Taurocholate prevents the loss of intrahepatic bile ducts due to vagotomy in bile duct-ligated rats

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Taurocholate prevents the loss of intrahepatic bile ducts due to vagotomy in bile duct-ligated rats. Am J Physiol Gastrointest Liver Physiol 284: G837–G852, 2003. First published January 29, 2003; 10.1152/ajpgi.00398.2002.—The aim of this study was to determine whether taurocholate prevents vagotomy-induced cholangiocyte apoptosis. After bile duct ligation (BDL) + vagotomy, rats were fed taurocholate for 1 wk in the absence or presence of wortmannin. Caspase involvement was evaluated by measurement of caspase 8, 9, and 3 activities. Proliferation was determined by morphometry and PCNA immunoblots. Changes in phosphatidylinositol 3-kinase (PI3-kinase) activity were estimated by the expression of the phosphorylated Akt protein. Apically located Na+/H+-dependent bile acid transporter (ABAT) expression and activity were evaluated by immunoblots and [3H]taurocholate uptake, respectively. Cholangiocyte apoptosis increased, whereas proliferation decreased in BDL + vagotomy rats. Taurocholate feeding prevented vagotomy effects on cholangiocyte functions, which were abolished by wortmannin. ABAT expression and activity as well as phosphorylated Akt protein expression were reduced by vagotomy but restored by taurocholate. The activities of caspase 8, 9, and 3 increased in BDL + vagotomy rats but were restored by taurocholate. The protective effect of taurocholate was associated with maintenance of ABAT activity, downregulation of caspase 8, 9, and 3, and activation of PI3-kinase. Bile acids are important in modulating cholangiocyte proliferation in denervated livers. Cholangiocytes are the target cells in several human cholestatic liver diseases (i.e., cholangiopathies), characterized by cholangiocyte proliferation and/or ductopenia (9). Cholangiocyte proliferation is involved, as a repair mechanism, in counteracting loss of bile ducts, thereby inhibiting the progression of cholangiopathies toward the final ductopenic stage (9). In animal models, activation of proliferation of cholangiocytes (which are normally mitotically dormant) (30) is triggered by a number of pathological events including bile duct ligation (BDL) (6, 8, 17) or feeding of certain bile acids (7, 10). Cholangiocyte loss can be achieved by acute administration of CCl4 (31, 32). Although cholangiocyte proliferation is coupled with increased secretin-stimulated choleresis (6, 8, 18, 32), ductopenia is associated with decreased secretin-induced ductal secretion (32).

We have previously shown (28) that interruption of the parasympathetic innervation by total vagotomy inhibits cholangiocyte proliferation in BDL rats and induces activation of cholangiocyte apoptosis leading to ductopenia. Vagotomy-induced cholangiocyte apoptosis and loss of proliferation are linked to reduced intracellular cholangiocyte cAMP levels, because maintenance of cAMP levels, by administration of forskolin, prevented the effects of vagotomy on cholangiocyte growth/ductopenia (28). The serum levels of gastrin, somatostatin, and insulin were unaffected by vagotomy in BDL rats, thus supporting the concept that our findings were not caused by changes in the release in the blood of these hormones, but rather by a direct effect of the cholinergic system on the intrahepatic biliary epithelium (28).

Bile acids enter cholangiocytes through the apically located Na+/H+-dependent bile acid transporter (ABAT) (5, 27). After uptake, bile acids modulate cholangiocyte secretion, proliferation, and apoptosis (4, 7, 10). Bile salts affect cell functions through the phosphatidylinositol 3-kinase pathway (PI3-kinase) pathway (35, 45, 52). For

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example, the hydrophobic bile acid taurochenodeoxycholate activates P13-kinase and inhibits caspase 8 activity in hepatocytes (45, 49), which prevent its inherent toxicity (45).

cAMP protects cultured rat hepatocytes from apoptosis in a P13-kinase-dependent manner (52, 53). In previous studies (2–4, 7), we have shown that taurocholate increases both basal and secretin-stimulated intracellular cAMP levels in cholangiocytes, both in vitro and in vivo, when given to rats by a dietary regimen containing this bile acid.

We posed the following questions: first, does taurocholate feeding prevent vagotomy-induced apoptosis of cholangiocytes, vagotomy inhibition of cholangiocyte proliferation, and secretin-stimulated ductal secretion? Second, are taurocholate effects on vagotomy-induced bile duct damage mediated by the P13-kinase/Akt pathway? And third, are changes in cholangiocyte apoptosis, proliferation, and secretion (following vagotomy and taurocholate feeding) dependent on ABAT activity?

**MATERIALS AND METHODS**

**Materials.** Reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Rat chow, containing 1% taurocholate or control diet (AIN-76), was prepared from Dyets (Bethlehem, PA). Control chow (AIN-76) has the same composition of the chow containing 1% taurocholate, but it does not contain taurocholate. The substrate for γ-glutamyltranspeptidase (γ-GT), N-(γ-L-glutamyl)-4-methoxy-2-naphthylamide, was purchased from Polysciences (Warrington, PA). The PKA inhibitor, Rp-cAMP (12), and the caspase 8 thylamide, was purchased from Polysciences (Warrington, NC). The antibody vs. ABAT (rabbit anti-rat ABAT) was a gift from Dr. P. Dawson (Bowman Gray School of Medicine, Winston-Salem, NC).

**Animal models.** Male Fischer 344 rats (150–175 g) were purchased from Charles River (Wilmington, MA), kept in a temperature-controlled environment (20–22°C) with a 12:12-h light-dark cycle, and fed ad libitum with the selected diet. The majority of the studies were performed in the following four groups of animals: 1) rats that, immediately after BDL (for cell isolation) (1, 3, 6–8, 10, 17, 24, 28, 29, 32) or bile duct cannulation (BDI; for bile collection) (8), were fed control diet or 1% taurocholate for 1 wk and rats that (immediately after BDL or BDI) underwent vagotomy and, subsequently, were fed control diet or 1% taurocholate diet for 1 wk.

To demonstrate the link between cholangiocyte apoptosis and the P13-kinase-signaling pathway, rats, immediately after BDL + vagotomy, were fed taurocholate and subsequently treated with Wortmannin, a specific P13-kinase inhibitor (35) (1 daily intraperitoneal injection of 0.7 mg/kg body wt) (38) in DMSO for 1 wk. To demonstrate the link between cholangiocyte apoptosis and the ERK-signaling pathway, rats, immediately after BDL + vagotomy, were fed taurocholate and subsequently treated with 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene (U-0126); an MEK inhibitor, 1 daily intraperitoneal injection of 5 mg kg/body wt) (14, 36) in DMSO for 1 wk. To demonstrate whether vagotomy-induced cholangiocyte apoptosis is mediated through the extrinsic or intrinsic apoptotic pathway, rats, immediately after BDL + vagotomy, were treated with Z-IETD-fmk (a specific caspase 8 inhibitor (44), by daily intraperitoneal injections, 4.6 μg/kg body wt–1·day–1 (15)) in DMSO for 1 wk. One week later, in these three groups of animals, we evaluated apoptosis in liver sections by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) analysis (29) and in purified cholangiocytes by both annexin-V staining (28, 29) and by the cell death-detection ELISA assay. Because we have previously shown (28) that chronic intraperitoneal injections of DMSO do not alter cholangiocyte apoptosis, proliferation, and secretion of BDL rats, we did not include this group in our study. Because we have previously shown (28) that vagotomy does not alter cholangiocyte apoptosis, proliferation, and basal or secretin-stimulated ductal secretion of normal rats, we did not evaluate the effects of taurocholate or control diet on vagotomy-induced changes in the biliary functions of normal rats. In addition, we have also shown (50) that sham surgery does not alter cholangiocyte function. Furthermore, we have shown (7, 33) that taurocholate feeding to normal or BDL rats increases cholangiocyte proliferation, which is devoid of cholangiocyte apoptosis.

