Taurocholate feeding prevents CCl₄-induced damage of large cholangiocytes through PI3-kinase-dependent mechanism

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¹Department of Internal Medicine and ²Medical Physiology, ³Division of Research and Education, Scott & White Hospital and The Texas A&M University System Health Science Center, College of Medicine and ⁴Central Texas Veterans Health Care System, Temple, Texas 76504; ⁵Division of Gastroenterology, University of Rome, La Sapienza, Rome, Italy 00100; and ⁶Department of Gastroenterology, University of Ancona, Ancona, Italy 60131

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Marucci, Luca, Gianfranco Alpini, Shannon S. Glaser, Domenico Alvaro, Antonio Benedetti, Heather Francis, Jo Lynne Phinizy, Marco Marzioni, Jeremy Mauldin, Julie Venter, Brandy Baumann, Laura Ugili, and Gene LeSage. Taurocholate feeding prevents CCl₄-induced damage of large cholangiocytes through PI3-kinase-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 284: G290–G301, 2003. First published October 2, 2002; 10.1152/ajpgi.00245.2002.—Bile acids are cytoprotective in hepatocytes by activating phosphatidylinositol-3-kinase (PI3-K) and its downstream signal AKT. Our aim was to determine whether feeding taurocholate to CCl₄-treated rats reduces cholangiocyte apoptosis and whether this cytoprotective effect is dependent on PI3-K. Cholangiocyte proliferation, secretion, and apoptosis were determined in cholangiocytes from bile duct ligation (BDL), CCl₄-treated BDL rats, and CCl₄-treated taurocholate-fed rats. In vitro, we tested whether CCl₄ induces apoptosis and whether loss of cholangiocyte proliferation and secretion is dependent on PI3-K. The CCl₄-induced cholangiocyte apoptosis and loss of cholangiocyte proliferation and secretion were reduced in CCl₄-treated rats fed taurocholate. CCl₄-induced cholangiocyte apoptosis, loss of cholangiocytes secretion, and proliferation were prevented by preincubation with taurocholate. Taurocholate cytoprotective effects were ablated by wortmannin. Taurocholate prevented, in vitro, CCl₄-induced decrease of phosphorylated AKT protein expression in cholangiocytes. The cytoprotective effects of taurocholate on CCl₄ effects on cholangiocyte proliferation and secretion were abolished by wortmannin. Taurocholate prevents cholangiocytes from CCl₄-induced apoptosis by a PI3-K-dependent mechanism. Bile acids are important in the prevention of drug-induced ductopenia in cholangiopathies.

Taurocholate feeding prevents CCl₄-induced damage of large cholangiocytes through PI3-kinase-dependent mechanism extending from the ducts of Hering to the septal bile ducts (9, 26). Cholangiocytes participate in hormone-regulated ductal secretion (8, 33, 35). Secretin stimulates ductal secretion (8, 33, 35) by interaction with basolateral secretin receptors [expressed only by cholangiocytes in rat liver (13)] through an increase in cAMP levels (6, 10, 12, 27, 33, 35). In the normal state, cholangiocytes are mitotically dormant (6, 26, 33) but proliferate in response to liver injury/toxins including bile duct ligation (BDL) (8, 22) or acute carbon tetra-chloride (CCl₄) administration (35). In these models of ductal hyperplasia, cholangiocyte proliferation is associated with changes in basal and secretin-stimulated ductal secretion (8, 22, 26, 35). In BDL rats, cholangiocyte proliferation is associated with an increase in basal and secretin-stimulated ductal secretion, whereas in rats with ductopenia (after acute CCl₄ administration), reduced cholangiocyte proliferation is coupled with inhibition of secretin-stimulated ductal secretion (8, 22, 26, 35). Thus the choleretic or lack of choleretic response to secretin may be an index to functionally evaluate cholangiocyte hyperplasia (6–9, 22, 33) or ductopenia (9, 35), respectively.

We have developed techniques for isolating subpopulations of small and large cholangiocytes (6, 10, 12, 35) and intrahepatic bile duct units (4) from different portions of the rat intrahepatic biliary epithelium and have shown that cholangiocytes are morphologically and functionally heterogeneous with secretin-stimulated ductal secretion restricted to large cholangiocytes (lining large ducts) but not small cholangiocytes (lining small bile ducts) (4, 6, 10, 12, 35). In BDL rats, cholangiocyte proliferation and increased ductal secretion occur in large but not small cholangiocytes (6, 26). A single dose of CCl₄ to BDL rats induces a transient cholangiocyte loss (by apoptosis) only in large ducts, an...
event associated with a reduction of proliferative and secretory activity in large cholangiocytes (35). In contrast, small cholangiocytes are resistant to apoptosis and, de novo, proliferate and secrete in response to CCl4 compensating for loss of large cholangiocyte function (35). The different sensitivity of small and large cholangiocytes to CCl4 toxicity may be due to the expression of the cytochrome P-4502E1 (the enzyme that initiates CCl4 hepatotoxicity) (15) in large but not small cholangiocytes (35). This unique animal model presents several features common to human pathology (9). In fact, loss and/or damage of large interlobular ducts by degenerative or inflammatory processes is a major factor in the pathogenesis of vanishing bile duct syndromes [e.g., primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC)] (9). Furthermore, several studies suggest that apoptosis is an important mechanism of cholangiocyte death resulting in ductopenia (35, 39).

