Tauroursodeoxycholate inhibits human cholangiocarcinoma growth via Ca\(^{2+}\), PKC-, and MAPK-dependent pathways

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Submitted 24 June 2003; accepted in final form 26 December 2003


Tauroursodeoxycholate inhibits human cholangiocarcinoma growth via Ca\(^{2+}\), PKC-, and MAPK-dependent pathways. Tauroursodeoxycholate (TUDCA) has been shown to be used for the treatment of cholestatic liver diseases including PBC (28, 52) and PSC (17, 55). The efficacy of UDCA or TUDCA is attributed to its cytotoxic effects against hepatocytes (48) by increasing bile flow and biliary acid secretion (9) and hepatocellular vesicular exocytosis (12). TUDCA has been shown to be more effective than UDCA in the enrichment of biliary UDCA and more effectively absorbed by the intestine (31).

PKC modulates the effects of bile acids on a number of epithelia, including cholangiocytes (1, 3, 13, 32, 44). For example, both primary and secondary bile acids have been shown to activate the expression of PKC isoenzymes, MAPK, and phosphoinositol 3-kinase in normal colonic epithelial cells and colorectal cancer tissue (50). Furthermore, sodium tauroliothocholate reduces hepatocyte canalicular secretion through activation of PKC-ε (13). In hepatocytes, glycocholate-induced apoptosis is associated with activation and membrane translocation of PKC-α, PKC-δ, and PKC-ε (32). In situ histological studies (51) have shown that feeding of UDCA to bile duct-ligated rats decreases the number of intrahepatic bile ducts. Both UDCA and TUDCA inhibit cholangiocyte proliferation of bile duct-ligated rats by activation of the Ca\(^{2+}\)-dependent PKC-α (1). Moreover, feeding of taurocholate and tauroliothocholate to normal rats increases cholangiocyte proliferation and the number of ducts by activation and membrane translocation of PKC-α (3). However, no information exists regarding the role and mechanism of action of TUDCA in the regulation of cholangiocarcinoma growth.

Three main distinct signaling cascades exist in the MAPK family of serine/threonine kinases: p38 MAPK, JNK, and p44 and p42 (encoded by ERK1 and ERK2, respectively) (14). The MAPK ERK1 and ERK2 are proline-directed kinases that are activated through concomitant phosphorylation of tyrosine and threonine residues (4). p38 is a member of the MAPK family with features most closely resembling those of the Saccharomyces cerevisiae protein HOG1 (15). The JNK family, which includes JNK1, JNK2, and JNK3, is distantly related to the MAPK family, members of which are activated by dual phosphorylation at a Thr-Pro-Tyr motif, specifically at Thr\(^{183}\) and Tyr\(^{185}\) residues, in response to ultraviolet (UV) light (60). JNK

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is phosphorylated by JNK-activating kinases (JNKK1 and JNKK2), which are members of the MEK family (68).

The cAMP-dependent PKA-MEK-MAPK pathway is modulated via cross-talk with other intracellular signaling pathways (64), including PKC, which studies have shown to be associated with activation of MAPK (50). The activation of the PKC pathway is not always associated with activation of MAPK (43). For example, PKC-γ inhibits UV-induced activation of caspase-3 in normal human keratinocytes by inhibition of p38 MAPK pathway (43). cAMP-dependent PKA is the major substrate of cAMP, and cAMP-dependent signaling is associated with a wide range of biological responses, including differentiation, survival, inhibition of growth, and apoptosis (19, 37, 45, 64). The activation of signal-transduction pathways by growth factors, hormones, and neurotransmitters is mediated through two closely related MAPK, p44 and p42, that are encoded by ERK1 and ERK2, respectively (14).