Total vagotomy was performed as described by our laboratory and others (28, 41). Briefly, after anesthesia with isoflurane (Ohmeda Caribe, Guayama, Puerto Rico), a midline incision was made just anterior to the nasopharyngeal opening, and anterior to the thoracic outlet, the right carotid artery was located and isolated. Then, the vagus nerve was blunt dissected out and ligated. The incision was closed with 3-0 Vicryl suture (Ethicon, Somerville, NJ) in an interrupted vertical mattress pattern. BDL or BDI was performed as described in detail by our laboratory in previous studies (8). Before terminal surgeries (e.g., liver perfusion for cell isolation or surgical preparation for bile collection), animals were anesthetized with pentobarbital sodium (50 mg/kg ip). Rats were fasted the night before the experiments. Study protocols were performed in compliance with the institution guidelines.

**Assessment of biliary bile acid concentration and composition.** The concentration of total bile salts in bile from rats that, immediately after BDL, were fed control diet or 1% taurocholate for 1 wk and rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate for 1 wk was assessed by the 3-α-hydroxyoestrogen dehydrogenase procedure by absorption spectrophotometry using a commercially available kit (Wako Chemicals, Richmond, VA) (34). Total bile acid concentration was measured in the bile that was collected within the first 10 min of bile flow. Aliquots of bile were analyzed for individual bile salt by reversed-phase HPLC (13).

**Purification of cholangiocytes.** Pure cholangiocytes were obtained from the selected group of animals by immunofluorescence (4, 6, 7, 10, 17, 18, 30, 32). Purity of cholangiocytes was evaluated by histology for γ-GT (46), a specific marker for cholangiocytes (8). Cell viability was >97%.

**Evaluation of apoptosis.** We evaluated cholangiocyte apoptosis by TUNEL analysis (32) in liver sections (n = 3) and by annexin-V staining (28, 32) and the cell death-detection ELISA assay in pure cholangiocytes from rats that, immediately after BDL, were fed control diet or 1% taurocholate for 1 wk and rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate diet for 1 wk. To evaluate whether vagotomy-induced
apoptosis is mediated through the extrinsic or intrinsic pathway, we measured apoptosis by TUNEL analysis in liver sections and by both annexin-V staining and cell death-detection ELISA assay in purified cholangiocytes from BDL, vagotomized rats that were fed taurocholate and simultaneously treated by intraperitoneal injections with Z-IETD-fmk for 1 wk. Similarly, to evaluate whether the protective effect of taurocholate on vagotomy-induced apoptosis is mediated by PI3-kinase or MEK pathways, we measured apoptosis by TUNEL analysis in liver sections and by both annexin-V staining and cell death-detection ELISA assay in purified cholangiocytes from rats that, immediately after BDL + vagotomy, were fed 1% taurocholate and simultaneously treated by intraperitoneal injections with wortmannin (a PI3-kinase inhibitor) or U-0126 (an MEK inhibitor), respectively, for 1 wk.

TUNEL analysis was performed using a commercially available kit (Wako Chemicals, Tokyo, Japan). After liver sections were counterstained with hematoxylin solution, they were examined by light microscopy with an Olympus BX-40 microscope (Olympus Optical) equipped with a camera. Appropriate kits according to the instructions supplied by the vendor were in 10 nonoverlapping fields. After annexin-V staining (28, 32), cells were counterstained with hematoxylin solution and examined by light microscopy with an Olympus BX-40 microscope equipped with a camera. Approximately 300 cells per slide were counted in a coded fashion.

Cell death detection by the Elisa assay was performed according to the vendor’s instructions (Boehringer-Mannheim, Frankfurt, Germany). The cell death ELISA assay is an assay that detects apoptosis. It is based on the binding of the anti-histone antibody with the core histones H2A, H2B, H3, and H4 that form the mono/oligonucleosomes, typical of the apoptotic degeneration of the nuclei. Briefly, 1 × 10^6 pure cholangiocytes from the selected group of animals were re-suspended in 500 μl of incubation buffer and incubated at 4°C for 30 min. Cells were then centrifuged, and the supernatant was rediluted in incubation buffer. The samples were incubated for 90 min at room temperature in microtiter plate (MTP) modules coated with a buffer containing an anti-histone antibody. The anti-histone antibody binds to the core histones H2A, H2B, H3, and H4 that form the mono/oligonucleosomes, typical of the apoptotic degeneration of the nuclei. After being washed, a solution containing anti-DNA-peroxi-dase was added and another incubation of 90 min followed. After removal of unbound peroxidase conjugate by washing, the amount of peroxidase retained in the immunocomplex was determined photometrically with 2,2'-azino-di[3-ethylbenzthiazoline sulphonate (6)] as a substrate.

To evaluate whether taurocholate protects from vagotomy-induced apoptosis through changes in the caspase cascade by a PI3-kinase-dependent mechanism, we measured the activities of caspase 3, 8, and 9 by proteolytic cleavage of substrates such as DEVD-pNA (caspase 3 substrate), IETD-pNA (caspase 8 substrate), and LEHD-pNA (caspase 9 substrate), respectively, added to each sample. The assay is based on the photometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the substrates. The pNA light emission was quantified using a microtiter plate reader at 406 nm.

**Evaluation of cholangiocyte proliferation.** Cholangiocyte proliferation was evaluated by quantitative immunohistochemistry for PCNA (32) or cytokertatin-19 (30) [CK-19, a cholangiocyte-specific marker (30)] in liver sections and by measurement of PCNA protein expression by immunoblot (17) in pure cholangiocytes from rats that, immediately after BDL, were fed control diet or 1% taurocholate for 1 wk, rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate diet for 1 wk, rats that, immediately after BDL, underwent vagotomy and subsequently fed 1% taurocholate and subsequently treated by intraperitoneal injections with wortmannin for 1 wk. After sections were stained for PCNA or CK-19, they were counterstained with hematoxylin and examined with a microscope (Olympus BX 40, Olympus Optical). Data were expressed as number of PCNA- or CK-19-positive cholangiocytes per each 100 cholangiocytes counted in seven different fields. DNA replication was evaluated by measurement of PCNA protein expression by immunoblot (17) in pure cholangiocytes from the aforementioned group of animals. 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 basal and secretin-stimulated ductal secretion.** Ductal secretion was evaluated by measurement of basal and secretin-stimulated bicarbonate-rich choleseresin, secretin receptor (SR) gene expression, and basal and secretin-induced cAMP levels in pure cholangiocytes from rats that, immediately after BDL, were fed control diet or 1% taurocholate for 1 wk and rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate diet for 1 wk.

