05$ vs. basal values; $^{\#}P < 0.05$ vs. secretin-stimulated cAMP levels. Data are mean ± standard error under the mean of at least 6 experiments.

ABAT expression may control cholangiocyte sensitivity to bile acid signaling. Our preliminary data show that experimental up- and down-regulation of cholangiocyte ABAT results in equivalent changes in bile acid–stimulated cholangiocyte proliferation (Alpini, Glaser, and LeSage, unpublished observations, November 2001).

In the current study, we have addressed the following question: During ductal hyperplasia, does elevated biliary bile acid concentration or altered ABAT expression trigger cholangiocyte proliferation? To determine whether increased biliary bile acid concentration or cholangiocyte ABAT expression initiates cholangiocyte hyperplasia associated with bile duct obstruction, we tested whether the manipulation of biliary bile acid concentration (bile acid depletion/repletion) may affect the proliferative and secretory properties of cholangiocytes of BDI rats. One-week BDI rats were submitted to external bile drainage for 24 hours to decrease biliary bile acid concentration (bile acid depletion) or to exogenous infusion of taurocholate (bile acid repletion) to maintain bile acid biliary concentration in bile similar to that of BDI rats. The proliferative and secretory activities of cholangiocytes were then evaluated in comparison to control BDI rats without external bile drainage. We measured the qualitative and quantitative bile acid biliary composition to confirm the efficacy of 24-hour external bile drainage in inducing a marked depletion of biliary bile acid concentration, which reflects exclusively the hepatic bile acid synthesis.[32] We also demonstrated that taurocholate infusion (1 μmol/min/kg body weight) was effective in restoring a total bile acid biliary concentration similar to that of BDI control rats. In these 3 models, we demonstrated that ABAT expression and activity in cholangiocytes was reduced when biliary bile acid concentration was decreased by external bile drainage and maintained (similar to BDI control) by taurocholate infusion. Bile acid depletion decreased cholangiocyte proliferation as evaluated in situ by PCNA immunohistochemistry and in purified cholangiocytes by PCNA and H_3 histone expression. Bile acid depletion reduced cholangiocyte secretory activities manifested by the abolishment of secretin-stimulated choleresis and secretin-induced intracellular cAMP levels. Bile acid repletion by intravenous taurocholate administration restored cholangiocyte proliferative and secretory activities to levels similar to that observed in BDI rats.[1,2,11] Taken together, the findings indicate that the secretory and proliferative activities of cholangiocytes are regulated by changes of biliary bile acid concentration in bile duct obstruction and are consistent with the hypothesis that elevated biliary bile acids due to bile duct obstruction initiates cholangiocyte proliferation. A number of effectors (e.g., hormones, estrogens, cytokines, nerve input) have been suggested to trigger cholangiocyte proliferation with bile duct obstruction.[33] Bile acids are attractive potential triggers, because they accumulate markedly in cholestasis and are known to directly stimulate (similar to estrogens[16]) cholangiocyte proliferation.[14,15] Furthermore, cholangiocyte proliferation is observed primarily with obstruction of large intrahepatic bile ducts or extrahepatic obstruction[7] (when biliary bile acids are increased) and is absent when cholestasis involves canalicular dysfunction (when biliary bile acids are not increased).[34] Although depletion in the BDI model may remove a number of potential cholangiocyte growth-promoting factors that are secreted in bile, the findings

that in BDI bile-depleted, taurocholate-infused rats in which cholangiocyte proliferation and secretion are maintained is a strong argument that bile acids may be important triggering signals for cholangiocyte proliferation in the development of bile duct obstruction.