In this study, we evaluated the role and mechanisms of action of TUDCA in the regulation of growth of the cholangiocarcinoma cell line Mz-ChA-1. We posed the following questions: 1) Does TUDCA inhibit the growth of the cholangiocarcinoma cell line, Mz-ChA-1? 2) Are TUDCA-inhibitory effects on Mz-ChA-1 growth associated with increases in intracellular Ca2+ concentration ([Ca2+]i) and activation of the Ca2+-dependent PKC-α, which plays an important role in the regulation of cholangiocyte functions (1, 3, 26, 27, 33, 41)? 3) Does TUDCA stimulation of Ca2+-dependent PKC lead to inhibition of MAPK activity? 4) Is TUDCA inhibition of MAPK activity associated with changes in Raf-1 and B-Raf proteins damaged by trypsin digestion (35). Subsequently, cells were incubated with 10 μM TUDCA (0.2–200 μM). After an initial incubation step of 4 h at 37°C in a 5% CO2 incubator, the cells were incubated at 37°C with TUDCA (0.2–200 μM). After incubation for 24–96 h, [3H]Thymidine was put into each well at 5.0 μCi/ml and incubated for additional 4 h. [3H]Thymidine incorporation was measured by a scintillation counter. At the end of each incubation period, we evaluated cell viability by trypan blue exclusion.

Measurement of [3H]Thymidine incorporation. Mz-ChA-1 cells (1.5 × 10^6) were seeded into flat-bottomed six-well plates and incubated in CCM until 70% confluence. Subsequently, cells were incubated with 1% 0.2% BSA (basal value) or TUDCA (200 μM) in the absence or presence of BAPTA-AM (a chelator of [Ca2+]i); 5 μM (33, 34) or 7 μM (33) for 48 h. After the selected treatment, cells were washed twice with ice-cold PBS and then lysis buffer (10 mM Tris, pH 7.4, 1 Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% BSA, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM Na3VO4) was added into each well. Culture plates were kept on ice for 30 min with gentle rocking, then cells were scraped, collected in a microcentrifuge tube, and centrifuged at 300 g for 10 min at 4°C. Following electrophoresis, protein samples (10 μg) were transferred to a nitrocellulose membrane. The membrane was immersed into a blocking solution consisting of 5% dry milk and 1% TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween-20) and incubated with gentle rocking for 2 h. The membrane was incubated with anti-PANC1 antibody diluted to 1:200 as the primary antibody over night at 4°C. After being washed, the membrane was incubated with HRP-conjugated anti-mouse IgG diluted to 1:3,000 for 1 h at room temperature. After washes, proteins were visualized by using chemiluminescence (ECL Plus kit; Amersham Life Science). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000 low-light imaging system (Alpha Innotech, San Leandro, CA).

Effect of TUDCA on [Ca2+]i, and Protein Expression and Membrane Translocation of the Ca2+-dependent PKC-α

After trypsinization, Mz-ChA-1 cells were transferred to a clean tube and incubated for 1 h at 37°C (33, 34) to regenerate membrane proteins damaged by trypsin digestion (35). Subsequently, cells were stimulated for 15 min at 22°C with 0.2% BSA (basal value) or TUDCA (200 μM) in the presence of 0.2% BSA. Mz-ChA-1 [Ca2+]i, levels were determined by a microfluorescent technique (1, 30) in Mz-ChA-1 cells previously loaded with the fluorescent Ca2+-indicator fluo-3 (1 μM for 10 min). The fluo-3 fluorescence was converted to [Ca2+]i levels by employing a calibration kit from Molecular Probes (Eugene, OR) (30). Cells (1.5 × 106) were seeded into flat-bottomed six-well plates and incubated in CCM until they were grown to 70% confluence.
Subsequently, cells were incubated with 0.2% BSA (basal) or TUDCA (200 µM) for 90 min as described by us and others (7, 25, 27, 33, 41). After two washes with ice-cold PBS, lysis buffer was added into each well (see Measurement of PCNA protein expression). Total protein expression for PKC-α in Mz-ChA-1 cells (treated with BSA or TUDCA for 90 min) was evaluated in whole cell lysate by immunoblots (25, 27, 33, 41).