After anesthesia, rats were surgically prepared for bile collection as described (8). One jugular vein was cannulated with a PE-50 cannula (Clay-Adams, New York, NY) to induce either Krebs-Ringer-Henseleit (KRH) buffer or secretin (100 nM) dissolved in KRH. The rate of fluid infusion was adjusted according to both the rate of bile flow and the value of the arterial hematocrit and ranged from 0.738 to 2.964 ml/h. Body temperature was monitored with a rectal thermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained at 37°C by using a heating pad. As soon as the bile duct was cannulated (a step that takes only a few seconds because we need only to connect the plastic tubing coming out from the bile duct with another tube of bigger diameter) (8), collection of bile started immediately. When steady spontaneous bile flow was reached (60–70 min from the infusion of KRH), rats were infused for 30 min with secretin followed by a final infusion of KRH for 30 min. After the rats were strictly prepared for bile-flow experiments, bile was collected every 10 min in preweighed tubes and immediately stored at −20°C before determining bicarbonate concentration. Bile volume was determined by weight, assuming a density of 1.0 g/ml. Bile flow was expressed as microliters per minute per kilogram of body weight. Bicarbonate concen-
Table 1. Bile acid composition in bile obtained from rats that, immediately after BDI, were fed control diet or 1% taurocholate for 1 wk and rats that, immediately after BDI, underwent vagotomy and subsequently were fed control diet or 1% taurocholate for 1 wk.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Bile Acid Concentration, mM</th>
<th>%Total</th>
<th>Muricholic Acid Concentration, mM</th>
<th>%Total</th>
<th>Taurocholic Acid Concentration, mM</th>
<th>%Total</th>
<th>Taurochenoehycolic Acid Concentration, mM</th>
<th>%Total</th>
<th>Taurodeoxycholic Acid Concentration, mM</th>
<th>%Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDI + control feeding 1 wk</td>
<td>7.85 ± 1.30</td>
<td>86.66 ± 1.62</td>
<td>6.87 ± 1.20</td>
<td>9.88 ± 1.38</td>
<td>0.76 ± 0.15</td>
<td>3.45 ± 0.73</td>
<td>0.23 ± 0.02</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
<tr>
<td>BDI + taurocholate 1 wk</td>
<td>15.91 ± 4.08*</td>
<td>46.86 ± 2.08</td>
<td>15.91 ± 4.08*</td>
<td>46.14 ± 1.58</td>
<td>7.8 ± 2.16*</td>
<td>8.00 ± 1.74</td>
<td>0.62 ± 0.12</td>
<td>detectable</td>
<td>detectable</td>
<td>Not detectable</td>
</tr>
<tr>
<td>BDI + control feeding + vagotomy 1 wk</td>
<td>1.52 ± 0.13*</td>
<td>84.96 ± 1.32</td>
<td>1.29 ± 0.11*</td>
<td>11.13 ± 1.11</td>
<td>0.18 ± 0.02*</td>
<td>3.91 ± 0.74</td>
<td>0.05 ± 0.007*</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
<tr>
<td>BDI + taurocholate + vagotomy 1 wk</td>
<td>15.69 ± 1.04*</td>
<td>54.5 ± 2.64</td>
<td>8.38 ± 0.46</td>
<td>43.54 ± 2.70</td>
<td>7.03 ± 0.85*</td>
<td>1.95 ± 0.30</td>
<td>0.28 ± 0.03</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 rats. After anesthesia, the selected groups of rats were surgically prepared for bile collection as follows. One jugular vein was cannulated with a PE 50-cannula to infuse either Krebs-Ringer-Henselet (KRH) or secretin (100 nM) in KRH. As soon as the bile duct was cannulated (a step that takes only a few seconds because we need only to connect the plastic tubing coming out from the bile duct with another tube of larger diameter), collection of bile started immediately. Bile was collected every 10 min in preweighed tubes and immediately stored at −20°C before determining total bile acid concentration. Total bile acid concentration was measured in the bile that was collected within the first 10 min of bile flow. Bile volume was determined by weight, assuming a density of 1.0 g/ml. 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-α-hydroxysteroid dehydrogenase procedure by a commercially available kit. *P < 0.05 vs. corresponding value of 1 bile duct ligated (BDL) rats fed control diet.

For the evaluation of intracellular cAMP levels, pure cholangiocytes from the selected group of animals were incubated for 1 h at 37°C (6, 7, 10, 17, 18, 28–32) in 1× HEPES buffer (140 mM NaCl, 5.4 mM KCl, 0.8 mM Na2HPO4, 25 mM HEPES, pH 7.4) containing 0.004% DNase I. Subsequently, cholangiocytes were incubated for 5 min at room temperature (6, 7, 10, 17, 18, 28–32) with 0.2% BSA (basal) or 100 nM secretin with 0.2% BSA. Intracellular cAMP levels were measured by commercially available RIA kits (Amersham Life Science).

Measurement of Akt phosphorylation. To determine that the PI3-kinase-Akt pathway regulates taurocholate preven-
tion of vagotomy-induced duct damage, we evaluated, by immunoblot (17) in whole cell lysate from purified cholangiocytes from rats that, immediately after BDL, were fed control diet or 1% taurocholate for 1 wk and rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate for 1 wk, the expression of the total protein and the phosphorylation (Ser473) of Akt (a downstream effector of the PI3-kinase-dependent cell-survival pathway responsible for antiapoptotic activity) (42). To determine whether taurocholate changes in Akt phosphorylation are PI3-kinase dependent, we evaluated total and phosphorylated Akt in cholangiocytes from rats that, immediately after BDL, underwent vagotomy, were fed taurocholate, and were subsequently treated with wortmannin (1 daily intraperitoneal injection of 0.7 mg·kg⁻¹·body wt⁻¹ (38)) for 1 wk.

Role of ABAT on taurocholate prevention of vagotomy-induced effects on cholangiocyte apoptosis, proliferation, secretion, and Akt phosphorylation. To determine whether changes in cholangiocyte apoptosis, proliferation, secretion, and Akt phosphorylation are dependent on ABAT activity, we incubated ABAT protein expression and bile acid transport activity (5, 10) in pure cholangiocytes from rats that, immediately after BDL, were fed control diet and rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate diet for 1 wk. Because we have previously shown (10) that taurocholate feeding increases ABAT protein expression and bile acid transport activity in rat cholangiocytes, we did not evaluate ABAT protein expression and bile acid transport activity in cholangiocytes from BDL rats fed taurocholate for 1 wk.