Bile acids are internalized into cholangiocytes by the apically located Na+-dependent bile acid transporter (ABAT) (5, 28) thus modulating cholangiocyte secretion and growth (3, 7). In vitro, taurocholate increased DNA synthesis, and secretin-stimulated cAMP levels in cholangiocytes (3). Taurocholate uptake into cholangiocytes was required for taurocholate stimulation of growth and secretion (3). Chronic in vivo feeding of taurocholate to normal rats increased secretin-stimulated ductal secretion and cholangiocyte proliferation (7).

The regulatory protein phosphatidylinositol-3-kinase (PI3-K) is involved in different signaling pathways and controls key functions of a number of cells (17, 36). PI3-K is considered one of the main intracellular factors responsible for the transmission of antipapoptotic signals and controlling the survival of cells (19, 24). For instance, overexpression of PI3-K in cells is accompanied by a marked antipapoptotic effect and a significant increase in cell survival following radiation (19, 24). PI3-K have also been implicated in controlling DNA synthesis and cell cycle (1). A number of studies has shown that bile acids may affect PI3-K activity (36, 42, 46). The hydrophobic bile acid taurochenodeoxycholate activates PI3-K-dependent survival pathways in hepatocytes, which prevent its inherent toxicity (42). In addition, cAMP-mediated cytoprotection against bile acid-induced apoptosis in cultured rat hepatocytes appears to involve PI3-K (46). In view of these findings, we tested the hypothesis that in BDL rats, taurocholate, both in vivo (by chronic feeding) and in vitro, prevents the CCl4-induced damage of large cholangiocyte functions through a PI3-K-dependent pathway.

MATERIALS AND METHODS

Materials

Reagents were purchased from Sigma (St Louis, MO) unless otherwise indicated. Control chow (i.e., AIN 76) and chow containing 1% taurocholate with AIN 76 were prepared from Dyets (Bethlehem, PA). Control chow (AIN 76) has the same composition of rat chow containing 1% taurocholate but it does not contain taurocholate. Porcine secretin was purchased from Peninsula Laboratories (Belmont, CA). RIA kits for the determination of intracellular cAMP levels were purchased from Amersham (Arlington Heights, IL). The mouse anti-cytkeratin 19 (CK-19) antibody was purchased from Amersham. The monoclonal mouse antibody against PCNA was purchased from DAKO (Kyoto, Japan). The monoclonal antibody (C-20) against total Akt was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The affinity-purified goat polyclonal antibody mapping at the COOH terminus of H3 histone of human origin was purchased from Santa Cruz Biotechnology. The monoclonal antibody against phosphorylated Akt (phosho-Akt, Ser473, 4E2) was purchased from Cell Signaling Technology (Beverly, MA). The nuclear dye 4,6-diamidino-2-phenylindole (DAPI) was purchased from Molecular Probes (Eugene, OR). A biotinylated antibody reacting with annexin-V was purchased from Boehringer-Mannheim (Mannheim, Germany).

Animal Model

Male Fisher 344 rats (150–175 g) were purchased from Charles River (Wilmington, MA), maintained in a temperature-controlled environment (22°C) with a 12:12-h light-dark cycle, and fed ad libitum standard rat chow. Studies were performed in 1) rats with BDL or bile duct incannulation (BDI; for bile collection (8)) for 9 days (controls); and 2) 1 wk BDL or BDI rats, which after the intragastric administration of a single dose of CCl4/mineral oil 1:1 (0.5 ml/100 g body wt), were fed control diet (AIN 76) or 1% taurocholate for 2 days for a total of 9 days of BDL or BDI. We omitted the group of BDL or BDI rats treated with mineral oil (0.5 ml/100 g body wt), because in previous studies, we have shown (35) that administration of a single dose of mineral oil (0.5 ml/100 g body wt) does not alter cholangiocyte proliferation and secretion. The animals were killed on day 2 after CCl4 administration plus taurocholate or control diet. BDL or BDI was performed as described (8). Before each experimental procedure, the animals were anesthetized with pentobarbital sodium (50 mg/kg weight ip).

Cholangiocyte Purification

Pure subpopulations of small and large cholangiocytes were purified from the selected group of animals as described (4, 6, 10) using a mouse monoclonal antibody against an unidentified membrane antigen expressed by all rat intrahepatic cholangiocytes (25). Cell number and viability were assessed by trypan blue exclusion. Cholangiocyte purity was assessed by histochemistry for γ-GT (43).