Increases or decreases of biliary bile acid concentrations in BDI rats produced proportional changes in cholangiocyte ABAT gene and protein expression and bile acid transport activity. These findings suggest that ligand concentration directly modulates ABAT expression in cholangiocytes. Although conflicting results have been reported,[35–37] it has been shown that in the ileum, activity and protein expression of the apical Na^+-dependent bile acid transporter (ASBT) displays similar regulation to what is found in the intrahepatic biliary epithelium. ASBT was down-regulated when intestinal bile acids where lowered by extrahepatic cholestasis,[37] whereas it was induced in the ileum by bile acids, such as cholate.[35] These studies indicate that both in the ileum and biliary epithelium, apical reabsorption of conjugated bile acids is increased proportionally to their lumenal concentration and that this occurs through a direct regulation of ABAT gene and protein expression by bile acids. Up-regulation of cholangiocyte ABAT may represent an important adaptation to bile duct obstruction. The increased number of bile ducts and ABAT with bile duct obstruction may promote cholehepatic shunting of bile acids, thus providing a pathway for continued flux of bile acid molecules within the hepatobiliary axis and preventing toxicity due to intracellular accumulation of bile acids. Bile acid signaling may not only trigger cholangiocyte proliferation, but also permit regression of bile duct hyperplasia after resolution of bile duct obstruction. Down-regulation of ABAT due to reversal of bile duct obstruction and decreased biliary bile acid concentration may reduce bile acid signaling of cholangiocyte proliferation, thus promoting regression of biliary hyperplasia.

We then demonstrated that taurocholate directly stimulates in vitro cholangiocyte proliferation (PCNA protein expression) and secretin-stimulated cAMP levels (an indirect index of ductal secretion) of purified cholangiocytes from BDI rats. The stimulatory effect of taurocholate on basal and secretin-stimulated cAMP levels in cholangiocytes from BDI rats, which confirmed our previous studies in normal cholangiocytes,[15] is different among the different cell types in the liver. In general, bile acids have been shown to influence basal and agonist-induced cAMP levels in different ways, depending on the cell types and the hydrophobicity of the tested bile acids.[38] Whereas in the perfused colon taurodeoxycholic acid stimulates cAMP synthesis, in hepatocytes as well as in dermal fibroblasts, endothelial cells, thyroid cells, and gastric adenocarcinoma, bile acids inhibit agonist-induced cAMP synthesis with no or little effect on basal cAMP levels.[38] Within the different liver cell types, in hepatocytes bile acids exert strong inhibitory effects on agonist-induced cAMP levels with a higher potency for the hydrophilic ursodeoxycholic acid than for hydrophobic bile acids.[38–40] Because bile acid uptake in hepatocytes is increased by cAMP, the inhibitory effects of bile acids on cAMP production has been suggested to represent a protective mechanism against bile acid intracellular accumulation during cholestasis.[38] In cholangiocytes, on the contrary, the stimulatory effect of bile acids on basal and secretin-induced cAMP levels and choleresis may have important physiologic implications. In fact, through the modulation of cAMP synthesis, bile acids may up-regulate biliary bicarbonate secretion in the intrahepatic biliary epithelium just when the need for bicarbonate for digestive purpose is maximal (i.e., during the postprandial phase), which corresponds to the maximal bile acid concentration in bile and reabsorption by cholangiocytes.

Taurocholate stimulatory effects on basal and secretin-induced cAMP levels required activation of PI3-K as an upstream event, because these effects were abolished by wortmannin. In addition, the effects of wortmannin were abolished by PI 3,4-bisphosphate but not by PI 4,5-bisphosphate (the inactive form of PI3-K[19]), thus confirming the specificity of the effect of wortmannin. Our findings indicate that activation of PI3-K is an early event in bile acid modulation of cholangiocyte proliferation and secretion. PI3-K has been implicated in controlling cell proliferation, actin cytoskeleton organization, the regulation of vesicle trafficking between intracellular organelles, and secretory processes in a number of cell types.[18] In hepatocytes, PI3-K is activated by bile acids and plays an important role in the choleretic and antiapoptotic effects of hydrophilic bile acids.[19,41–43] In light of the present findings, further studies are needed to evaluate the role of bile acids with different hydrophobicity in the modulation of PI3-K and cAMP in cholangiocytes in normal and pathologic conditions.

In conclusion, we have provided evidence that in a model of bile duct obstruction, the increased proliferative and secretory activities of cholangiocytes is associated with high biliary bile acid concentration, a finding suggesting that increased biliary bile acid concentration triggers cholangiocyte proliferation. Down-regulation of ABAT following resolution of bile duct obstruction may promote regression of ductal hyperplasia due to a reduc-

tion of bile acid signaling of proliferation and secretion. The modulatory effects of bile acids on cholangiocyte proliferation and secretion occur through intracellular pathways involving both cAMP production and PI3-K activity. These findings have important implications in understanding the changes of proliferative and secretory activities of cholangiocytes in human chronic cholestatic liver diseases characterized by marked qualitative and quantitative alterations of bile acid biliary composition.

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