To determine whether the taurocholate-induced changes in APAT expression are PI3-kinase dependent, we evaluated ABAT protein expression in cholangiocytes from rats that, immediately after BDL + vagotomy, were fed taurocholate and were subsequently treated with wortmannin (1 daily intraperitoneal injection of 0.7 mg·kg⁻¹·body wt⁻¹ (38)) for 1 wk. Immunoblots for ABAT were performed as described (10). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low-light imaging system.

ABAT transport activity was determined by measuring the Na⁺-dependent [³H]taurocholate uptake in pure cholangiocytes from rats that, immediately after BDL, were fed control diet, and rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate diet for 1 wk as described by our laboratory (5, 10). Uptake was determined at 30 s from 1–200 μM taurocholate. Results were expressed as picomoles of taurocholate uptake per microgram of cholangiocyte protein. Estimates of Kₐ and Vₘₐₓ were determined by a weighted least-squares fit of the sigmoidal curve according to the method of Vaughan et al. (51).

In in vitro studies to directly evaluate the role of ABAT and PI3-kinase in taurocholate regulation of cholangiocyte proliferation and to demonstrate that the effects of taurocholate on PI3-kinase and proliferation are mediated by the cAMP/PKA pathway, pure cholangiocytes (5 × 10⁶) from rats that, immediately after BDL, were fed control diet and rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate diet for 1 wk were incubated for 2 h at 37°C with 0.2% BSA (basal) or taurocholate (20 μM) (4) with 0.2% BSA in the presence or absence of 20-min preincubation with the PI3-kinase inhibitor wortmannin (100 nM) or the PKA inhibitor Rp-cAMP (100 μM) (12). Subsequently, by immunoblot, we measured the PCNA protein expression (an index of cholangiocyte proliferation) (17), total protein expression, and the phosphorylation (Ser473) of Akt.

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 were analyzed.

RESULTS

Assessment of biliary bile acid concentration and composition. Bile acid composition of bile from the selected groups of rats is shown in Table 1. After vagotomy, there was a significant decrease in biliary bile acid concentration of muricholic, taurocholic, and taurochenodeoxycholic acid compared with BDL control rats (Table 1). Taurocholate feeding to BDL + vagotomy rats increased total biliary bile acid concentration to levels higher than those of BDL control rats (Table 1). Biliary bile acid concentration and composition of rats that, immediately after BDL, were fed 1% taurocholate for 1 wk are also shown in Table 1.

Measurement of apoptosis. Taurocholate prevention of vagotomy-induced cholangiocyte apoptosis is blocked by wortmannin. In agreement with previous studies (33), TUNEL analysis showed only a few apoptotic bodies in the liver sections of BDL rats fed control diet or 1% taurocholate for 1 wk (Fig. 1A). The number of cholangiocytes undergoing apoptosis increased in liver sections from BDL with vagotomy compared with BDL control rats (Fig. 1A). Taurocholate feeding prevented the increase in cholangiocyte apoptosis induced by vagotomy (Fig. 1A).

Staining of purified cholangiocytes for annexin-V showed that vagotomy increased the percentage of annexin-V-positive cholangiocytes compared with cholangiocytes from BDL rats fed control diet or 1% taurocholate for 1 wk (Fig. 1B). Taurocholate feeding to BDL + vagotomy rats prevented the increase in the number of annexin-V-positive cholangiocytes, which were not statistically different from those of BDL control rats (Fig. 1B). Similarly, cell death detection by ELISA assay showed that apoptosis increased in cholangiocytes from BDL-vagotomized rats but was inhibited by taurocholate feeding (Fig. 1C). We also found that taurocholate prevention of vagotomy-induced cholangiocyte apoptosis was blocked by wortmannin but not U-0126 (Fig. 1, A–C). Z-IETD-fmk did not alter vagotomy-induced increase in cholangiocyte apoptosis (Fig. 1, A–C).

Taurocholate prevention of vagotomy-induced apoptosis is mediated by reduction of caspase activity. Vagotomy induced an increase in the activity of caspases 8, 9, and 3 in purified cholangiocytes compared with cholangiocytes isolated from BDL rats fed control diet or taurocholate (Fig. 1D). Feeding taurocholate to BDL, vagotomized rats resulted in a decrease in the activity of caspases 8, 9, and 3, which were not statistically different from that of cholangiocytes from BDL rats fed control diet or taurocholate (Fig. 1D). However, the in vivo administration of wortmannin blocked the protective effect of taurocholate, because the activity of caspases 8, 9, and 3 increased again to levels not statis-
Fig. 2. Quantitative immunohistochemistry for PCNA (A; original magnification, ×1,000) or CK-19 (B; original magnification, ×250) in liver sections from 1) rats that, immediately after BDL, were fed control diet or 1% taurocholate for 1 wk, 2) rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate diet for 1 wk, and 3) rats that, immediately after BDL + vagotomy, were fed 1% taurocholate and subsequently were treated with intraperitoneal injections with wortmannin for 1 wk. After vagotomy, the number of PCNA- or CK-19-positive cholangiocytes decreased compared with liver sections from BDL rats fed control diet or 1% taurocholate for 1 wk. Taurocholate feeding prevented the inhibitory effect of vagotomy on the number of PCNA- or CK-19-positive cholangiocytes. Taurocholate prevention of vagotomy-induced inhibition of the number of PCNA- and CK-19-positive cholangiocytes was blocked by wortmannin (A and B). *P < 0.05 vs. the other groups of rats. Data are means ± SE of 3 experiments.

C: measurement of PCNA protein expression of cholangiocytes from 1) rats that, immediately after BDL, were fed control diet or 1% taurocholate for 1 wk, 2) rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate diet for 1 wk, and 3) rats that, immediately after BDL + vagotomy, were fed 1% taurocholate and subsequently were treated by intraperitoneal injections with wortmannin for 1 wk. Vagotomy decreased PCNA protein expression compared with cholangiocytes from BDL control rats. Taurocholate feeding prevented vagotomy-induced decrease in PCNA protein expression. Taurocholate prevention of vagotomy-induced inhibition of PCNA protein expression was blocked by wortmannin. *P < 0.05 vs. all the other groups. Data are means ± SE of at least 3 experiments.
tically different from those of cholangiocytes from BDL, vagotomized rats (Fig. 1D). Because the injection of Z-IETD-fmk into BDL, vagotomized rats and of U-0126 into the BDL, vagotomized, taurocholate-fed rats did not determine any change in apoptosis (Fig. 1, A–C), we did not evaluate the caspase activity in those groups.