Studies in BDL Rats Treated in Vivo With CCl4 Plus Taurocholate or Control Feeding

Measurement of transaminase serum levels. We evaluated the effect of taurocholate or control feeding on CCl4-induced liver damage assessed by transaminase serum level determination. In BDL rats and 1-wk-old BDL rats treated with CCl4 and subsequently fed 1% taurocholate or control diet, the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by commercially available kits (Sigma).

In Situ Morphological Studies and TUNEL Analysis

After anesthesia, small pieces of liver tissue were taken from each animal and fixed in 10% formalin (pH 7.4). Sections (5-μm thick) were cut from two liver blocks for each animal, stained for hematoxylin and eosin, and examined under a light microscope equipped with a reticular frame.
inserted in the ocular lens (35). An in situ cell death detection kit (Boehringer-Mannheim, Monza, Italy) was also used. The kit (previously used by us for determining cholangiocyte apoptosis) (35) was used according to the instructions of the vendor. In each liver section, the number of apoptotic bodies present in cholangiocytes and the diameter (distance between basement membranes) of the bile duct (in which apoptosis was observed) were evaluated. In each liver section, at least 50 nonoverlapping fields were analyzed. The incidence of apoptosis was expressed as number of apoptotic bodies per 100 cholangiocytes.

Measurement of Caspase 8, 9, and 3 Activities

Intracellular activity of caspase 8, 9, and 3 of small and large cholangiocytes from the selected groups of animals was measured by enzymatic kits according to the instructions supplied by the vendor (Medical & Biological Laboratories, Nagoya, Japan). After isolation, cholangiocytes were centrifuged at 1,500 rpm for 10 min, incubated in lysis buffer on ice for 10 min, and centrifuged at 10,000 g for 10 min. After the supernatant was centrifuged, that containing the cytosolic fraction, was transferred to a clean tube. For each sample, supernatant was centrifuged, that containing the cytosolic fraction, was transferred to a clean tube. For each sample, 100 μg of proteins or BSA (negative control) were added to 50 μl of 2X reaction buffer. The activity of caspase 8, 9, and 3 was measured by proteolytic cleavage of substrates such as DEVD-p-nitroanilide (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 pNA after cleavage from the substrates. The pNA light emission was quantified using a microtiter plate reader at 406 nm. Values are expressed as fold increase compared with negative control.

Assessment of Cholangiocyte Proliferation

In situ morphometric analysis of bile duct mass. In coded frozen liver sections (n = 6 for each group) from the selected group of animals, intrahepatic mass of large ducts was evaluated by point count analysis (33, 48) by determining the number of ducts stained for CK-19, a specific marker for rat cholangiocytes (33). Immunohistochemistry for CK-19 was performed as described (33). After sections were stained, they were counterstained with hematoxylin and examined in a coded fashion with a microscope (Olympus BX 40, Olympus Optical, Tokyo, Japan). The duct diameter was estimated from the distance between basement membranes. The data were expressed for each duct as the mean of two diameters taken perpendicular to each other.

Assessment of Ductal Secretion

Bile flow. After anesthesia, BDI rats were surgically prepared for bile collection (8). When steady-state bile flow was achieved, secretin (100 nM) was infused for 30 min followed by a final infusion of Krebs-Ringer Henseleit for 60 min. Bile was collected at 10-min intervals, placed in preweighed tubes and immediately stored at −70°C before determining bicarbonate concentration. Biliary bicarbonate concentration (measured as total CO₂) was determined by a Natelson microgasometer apparatus (Scientific Industries, Bohemia, NY).

Intracellular cAMP levels. Small and large cholangiocytes were incubated for 1 h at 37°C to restore surface proteins damaged by treatment with proteolytic enzymes (27) and subsequently stimulated with 0.2% BSA (basal) or secretin (100 nM) in the presence of 0.2% BSA for 5 min at 22°C (6, 10, 22, 27, 33, 35, 45). Following ethanol extraction, cAMP levels were measured by RIA using a commercially available kit (Amersham).

In Vitro Studies in Large Cholangiocytes from BDL Rats

PI3-K regulation of apoptosis, proliferation and secretion. To evaluate the intracellular mechanisms by which taurocholate protects against CCl₄-induced damage of large cholangiocytes, we evaluated in vitro the effect of taurocholate on CCl₄-induced apoptosis (assessed by annexin-V (a specific marker of early stage apoptosis) (23) or DAPI staining (29, 32, 35)), proliferation (measured by both H3 histone and PCNA protein expression) (32), and secretory capacity (by measurement of cAMP levels) (4, 6, 10–12, 22, 29, 32, 35, 45) of large cholangiocytes in the presence or absence of wortmannin, a specific PI3-K inhibitor (36). Because downstream effectors of PI3-K-dependent cell survival include Akt (which may inactivate Bad phosphorylation and upregulate Bel-2 by transcriptional activation (38)), we evaluated whether taurocholate protection of CCl₄-induced damage of large ducts (35) was associated with increased protein expression of phosphorylated Akt. The comparability of the protein used was assessed by immunoblots for β-actin (11). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low-light imaging system (Alpha Innotech, San Leandro, CA).