TAUROCHOLATE PREVENTION OF VAGOTOMY-INHIBITION OF CHOLANGIOCYTE PROLIFERATION IS BLOCKED BY WORTMANNIN. After vagotomy, the number of PCNA- or CK-19-positive cholangiocytes decreased compared with liver sections from BDL rats fed control diet or 1% taurocholate for 1 wk (Fig. 2, A and B). Taurocholate feeding prevented the inhibitory effect of vagotomy on the number of PCNA- or CK-19-positive cholangiocytes, which was not statistically different from that of BDL control rats (Fig. 2, A and B). In purified cholangiocytes, vagotomy decreased PCNA protein expression compared with cholangiocytes from BDL rats fed control or 1% taurocholate for 1 wk (Fig. 2C). In agreement with previous studies (7), taurocholate feeding increased DNA synthesis compared with cholangiocytes from BDL control rats (Fig. 2C). Taurocholate feeding prevented vagotomy-induced decrease in PCNA protein expression, which was not statistically different from that of BDL control rats (Fig. 2C). Taurocholate feeding prevented vagotomy-induced inhibition of cholangiocyte PCNA protein expression was blocked by wortmannin (Fig. 2, A–C).

TAUROCHOLATE FEEDING PREVENTS VAGOTOMY-INHIBITION OF SECRETIN-STIMULATED DUCTAL SECRETION. Secretin stimulated bile flow, bicarbonate concentration, and secretion of BDI rats fed control diet or 1% taurocholate for 1 wk (Table 2). Vagotomy ablated the stimulatory effect of secretin on bile flow and bicarbonate concentration and secretion of BDI rats (Table 2). Feeding taurocholate to BDI, vagotomized rats prevented the vagotomy-induced inhibition of secretin-stimulated bicarbonate-rich choleretic, which was not statistically different from that of BDL control rats (Table 2). There was a significant decrease in SR gene expression in cholangiocytes from BDL, vagotomized compared with cholangiocytes from BDL rats fed control diet or 1% taurocholate for 1 wk (Fig. 3A). Feeding taurocholate to BDL, vagotomized rats prevented the vagotomy-induced inhibition of SR gene expression, which was not statistically different from that of BDL control rats (Fig. 3A). Secretin increased intracellular cAMP levels of cholangiocytes from BDL rats fed control diet or 1% taurocholate for 1 wk (Fig. 3B). Vagotomy significantly ($P < 0.05$) reduced basal cholangiocyte cAMP levels and inhibited secretin-stimulated cAMP levels of cholangiocytes compared with cholangiocytes from BDL rats fed control diet (Fig. 3B). Taurocholate feeding increased basal cAMP levels compared with cholangiocytes from BDL rats fed control diet (Fig. 3B). Feeding taurocholate to BDL, vagotomized rats prevented the inhibitory effect of vagotomy on basal and secretin-stimulated cAMP levels, which were not statistically different from those of cholangiocytes isolated from BDL rats (Fig. 3B).

TAUROCHOLATE PREVENTION OF VAGOTOMY-INHIBITED APOPTOSIS AND LOSS OF PROLIFERATION IS ASSOCIATED WITH INCREASED AKT PHOSPHORYLATION. In cholangiocytes from BDL, vagotomized rats, Akt phosphorylation decreased compared with phosphorylated Akt expression of cholangiocytes from BDL rats fed control diet or taurocholate (Fig. 4). After BDL, vagotomized rats were fed taurocholate, there was an increase in phosphorylated Akt, which was not statistically different from that of cholangiocytes from BDL rats fed control diet or taurocholate (Fig. 4). Feeding taurocholate to BDL, vagotomized rats prevented the inhibitory effect of vagotomy on basal and secretin-stimulated cAMP levels (Fig. 4). No changes in the Akt total protein expression were observed (Fig. 4).

Changes in cholangiocyte apoptosis, proliferation, secretion, and Akt phosphorylation are dependent on ABAT activity. We evaluated ABAT protein expression and bile acid transport activity in pure cholangiocytes from BDL rats fed control diet and BDL + vagotomy rats fed control diet or taurocholate for 1 wk. In agreement with previous studies (5), ABAT (with a band migrating at ~42 kDa) was expressed by cholangio-

<table>
<thead>
<tr>
<th>Table 2. Basal and secretin-stimulated bile flow, bicarbonate concentration, and secretion in rats that, immediately after BDI, were fed control diet or 1% taurocholate for 1 wk and rats that, immediately after BDI, underwent vagotomy and subsequently were fed control diet or 1% taurocholate for 1 wk</th>
<th>Bile Flow</th>
<th>Bicarbonate Concentration</th>
<th>Bicarbonate Secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Basal, μEq min⁻¹·kg body wt⁻¹</td>
<td>Secretin, μEq min⁻¹·kg body wt⁻¹</td>
<td>Basal, mEq/l</td>
</tr>
<tr>
<td>BDI + control feeding 1 wk</td>
<td>106.31 ± 3.12</td>
<td>155.64 ± 8.22*</td>
<td>38.79 ± 1.72</td>
</tr>
<tr>
<td>BDI + taurocholate 1 wk</td>
<td>111.50 ± 10.58</td>
<td>152.44 ± 17.70*</td>
<td>46.44 ± 3.10</td>
</tr>
<tr>
<td>BDI + control feeding + vagotomy 1 wk</td>
<td>98.55 ± 6.07</td>
<td>109.76 ± 7.53§</td>
<td>41.68 ± 3.55</td>
</tr>
<tr>
<td>BDI + taurocholate + vagotomy 1 wk</td>
<td>117.36 ± 8.75</td>
<td>144.39 ± 8.58*</td>
<td>35.63 ± 2.77</td>
</tr>
</tbody>
</table>

Data are means ± SE of 8–20 rats. *$P < 0.05$ vs. corresponding basal value of bile flow. †$P < 0.05$ vs. corresponding basal value of bicarbonate secretion. ‡$P < 0.05$ vs. corresponding basal value of control rats. §Not significant.
cytes from BDL rats fed control diet (Fig. 5A). Vagotomy induced a marked decrease in ABAT protein expression in purified cholangiocytes compared with cholangiocytes from BDL control rats (Fig. 5A). Feeding taurocholate to BDL, vagotomized rats prevented the inhibitory effect of vagotomy on ABAT protein expression, which was not statistically different from that of 1-wk-old BDL rats (Fig. 5A). Consistent with the concept that taurocholate effects on cholangiocyte functions are regulated by the PI3-kinase system, the in vivo administration of wortmannin to BDL, vagotomized rats blocked the protective effects of taurocholate against vagotomy inhibition of cholangiocyte ABAT protein expression (Fig. 5A).

Na+-dependent ABAT transport activity (the $V_{\text{max}}$) determined from $[3H]$taurocholate uptake) was decreased in cholangiocytes from BDL + vagotomy rats compared with cholangiocytes from BDL control rats (Fig. 5B). Na+-dependent cholangiocyte ABAT bile acid transport activity in taurocholate fed BDL + vagotomy rats was not statistically different from that of cholangiocytes from 1-wk-old BDL rats (Fig. 5B). Consistent with vagotomy and bile acid feeding alternating ABAT content rather than ABAT transport activity, the $K_m$ for $[3H]$taurocholic acid uptake in cholangiocytes was similar in BDL, BDL + vagotomy, and taurocholate-fed BDL + vagotomy rats (data not shown).

To directly evaluate the role of ABAT and PI3-kinase in taurocholate regulation of cholangiocyte proliferation, rats that, immediately after BDL, were fed control diet for 1 wk, and rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate diet for 1 wk were treated in vivo with taurocholate in the absence or presence of wortmannin. Subsequently, we isolated pure cholangiocytes from these groups of animals and evaluated, by immunoblots, cholangiocyte PCNA protein expression (an index of proliferation), total Akt protein expression, and Akt (Ser473) phosphorylation. We anticipated that cholangiocytes isolated from BDL, vagotomized rats (expressing less ABAT) respond minimally to taurocholate, whereas cholangiocytes isolated from BDL rats fed control diet or taurocholate, and from BDL, vagotomized, taurocholate-fed rats (expressing more ABAT), show a higher response to taurocholate. Taurocholate increased in vitro PCNA protein expres-
Fig. 4. Measurement of phosphorylated Akt protein expression in cholangiocytes from 1) rats that, immediately after BDL, were fed control diet or 1% taurocholate for 1 wk; 2) rats that, immediately after BDL, underwent vagotomy and subsequently were fed control diet or 1% taurocholate diet for 1 wk; and 3) rats that, immediately after BDL + vagotomy, were fed 1% taurocholate and subsequently were treated by intraperitoneal injections with wortmannin for 1 wk. In cholangiocytes from BDL, vagotomized rats, the phosphorylation of Akt (measured as ratio to β-actin) decreased compared with the phosphorylation of Akt of cholangiocytes isolated from BDL rats fed control diet or taurocholate. After feeding taurocholate to BDL, vagotomized rats, there was an increase in phosphorylated Akt, which was not statistically different from that of cholangiocytes from BDL rats fed control diet or taurocholate. In vivo administration of wortmannin blocked taurocholate prevention of vagotomy inhibition of phosphorylated Akt. No changes in the total Akt protein expression were observed. *P < 0.05 vs. all the other groups. Data are means ± SE of 17 experiments.

Fig. 5. Measurement of apically located Na⁺-dependent bile acid transporter (ABAT) protein expression (A) and Na⁺-dependent ABAT transport activity (B) in pure cholangiocytes from BDL control rats and BDL rats that, immediately after BDL, underwent vagotomy + control or taurocholate feeding. A: vagotomy induced a decrease in ABAT protein expression in cholangiocytes compared with cholangiocytes from BDL rats. Feeding taurocholate to BDL, vagotomized rats stimulated Na⁺-dependent cholangiocyte ABAT bile acid transport activity, which was not statistically different from that of cholangiocytes from BDL rats. *P < 0.05 vs. all the other groups. Data are means ± SE of 3 experiments.

sion of isolated cholangiocytes from BDL rats fed control diet (Fig. 6A) and BDL, vagotomized taurocholate-fed rats (Fig. 6C), whereas PCNA protein expression of cholangiocytes isolated from BDL, vagotomized rats (fed control diet) was not affected by in vitro taurocholate treatment (Fig. 6B). Taurocholate increase in PCNA protein expression of cholangiocytes from BDL control rats and BDL + vagotomy rats fed taurocholate was blocked by the PI3-kinase inhibitor wortmannin (Fig. 6, A and C), which parallels our previous studies (3, 33). Wortmannin alone did not change the effect of taurocholate on PCNA protein expression of purified cholangiocytes (Fig. 6, A-C). The dependence of taurocholate effects on ABAT and PI3-kinase was further demonstrated by the fact that when purified cholangiocytes were treated in vitro with 20 μM taurocholate, we found increased Akt phosphorylation in cholangiocytes expressing higher levels of ABAT, e.g., isolated from BDL rats fed control diet for 1 wk (Fig. 7A) and BDL,
vagotomized rats fed taurocholate for 1 wk (Fig. 7C). Such an effect, however, was prevented by the preincubation of cholangiocytes with the PI3-kinase inhibitor wortmannin (Fig. 7, A and C). To the contrary, in vitro exposure to taurocholate did not increase Akt phosphorylation in cholangiocytes purified from BDL, vagotomized rats fed control diet, which minimally express ABAT (Fig. 7B). The effect of taurocholate on cholangiocyte PI3-kinase and proliferation was found to be mediated by the cAMP/PKA pathway, because both the taurocholate-induced increases in Akt phosphorylation and proliferation were absent in the presence of the PKA inhibitor Rp-cAMP (Figs. 6 and 7).

**DISCUSSION**

The study demonstrates that, in the BDL rat, taurocholate feeding 1) prevents cholangiocyte apoptosis due to vagotomy (28); 2) prevents vagotomy-induced inhibition of cholangiocyte proliferation and secretin-stimulated ductal secretion (28); 3) prevents vagotomy-induced inhibition of PI3-kinase and activation of caspase 8, 9, and 3 activities; and 4) prevents vagotomy-induced inhibition of ABAT protein expression and bile acid transport activity. Taurocholate prevention of vagotomy-induced apoptosis was regulated by the PI3-kinase but not by the MEK pathway, because wortmannin (but not U-0126) blocked taurocholate protective effects against vagotomy-induced cholangiocyte apoptosis. Consistent with the concept that vagotomy-induced apoptosis is mediated by the intrinsic rather than the extrinsic pathway, the caspase 8 inhibitor Z-IETD-fmk did not alter vagotomy-induced increase in cholangiocyte apoptosis. Taurocholate prevention of vagotomy inhibition of cholangiocyte proliferation was regulated by the PI3-kinase, since wortmannin blocked taurocholate protective effects against vagotomy inhibition of cholangiocyte proliferation. The modulatory effect of bile salts on cholangiocyte proliferation and secretion correlates with the protein expression and functional activity of ABAT (3, 7, 10), the transporter localized in the apical pole of cholangio-

Fig. 6. Measurement of basal and taurocholate-stimulated PCNA protein expression in cholangiocytes that, after the purification from BDL rats fed control diet for 1 wk (A) and BDL rats that, immediately after BDL, underwent vagotomy and control (B) or taurocholate feeding (C), were treated in vitro at 37°C with 0.2% BSA (basal) for 2 h, taurocholate (20 μM) for 2 h, wortmannin (100 nM), or Rp-cAMP (100 μM) for 20 min before treatment with taurocholate (20 μM) for 2 h or intravenous wortmannin (100 nM) or Rp-cAMP (100 μM) for 20 min. Taurocholate stimulated PCNA protein expression of isolated cholangiocytes from BDL rats fed control diet (A) or taurocholate and BDL, vagotomized taurocholate-fed rats (C), whereas PCNA of cholangiocytes from BDL, vagotomized rats was not affected by taurocholate treatment (B). Taurocholate increase in PCNA protein expression of cholangiocytes from BDL control or taurocholate-fed rats and BDL + vagotomy rats fed taurocholate was blocked by wortmannin and by Rp-cAMP (A and C). Wortmannin and Rp-cAMP alone did not change the effect of taurocholate on PCNA protein expression of purified cholangiocytes (A–C). *P < 0.05 vs. the other groups. Values are expressed as % increase compared with unstimulated cells. Data are means ± SE of 9 experiments.
cytes, and is responsible for bile salt entrance into cholangiocytes (5, 27).