Large cholangiocytes from BDL rats were treated at 37°C with 1) 0.2% BSA (basal) for 2 h; 2) 5 μM CCl₄ for 2 h with 0.2% BSA; 3) taurocholate (20 μM for 2 h) plus CCl₄ (5 μM for 2 h) with 0.2% BSA; 4) wortmannin (100 nM for 1 h) plus taurocholate (20 μM for 2 h) plus CCl₄ (5 μM for 2 h) with 0.2% BSA; or 5) wortmannin (100 nM for 1 h) with 0.2% BSA. Cholangiocyte apoptosis was determined by observation of nuclear fragmentation by staining with both annexin-V and DAPI as described by LeSage et al. (35) and Que et al. (39). In this set of cells, we also evaluated cholangiocyte proliferation by measurement of both H3 histone and PCNA protein expression (32). In separate sets of experiments, large cholangiocytes were incubated for 1 h at 37°C to restore surface proteins damaged by treatment with proteolytic enzymes (27) and subsequently were treated at room temperature with 1) 0.2% BSA (basal) for 5 min; 2) taurocholate (20 μM for 10 min, positive control) with 0.2% BSA; 3) 5 μM CCl₄ for 10 min in the absence or presence of secretin (100 nM for 5 min) with 0.2% BSA; 4) taurocholate (20 μM for 10 min)
plus CCl₄ (5 μM for 10 min) in the absence or presence of secretin (100 nM for 5 min) with 0.2% BSA; 5) wortmannin (100 nM for 10 min) plus CCl₄ (5 μM for 10 min) in the absence or presence of secretin (100 nM for 5 min) with 0.2% BSA; or 6) wortmannin (100 nM for 10 min) in the absence or presence of CCl₄ (5 μM for 10 min) or taurocholate (20 μM for 10 min) with 0.2% BSA. Intracellular cAMP levels were measured by RIA (6, 10, 22, 27, 33, 35, 45).

Total and phosphorylated AKT protein expression was evaluated by immunobots in large cholangiocytes from BDL rats treated at 37°C with 1) 0.2% BSA (basal) for 2 h; 2) 5 μM CCl₄ for 2 h with 0.2% BSA; 3) taurocholate (20 μM for 2 h) plus CCl₄ (5 μM for 2 h) with 0.2% BSA; 4) wortmannin (100 nM for 1 h) plus taurocholate (20 μM for 2 h) plus CCl₄ (5 μM for 2 h) with 0.2% BSA; or 5) wortmannin (100 nM for 1 h) with 0.2% BSA. The comparability of the protein used was assessed by immunobots for β-actin (11). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low-light imaging system (Alpha Innotech).

**Statistical Analysis**

All data are expressed as means ± SE. The differences between groups were analyzed by Student’s t-test when two groups were analyzed or by ANOVA if more than two groups were analyzed.

**RESULTS**

**Studies in BDL Rats Treated in Vivo with CCl₄ Plus Taurocholate or Control Feeding**

**Measurement of transaminase serum levels.** Transaminase serum levels of BDL rats and BDL rats treated with CCl₄ and subsequently fed taurocholate or control diet are shown in Table 1. Consistent with the concept that acute CCl₄ administration induces hepatic damage, CCl₄ administration induced a marked increase in the serum levels of AST and ALT compared with 1 wk BDL rats (Table 1). Taurocholate feeding prevented CCl₄-induced increases in AST and ALT serum levels, which were similar to those of BDL rats (Table 1).

**In Situ Morphological Studies and TUNEL Analysis**

Low-power view of a rat liver section from a rat with BDL for 9 days shows normal parenchyma surrounding the terminal hepatic vein (Fig. 1A). Two days after CCl₄ treatment plus control diet feeding, we found necrosis restricted to zone 3 (Fig. 1B). Taurocholate feeding protected the liver from CCl₄ toxicity, whereas the hepatocytes surrounding the central vein did not appear damaged (Fig. 1C).

Morphological analysis of liver sections from BDL rats showed a few apoptotic bodies (0.18 ± 0.06%), which appeared as small acidophilic globules often containing nuclear material localized between two adjacent cholangiocytes, engulfed within their cytoplasm, or released into the biliary lumen without an associated inflammatory response (not shown). The percentage of apoptotic bodies per 100 cholangiocytes observed in large (diameter of >15 μm) bile ducts appears higher in liver sections from BDL, CCl₄-treated rats (0.50 ± 0.06%; Fig. 2A) compared with BDL rats (0.18 ± 0.06%) and BAL, taurocholate-fed, CCl₄-treated rats (0.23 ± 0.05%; Fig. 2B). Similarly, terminal deoxynucleotidyl transferase-mediated dUTP
nick-end labeling (TUNEL) analysis showed that CCl4 increased the number of cholangiocytes undergoing apoptosis compared with BDL rats (Fig. 3). Taurocholate feeding prevented CCl4-induced increase in cholangiocyte apoptosis, which remained at levels similar to that of BDL rats (Fig. 3). Taurocholate feeding did not alter apoptosis in cholangiocytes from BDL rats (not shown). In agreement with previous studies (35),
apoptosis was never observed in small (diameter of <15 µm) bile ducts. In agreement with our previous studies (34, 35), acute CCl4 treatment did not result in morphological evidence of necrosis in cholangiocytes.

**Measurement of Caspase 8, 9, and 3 Activities**

The activities of caspase 8, 9, and 3 in small and large cholangiocytes from BDL rats was low (Fig. 4). Consistent with the concept that CCl4 damages large but not small ducts (34, 35), acute CCl4 administration induces an increase in the activities of caspase 8, 9, and 3 in large but not small cholangiocytes (Fig. 4). Taurocholate feeding prevented CCl4-induced increases in caspase 8, 9, and 3 activities in large cholangiocytes, which remained at values similar to those of BDL rats (Fig. 4).

**Measurement of Cholangiocyte Proliferation**

*In situ morphometry.* In agreement with previous studies (8), in BDL rats, there was a marked increase in the number of large intrahepatic bile ducts restricted to portal areas (volume of liver occupied by bile ducts, normal control 0.53 ± 0.1% vs. BDL 6.9 ± 0.2%; P < 0.001). Parallel with other studies (8), in rats fed control diet after CCl4 treatment, we found a reduction in the liver volume occupied by large bile ducts compared with BDL rats (3.2 ± 0.5% vs. 6.9 ± 0.2%, respectively; P < 0.05). Taurocholate feeding prevented the CCl4-induced loss of large bile ducts, because liver volume occupied by large bile ducts was similar in taurocholate-fed, CCl4-treated rats and BDL rats (5.9 ± 0.2% vs. 6.9 ± 0.2%, respectively).

**Measurement of Ductal Secretion**

We evaluated the in vitro (i.e., cAMP) and in vivo (i.e., bile flow and bicarbonate secretion) responses to secretin as functional assays of cholangiocyte damage/integrity of bile ducts following CCl4 administration with and without taurocholate feeding.

Similar to other studies (35), secretin increased intracellular cAMP levels of large (but not small) cholangiocytes from BDL rats (Fig. 5). CCl4 treatment inhibited secretin-induced cAMP levels of large cholangiocytes from BDL rats fed control diet, whereas small cholangiocytes (normally unresponsive to secretin) (6, 35) de novo responded to secretin with increases in cAMP levels (Fig. 5). Taurocholate but not control feeding prevented CCl4-induced decreases of large cholangiocyte secretin-stimulated cAMP levels and prevented the de novo cAMP response of small cholangiocytes to CCl4 (Fig. 5).

The effect of control or taurocholate feeding on CCl4-induced effects on basal and secretin-stimulated bile flow and bicarbonate concentration and secretion is shown in Table 2. Basal bile flow and bicarbonate concentration and secretion of BDL rats were similar to those of previous studies (Table 2) (8, 35, 45). Secretin increased bile flow and bicarbonate concentration and secretion in BDL rats (Table 2). In rats fed control diet and treated with CCl4, secretin did not increase bile flow, bicarbonate concentration, or secretion (Table 2). Secretin-induced bile flow and bicarbonate concentration and secretion were similar to control BDL values after CCl4 administration and taurocholate feeding (Table 2), indicating restoration of ductal secretion by taurocholate. The data show that after CCl4 administration, there is a lack of secretin secretory responses, whereas taurocholate prevention of CCl4-induced duct damage is associated with intact duct functional integrity and secretin response.

**Studies in Large Cholangiocytes Treated in Vitro with CCl4 and Taurocholate**

Quantitative evaluation of apoptosis by annexin-V and DAPI staining. When large cholangiocytes were treated in vitro with 5 µM CCl4, we found an increase in apoptosis (evaluated by both annexin-V and DAPI staining) compared with large cholangiocytes treated with 0.2% BSA (Fig. 6, A and B). In vitro taurocholate pretreatment (20 µM) reduced large cholangiocyte apoptosis induced by CCl4 treatment (Fig. 6, A and B). The protective effect of taurocholate on CCl4-induced apoptosis of large cholangiocytes was abolished by wortmannin (100 nM), a PI3-K inhibitor (Fig. 6, A and B). Taurocholate treatment did not alter cholangiocyte apoptosis of BDL rats (not shown). Wortmannin alone did not alter apoptosis of large cholangiocytes (Fig. 6, A and B). Normal cholangiocytes treated with 10 µM beauvericin (the positive control, not shown) (39) displayed the same morphological features of apoptosis as we observed in cholangiocytes from CCl4-treated rats.