We measured biliary bile acid concentration and composition and showed that vagotomy effects on cholangiocyte functions and taurocholate prevention of vagotomy effects on cholangiocyte functions are associated with altered bile acid composition. We found that after vagotomy, the biliary concentration of taurocholate decreases compared with BDL rats, whereas feeding taurocholate to BDL + vagotomy rats increased biliary concentration of taurocholate.

Intracellular accumulation of bile acids may induce apoptosis and liver injury (40). Not all the bile acids, however, are toxic. For example, some bile acids protect hepatocytes (40, 45, 49) from apoptosis. Taurocholate prevents the CCl4-induced apoptosis of cholangiocytes through phosphorylation of Akt (33). Bile acids accumulate in bile during cholestasis, thus representing a possible trigger for cholangiocyte proliferation (8, 10). In support of this concept, previous studies have shown that bile acids directly stimulate ductal proliferation (4, 7, 10). The current study extends this concept by showing that taurocholate is also able to sustain proliferation in a condition characterized by bile duct loss because of the lack of cholinergic innervation. Together, the reduced apoptosis and the increased proliferation, due to taurocholate feeding, maintained bile duct mass at levels not statistically different from those of the BDL rat (18). The importance of this protective effect is also demonstrated by our finding that, after taurocholate feeding, cholangiocytes maintain their functional activity, i.e., the enhanced ductal secretory response to secretin, SR gene expression, and increased basal and secretin-stimulated intracellular cAMP levels, which are lost in the vagotomized rats (28).

Because bile acids exert their functions in hepatocytes through the activation of the PI3-kinase (35, 45, 52), we evaluated the role of its transduction pathway in taurocholate prevention of vagotomy-induced damage of cholangiocytes. We found that the simultaneous administration of the PI3-kinase inhibitor wortmannin...
blocked the protective effect of taurocholate on the vagotony-induced apoptosis and inhibition of proliferation. Consistently, we also observed that vagotomy resulted in diminished phosphorylation of Akt [an immediate downstream of PI3-kinase (21, 42)], which was prevented by taurocholate feeding. The simultaneous administration of wortmannin, however, abolished the increase in Akt phosphorylation observed after taurocholate feeding. Therefore, our study provides evidence that the activation of the PI3-kinase/Akt pathway triggers, similar to other cells (19, 26, 37), an antiapoptotic and proproliferative signaling in cholangiocytes.

A number of studies has shown that changes in the PI3-kinase/Akt cascade modulate the activity of caspases (20, 45). For instance, the monocyte survival factor stimulation of PI3-kinase/Akt pathway reduces caspase 8, 9, and 3 activation (20, 48). Renal tubular cell injury by cisplatinum is associated with an enhanced phosphorylation of Akt, inhibition of which enhances activation of caspase 3 and caspase 9 (25). In accordance with these previous studies, we show that vagotomy reduces PI3-kinase/Akt and enhances the caspase activity. In addition, we demonstrated that the vagotomy-induced apoptosis of cholangiocytes likely occurs through the mitochondrial or intrinsic pathway (47, 54), because we did not observe any changes in the number of apoptotic cholangiocytes after vagotomy in the presence of the caspase 8 inhibitor Z-IETD-fmk. The extrinsic pathway, in fact, has an essential upstream event: the activation of caspase 8 after ligands bind to death receptors (54). Thus the absence of changes in apoptosis after the inhibition of caspase 8 suggests that the vagotomy-induced apoptosis is likely to happen through the intrinsic rather than the extrinsic pathway of apoptosis. Moreover, the intrinsic pathway is involved in the induction of apoptosis by stress signals, among which hormone or growth factor withdrawal are considered (47, 54). Therefore, the finding that the vagotomy-induced apoptosis is mediated through the intrinsic pathway is in accordance with the concept that the deprivation of cholinergic innervation represents the loss of an important element for the growth of the biliary tree (28). The intrinsic pathway leads to apoptosis through the release of the cytochrome c from the mitochondria and the subsequent activation of caspase 9 and caspase 3 but not caspase 8 (47, 54). In our study, in fact, caspase 9 and caspase 3 were found significantly increased in cholangiocytes after the vagotomy of the BDL rat, compared with cholangiocytes from BDL rats treated with control or 1% taurocholate diet. Surprisingly, in these cells, an upregulated activity of caspase 8 was also observed. If our data do not provide any direct explanation for this phenomenon, they suggest that the vagotomy-induced triggering of the intrinsic pathway of apoptosis may directly or indirectly activate caspase 8 in the same fashion as what has been previously shown in other cells (47). Taurocholate feeding prevented the increase of the caspase activity due to vagotomy, an effect that was abolished by the simultaneous administration of the PI3-kinase inhibitor wortmannin. These data suggest that taurocholate, by sustaining the activation of the PI3-kinase/Akt cascade, reduces the activation of caspases, thus preventing the vagotomy-induced apoptosis. Similar findings have been described in hepatocytes (40, 45), in which taurochenodeoxycholate prevents apoptosis through the activation of PI3-kinase and the consequent inhibition of the caspase activity (45, 49). In hepatocytes, however, the protective effect of PI3-kinase occurs without activation of Akt but requires recruitment of one of the atypical PKC isoforms, PKCζ (45). The discrepancy between this (45) and our present results is likely due to different mechanisms for regulation of apoptosis in cholangiocytes and hepatocytes. Recently, it has been shown that the antiapoptotic message of the bile acid-activated PI3-kinase is mediated, at least in part, by the MAP-kinase cascade (43, 44). Therefore, we evaluated the role of the MAP-kinase cascade in the taurocholate protection of vagotony-induced bile duct damage, treating BDL, vagotomized and taurocholate-fed animals with the MAP-kinase inhibitor U-0126. However, the presence of U-0126 did not diminish the protective effect of taurocholate on the vagotony-induced apoptosis. The MAP-kinase activation by bile acids is secondary to the interaction of the bile acid itself with the epidermal growth factor (EGF) receptor (43, 44). Thus the fact that the effects of taurocholate seem not to depend on the MAP-kinase cascade might be ascribed to the lack of interaction of this bile acid with the EGF receptor in cholangiocytes. Further studies to elucidate the role of the links among bile acids, EGF receptor, and the MAP kinases in cholangiocytes are necessary.