**Proliferative capacity of large cholangiocytes.** In vitro, taurocholate increased both H3 histone and
PCNA protein expression of large cholangiocytes compared with large cholangiocytes stimulated with BSA [93.60 ± 8.65 (taurocholate-stimulated value for PCNA) vs. 54.31 ± 5.16 (basal value for PCNA) arbitrary units and 198.88 ± 28.84 (taurocholate-stimulated value for H3 histone) vs. 68.01 ± 6.92 (basal value for H3 histone) arbitrary units]. Taurocholate-induced increases in H3 histone and PCNA protein expression were inhibited by wortmannin [50.24 ± 5.73 vs. 54.31 ± 5.16 (basal value) arbitrary units for PCNA and 90.41 ± 19.80 vs. 68.01 ± 6.92 (basal value) arbitrary units for H3 histone]. In vitro, 5 μM CCl4 decreased both H3 histone and PCNA protein expression of large cholangiocytes from BDL rats compared with large cholangiocytes treated with BSA (Fig. 7). The CCl4-induced loss of H3 histone and PCNA protein expression of large cholangiocytes was prevented by in vitro 20 μM taurocholate treatment (Fig. 7). The protective effect of taurocholate on CCl4-induced inhibition of H3 histone and PCNA protein expression of large cholangiocytes was prevented by pretreatment of large cholangiocytes with wortmannin (Fig. 7), a specific PI3-K inhibitor (36). Wortmannin did not alter basal PCNA protein expression of large cholangiocytes (Fig. 7).

**Intracellular cAMP levels.** Secretin increased cAMP levels of large cholangiocytes from BDL rats (Fig. 8). In agreement with previous studies (3), taurocholate increased both basal (69.8 ± 5.44 vs. 41.27 ± 4.64 fmol/100,000 cells; \( P < 0.05 \)) and secretin-stimulated (141.65 ± 40.54 vs. 72.53 ± 6.01 fmol/100,000 cells; \( P < 0.05 \)) cAMP levels of large cholangiocytes from BDL rats. CCl4 decreased both basal (19.9 ± 2.39 vs. 41.27 ± 4.64 fmol/100,000 cells) and secretin-stimulated cAMP levels of large cholangiocytes (Fig. 8). When large purified cholangiocytes were pretreated in

![Image](http://ajpgi.physiology.org/)

**Table 2.** Basal and secretin-stimulated bile flow, bicarbonate concentration, and secretion in 1-wk BDL rats, 1-wk BDL rats fed bile acid control diet after a single dose of CCl4, and 1-wk BDL rats fed with TC before a single administration of CCl4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bile Flow, μl·min⁻¹·kg⁻¹</th>
<th>Bicarbonate Concentration, μEq·min⁻¹·kg⁻¹</th>
<th>Bicarbonate Secretion, μEq·min⁻¹·kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDL 1 wk (control)</td>
<td>98.94 ± 9.73</td>
<td>26.64 ± 2.34</td>
<td>2.67 ± 0.40</td>
</tr>
<tr>
<td>BDL 1 wk + bile acid control diet + CCl4 2 days</td>
<td>106.97 ± 5.43</td>
<td>35.21 ± 1.10</td>
<td>3.78 ± 0.25</td>
</tr>
<tr>
<td>BDL 1 wk + TC + CCl4 2 days</td>
<td>117.77 ± 10.01</td>
<td>31.59 ± 3.50</td>
<td>3.84 ± 0.84</td>
</tr>
</tbody>
</table>

Data are means ± SE of 3–12 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.

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vitro with 20 μM taurocholate, CCl₄ did not abolish secretin-stimulated cAMP levels of large cholangiocytes (Fig. 8). The protective effect of taurocholate on CCl₄-induced decrease in secretin-stimulated cAMP levels was abolished by in vitro pretreatment of large cholangiocytes with 100 nM wortmannin (Fig. 8). Wortmannin (100 nM) did not alter basal or secretin-stimulated cAMP levels of large cholangiocytes (not shown).

**Fig. 6.** Assessment of apoptosis by annexin-V (A) and DAPI staining (B) in large cholangiocytes from BDL rats treated for 2 h at 37°C with 1) 0.2% BSA (basal); 2) 5 μM CCl₄ in the absence or presence of 20 μM TC ± 100 nM wortmannin with 0.2% BSA; or 3) wortmannin (100 nM) with 0.2% BSA. There was a significant (P < 0.05) increase in apoptosis in large cholangiocytes treated with CCl₄ compared with large cholangiocytes treated with 0.2% BSA. TC in vitro pretreatment reduced large cholangiocyte apoptosis induced by CCl₄. The protective effect of TC on CCl₄-induced apoptosis of large cholangiocytes was abolished by wortmannin (100 nM), a phosphatidylinositol-3-kinase inhibitor (PI3-K). Data are means ± SE of at least 3 experiments. DAPI, 4,6-diamidino-2-phenylindole.

**Fig. 7.** Measurement of PCNA and H3 histone protein expression in large cholangiocytes from BDL rats treated for 37°C with 1) 0.2% BSA (basal) for 2 h; 2) 5 μM CCl₄ for 2 h with 0.2% BSA; 3) TC (20 μM for 1 h) + CCl₄ (5 μM for 2 h) with 0.2% BSA; 4) wortmannin (100 nM for 1 h) + TC (20 μM for 1 h) + CCl₄ (5 μM for 2 h) with 0.2% BSA; or 5) wortmannin (100 nM for 1 h) with 0.2% BSA. There was a significant (P < 0.05) decrease in both PCNA and H3 histone protein expression of large cholangiocytes treated with CCl₄ compared with large cholangiocytes treated with 0.2% BSA. TC treatment prevented the decrease in both PCNA and H3 histone protein expression of large cholangiocytes induced by CCl₄. The effect of TC on CCl₄-induced decrease in both PCNA and H3 histone protein expression of large cholangiocytes was abolished by wortmannin (100 nM), a PI3-K inhibitor. Data are means ± SE of at least 3 experiments. *P < 0.05 vs. H3 histone and PCNA protein expression of large cholangiocytes treated with 0.2% BSA.
Phosphorylated AKT protein expression. When large cholangiocytes from BDL rats were treated in vitro with 5 μM CCl₄, we found a significant decrease in phosphorylated AKT protein expression compared with large cholangiocytes treated with BSA (Fig. 9). In vitro taurocholate treatment prevented CCl₄-induced decrease of phosphorylated AKT protein expression of large cholangiocytes (Fig. 9). The effect of taurocholate on CCl₄-induced decrease in phosphorylated AKT protein expression of large cholangiocytes was abolished by wortmannin (100 nM; Fig. 9). Taurocholate or wortmannin did not alter phosphorylated AKT protein expression (not shown). The expression of total AKT and β-actin was similar among purified cholangiocytes (Fig. 9).

DISCUSSION

In a previous study, LeSage et al. (35) showed that administration of a single dose of CCl₄ to BDL rats induces (after 2 days) a transient damage (by apoptosis) and loss of functions of large cholangiocytes. In contrast, small cholangiocytes were resistant to apoptosis and de novo proliferated and transiently acquired phenotypes of large cholangiocytes in response to CCl₄ (35). In this study, we showed that feeding of taurocholate to BDL rats prevents CCl₄-induced apoptosis of large cholangiocytes and loss of proliferative and secretory capacity of large cholangiocytes in large ducts. After taurocholate feeding, small cholangiocytes [which are de novo activated in this model of CCl₄-induced damage of large ducts (35)] remained mitotically quiescent (6) and unresponsive to secretin (4, 6, 10, 12, 35). In vitro studies in purified large cholangiocytes from BDL rats showed that 1) taurocholate (at 20 μM) prevents the effects of CCl₄ (5 μM) on apoptotic, proliferative, and secretory capacity of large cholangiocytes; 2) taurocholate protective effects against apop-
Bile acids are internalized into cholangiocytes by the Na\(^+\)-dependent ABAT (5, 28), thus modulating secretory and proliferative activities of cholangiocytes (3, 5, 7). Alpini et al. (5) previously demonstrated that ABAT and bile acid transport activity are expressed by large but not small cholangiocytes of normal rats. Taurocholate, for example, increases proliferative and secretory activities of normal, large cholangiocytes (3). Inhibition of ABAT-mediated bile acid uptake ablates the taurocholate effects on cholangiocyte proliferation and secretion (3), so it is likely that bile acid internalization by ABAT is required for bile acid signaling in cholangiocytes. Thus the taurocholate cytoprotective effects in CCl\(_4\)-induced cholangiocyte injury may require ABAT-mediated bile acid uptake, and this issue will be addressed in future studies by examining ABAT expression and the effects of ABAT inhibitors.

Studies showed that bile acids affect PI3-K activity in hepatocytes (36, 42, 46); however, no information exists regarding the role of PI3-K on the regulation of cholangiocyte functions. Taurochenodeoxycholate, for example, activates PI3-K-dependent survival pathways in rat hepatocytes, which prevent its inherent toxicity (42). In addition, cAMP-mediated cytoprotection against bile acid-induced apoptosis in cultured rat hepatocytes is mediated by PI3-K (46). PI3-K (which is activated by taurocholate) (49) represents a potent activator of survival signals, raising the possibility that nontoxic, hydrophobic bile acids do not trigger apoptosis in hepatocytes because they activate a PI3-K-dependent survival signaling pathway (42). Recently, Webster and Anwer (47) showed that hepatocyte growth factor (HGF) inhibits bile acid-induced hepatocyte apoptosis in primary cultures of rat hepatocytes. This antiapoptotic effect was completely reversed by PI3-K inhibitors, indicating that HGF-induced survival from bile acid-induced apoptosis in hepatocytes is mediated via the PI3-K signaling pathway. Consistent with these previous studies, we found that taurocholate feeding may reduce CCl\(_4\)-induced hepatocyte injury, because markers of hepatocyte injury (serum ALT and AST levels), which were significantly elevated in CCl\(_4\)-treated BDL rats, were similar in taurocholate-fed CCl\(_4\)-treated and BDL control rats. The present data demonstrate that in cholangiocytes, taurocholates exert a protective effect against CCl\(_4\)-induced apoptosis of large cholangiocytes through a PI3-K mechanism. Downstream effectors of PI3-K-dependent survival include PKC isoforms and Akt, which seems to inactivate Bad phosphorylation and upregulate Bcl-2 by transcriptional activation (38). In support of this, we have shown that in vitro CCl\(_4\)-induced apoptosis of large cholangiocytes was associated with decreased phosphorylated Akt protein expression and that taurocholate protection against CCl\(_4\)-induced damage of large cholangiocytes is associated with maintained phosphorylated Akt protein expression.