To clarify the intracellular mechanisms by which taurocholate prevented the effects of vagotomy on cholangiocyte apoptosis, proliferation, and secretion, we studied the changes in ABAT protein expression and cholangiocyte bile acid transport activity. The rationale for these studies is based on the fact that bile acids, to exert their effects on cholangiocytes, must be internalized through ABAT (5, 27). Thus we anticipated that the effects of bile acids on cholangiocyte proliferation are determined by ABAT expression and bile acid transport activity in cholangiocytes. First, we observed, for the first time, that the bile acid transport in the biliary epithelium is also controlled by cholinergic innervation, because both the expression and the activity of ABAT were reduced after vagotomy. The impaired ABAT protein expression and activity in cholangiocytes could be the consequence of reduced biliary bile salt concentration caused by vagotomy. Chronic taurocholate feeding, in contrast, prevents the loss of ABAT. In a fashion similar to what has been previously shown in the ileum (22), the apical uptake of bile acids by cholangiocytes increased in proportion to bile acid luminal concentration through direct regulation of ABAT gene and protein expression (10). Consistent with the concept that taurocholate effects are PI3-kinase dependent, the restoration of ABAT protein expression by this bile acid was abolished in the presence of wortmannin. For further confirmation of the key role played by ABAT, we have also shown that in...
vitro exposure of cholangiocytes to taurocholate strongly increased proliferation in cells expressing higher levels of ABAT (i.e., cholangiocytes from BDL rats and BDL, vagotomized, taurocholate-fed rats) compared with cholangiocytes from vagotomized rats, which express low levels of ABAT. This effect also was PI3-kinase dependent, because the preincubation with wortmannin prevented the increase of cholangiocyte proliferation induced by taurocholate. Other important evidence of the link among ABAT, taurocholate, and PI3-kinase is that the in vitro exposure to taurocholate induced a significant increase of Akt phosphorylation in cholangiocytes with a higher expression and activity of ABAT (e.g., purified from BDL rats or rats subjected to BDL, vagotomy, and taurocholate feeding) but not in cholangiocytes with low ABAT expression and activity (e.g., purified from rats subjected to BDL and vagotomy). The changes in Akt phosphorylation after the in vitro exposure to taurocholate were PI3-kinase dependent, because they were abolished by the preincubation with wortmannin. Hence, the loss of ABAT expression and activity observed after vagotomy might determine the lack of cytoprotective effects by bile acids. When ABAT expression is restored, the presence of high concentrations of cytoprotective bile acids (taurocholate in the current study) prevents vagotomy-induced cholangiocyte damage. The novelty of these data is based on the demonstration of the existence of a dual relationship between bile acids and their apical transporter. On one side, we have the modulation (PI3-kinase mediated) of ABAT expression and activity by bile acids; on the other, we have the dependency of bile acid effects on ABAT expression. These findings candidates ABAT as a possible major regulator of cholangiocyte proliferation/loss in cholestatic liver diseases in response to injury/toxins. In support of this concept, we have shown (3) that depletion of endogenous bile acids, by prolonged external bile drainage, leads to downregulation of ABAT and decreased cholangiocyte proliferation and secretin-stimulated ductal secretion, whereas feeding taurocholate to normal rats increases ABAT expression and bile acid transport activity that leads to enhanced cholangiocyte proliferation and secretin-stimulated ductal secretion (10).

Our findings also indicate that taurocholate and cholinergic innervation cooperatively modulate the PI3-kinase/Akt survival pathway by regulating cholangiocyte intracellular cAMP levels. We have previously shown (28) that maintenance of cAMP levels by forskolin administration counteracts the effects of vagotomy on cholangiocyte apoptosis, proliferation, and secretion. In the current study, taurocholate feeding determined a restoration of both basal and secretin-stimulated cAMP synthesis (at levels not statistically different from those of cholangiocytes from BDL rats), thus preventing the effects of vagotomy. In our studies, intracellular cAMP levels change in parallel with PI3-kinase/Akt activity. In fact, after vagotomy, decreased cAMP levels were coupled with diminished activation of the PI3-kinase/Akt pathway, followed by increased caspase activity, increased apoptosis, and diminished proliferation. In contrast, when vagotomized rats were fed with taurocholate, increased cAMP levels were associated with enhanced PI3-kinase/Akt activation, diminished caspase activity and apoptosis, and enhanced proliferation. The fundamental role of cAMP in mediating the effects of taurocholate is also demonstrated by the finding that the increase of cholangiocyte proliferation induced by the in vitro exposure to taurocholate is abolished when PKA [known to be an immediate downstream of cAMP (12, 16)] is inhibited by Rp-cAMP. In addition, the preincubation with Rp-cAMP also blocked the taurocholate-induced increase of PI3-kinase activity, as testified by the lack of the enhancement of Akt phosphorylation observed after the in vitro exposure to taurocholate. Our data are in accordance with previous studies in hepatocytes (52, 53), in which cAMP has been found to exert prosurvival effects through the modulation of the PI3-kinase-signalling pathway. Therefore, the modulation of cAMP synthesis appears to be the intracellular step at which the cholinergic and taurocholate pathways may merge.

The proposed intracellular pathway that allows taurocholate to exert its protective effect against vagotomy-induced bile duct damage is shown in Fig. 8. Taurocholate is internalized in cholangiocytes through ABAT; once inside the cell, taurocholate enhances the cAMP synthesis, which is responsible for the increased PI3-kinase activity. PI3-kinase stimulates proliferation and inhibits the caspase activity, thus leading to diminished apoptosis. Taurocholate/cAMP-stimulated PI3-kinase is also responsible for changes in the expression of the ABAT protein, which represents the last step in a circle of events that are positively regulated by the chronic exposure to high levels of taurocholate. This pathway seems instead to be downregulated by vagotomy.

Fig. 8. Working model related to the intracellular pathway that allows taurocholate to exert its protective effect against vagotomy-induced bile duct damage. Taurocholate is internalized in cholangiocytes through ABAT; once inside the cell, taurocholate enhances the cAMP synthesis, which is responsible for the increased PI3-kinase activity. PI3-kinase stimulates proliferation and inhibits the caspase activity, thus leading to diminished apoptosis. Taurocholate/cAMP-stimulated PI3-kinase is also responsible for changes in the expression of the ABAT protein, which represents the last step in a circle of events that are positively regulated by the chronic exposure to high levels of taurocholate. This pathway seems instead to be downregulated by vagotomy.
lated by the chronic exposure to high levels of taurocholate.

Previous studies (1, 10, 11) have observed how both bile acids and acetylcholine are able to modulate the intracellular Ca$^{2+}$/release in cholangiocytes. Thus the prevention of vagotomy effects by taurocholate could involve also the Ca$^{2+}$/dependent PKC signaling, likely as an upstream regulator of the cAMP synthesis.

These findings have important implications for the pathophysiology of the transplanted (denervated) liver (8), in which ischemic (39) or infectious (23) insults against intrahepatic bile ducts may not be adequately counteracted, during the immediate posttransplant period. With an inadequate repair mechanism due to the lack of cholinergic modulation of cholangiocyte proliferation, bile duct loss ensues (28). Supporting this hypothesis, biliary complications after liver transplant occur most often during the first 3 mo while reinnervation of the transplanted liver is occurring.

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