A number of studies has shown that the secretin receptor and cAMP levels play an important role in the regulation of cholangiocyte apoptosis/proliferation (3, 6, 7, 9, 22, 29, 30, 32–35). This concept is supported by several studies showing that there is upregulation of secretin receptor and basal and secretin-stimulated cAMP levels in all of the hyperplastic models of cholangiocyte proliferation so far studied (3, 6, 7, 9, 22, 29, 30, 32–35), whereas reduced cholangiocyte replication [e.g., following total vagotomy (29), ursodeoxycholate, or tauoursodeoxycholate feeding (2) or gastrin treatment (22), or acute gavage CCl\(_4\) administration (34, 35)] is associated with depressed secretin receptor and secretin-stimulated cAMP levels. Furthermore, upregulation of cAMP levels (by chronic forskolin treatment) increases cholangiocyte proliferation and the number of bile ducts of normal rats through a PKA-dependent mechanism (31). In BDL, vagotomized rats (29), chronic forskolin treatment prevented the decrease in cAMP levels, maintained cholangiocyte proliferation and secretion, and decreased cholangiocyte apoptosis induced by vagotomy in BDL rats (29). Finally, recent studies (40) have shown that changes in the activity of G\(_\alpha\) and G\(_\beta\) protein subunits are associated with alteration of cholangiocytes of BDL rats. In primary hepatocyte cultures, elevated cAMP intracellular levels not only protected against Fas-induced apoptosis, but also rescued the cells from functional deterioration (41). Furthermore, forskolin and cAMP analogs have been shown to prevent apoptosis induced by hydrophobic bile acids in cultured rat hepatocytes (46). In neuronal tissues, the intracellular cAMP system is fundamental for tissue differentiation and survival, for promotion of regeneration of injured tissue, and for prevention of apoptosis (14, 16, 18). Several growth factors (e.g., nerve growth factor and pituitary adenylate cyclase-activating protein) that promote neural tissue differentiation and prevent apoptosis act via cAMP pathways through yet unidentified signaling factors (e.g., mitogen-activated protein kinases and cAMP-dependent transcription factors) (44). In support of the fact that cAMP system plays an important role in the regulation of cell proliferation/apoptosis, our data show that taurocholate, by maintaining basal and secretin-stimulated cAMP levels of cholangiocytes from BDL rats, prevents CCl\(_4\)-induced cholangiocyte apoptosis (35) and the inhibition of cholangiocyte proliferation and secretion (35).

Cytoprotection provided by activation of cAMP and AKT appear to occur through cooperative effects of these two pathways. cAMP does not lead to direct activation of PI3-K (46). The dual requirement for PI3-K and cAMP for cytoprotection against apoptosis may therefore reflect the need for cooperative action on a single downstream effector. Because PI3-K activates protein translocation, the movement of some effector molecule to a subcellular location where cAMP exerts its effect, may be required. The lipid products of PI3-K hydrolysis are necessary to promote membrane interaction of PKB, where it can subsequently activate cAMP's cytoprotective actions (46). Studies (21) have
also shown that PI3-K is involved in taurocholate regulation of bile secretion. PI3-K is involved in the regulation of vesicle trafficking and cytoskeleton organization in the process of bile formation. The increase in canalicular bile acid secretion after taurocholate administration in vivo results from rapid transporter activation or recruitment to the canalicular domain, and PI3-K is required for intracellular trafficking of ATP-dependent transporters to the canalicular membrane.

Defining the signaling pathways involved in cholangiocyte apoptosis and cytoprotection in specifically sized ducts has important pathophysiological implications, because several human cholestatic liver diseases (e.g., PBC) are characterized by spotty rather than diffuse proliferation/loss of certain sized ducts (i.e., small interlobular bile ducts) (9). The present studies allow a better understanding of the mechanisms by which different-sized ducts are damaged and how certain bile acids protect against toxin-induced ductopenia in cholestatic liver diseases (e.g., PBC and PSC) (9).

Failure of bile acids to activate cytoprotective mechanisms in cholangiocytes may promote apoptosis and loss of bile ducts in these diseases. Because ursodeoxycholic acid is a major therapeutically used bile acid with antiapoptotic effects (37), a direct outgrowth of the studies is to evaluate the potential protective effects of this bile in CCl4 (34, 35) - vagotomy (29) - induced duct damage.

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