PKC-α membrane translocation was evaluated by immunoblots (25, 27, 33, 41) in a cytosol or membrane fraction (54) isolated from Mz-ChA-1 cells treated with BSA or TUDCA for 90 min. The cytosol and membrane fractions were obtained from Mz-ChA-1 as described previously (54). Briefly, Mz-ChA-1 cells in 100-mm dishes were washed with PBS, extracted in 1 ml of buffer A (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 25 µg/ml each aprotinin and leupeptin), and homogenized with 30 strokes of a Dounce homogenizer. The homogenate was transferred to a microcentrifuge tube and centrifuged in a microcentrifuge at 4°C at 10,000 g for 2 min to clarify. The clarified homogenate was centrifuged at 4°C in an ultracentrifuge at 45,000 g for 30 min. The supernatant was collected as the cytosol fraction. The pellet was washed twice with buffer A and resuspended in 500 µl of buffer A with 0.5% Triton X-100. The pellet solution was vortexed, incubated on ice for 30 min, and centrifuged in a microcentrifuge at 4°C at 10,000 g for 2 min. The supernatant was collected as the membrane fraction. Immunoblots for PKC-α were performed as described above for Western immunoblotting for PCNA expression except for the use of mouse anti-PKC-α (diluted to 1:10,000) as the primary antibody and HRP-conjugated anti-mouse IgG diluted to 1:3,000 as a secondary antibody. The intensity of the bands was determined by scanning video densitometry using the Chemi-Imager 4000. The effects of TUDCA on [Ca^{2+}]i, and PKC-α protein expression were performed at different incubation times with TUDCA (15 min for Ca^{2+} and 90 min for PKC), because we anticipated that the Ca^{2+}-dependent activation of PKC-α protein expression would occur at a later time than the increase in [Ca^{2+}]i. This is due both to the fact that the increased calcium signal is upstream to the increase in protein expression as well as the time lag required for synthesis of new proteins (90 min for activation of PKC (7, 27, 41)). The same time period (15 min for Ca^{2+} and 90 min for PKC) has been used by us in studies aimed to evaluate the effect of bile acids (e.g., UDCA and TUDCA) (1), gastrointestinal hormones (e.g., insulin) (41), or nerve receptor agonists (e.g., the D2 dopaminergic receptor agonist quinelorane) (25) on intracellular Ca^{2+} levels and the expression and membrane translocation of Ca^{2+}-dependent PKC isoforms. Furthermore, other studies have shown that prolonged incubation time (i.e., 90 min) is necessary for the activation of PKC-γ and PKC-δ, which are involved in insulin-like growth factor-I migration of colonic epithelial cells (7).

Expression of Raf-1 and B-Raf in Mz-ChA-1 Cells

The protein expression of Raf-1 and B-Raf in Mz-ChA-1 cells was evaluated by immunoblotting (25, 27, 34) using anti-Raf-1 and anti-B-Raf primary antibodies and the corresponding secondary antibod-
ies. After washes, bands were visualized by using chemiluminescence (ECL Plus). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000.

Evaluation of the Transduction Pathways by Which TUDCA Regulates Cholangiocarcinoma Growth: Effect of TUDCA on Raf-1, B-Raf, and MAPK Activities and on Phosphorylation of MAPK p42/44, p38, and JNK/SAPKs

The effect of TUDCA (200 μM) on the activity of Raf-1, B-Raf, and MAPK was performed as previously described by us (34). Cells in the culture medium were seeded into flat-bottomed six-well plates and incubated until they became 70% confluent. Subsequently, cells were placed in serum-free medium (CMRL Medium-1066 supplemented with 0.1% BSA, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) and cultured for an additional 24 h. After serum starvation, Mz-ChA-1 cells were incubated for 24 h with 100 nM EGF or TUDCA (200 μM containing 100 nM EGF) in the absence or presence of BAPTA-AM (5 μM) or H7 (2 μM). The effect of TUDCA on MAPK activity (using an antibody against MAPK1/2, ERK1/2) in Mz-ChA-1 cells was also evaluated in the presence of serum. Mz-ChA-1 cells (1.5 × 10⁶) were seeded into flat-bottomed six-well plates and incubated in CCM until 70% confluence. Subsequently, cells were incubated with 0.2% BSA (basal value) or TUDCA (200 μM) for 24 h. Subsequently, cells were washed twice with ice-cold PBS and incubated with lysis buffer (10 mM Tris, pH 7.4, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% BSA, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM Na3VO4) for 30 min on ice. Samples were collected, and total protein concentration was evaluated by using the Pierce protein assay kit. The protein expression of total PKC-α was evaluated by using a scintillation counter. The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000.

Fig. 2. Effect of TUDCA on intracellular Ca²⁺ concentration ([Ca²⁺]i) (A), total protein expression (B), and membrane translocation (C) for PKC-α in Mz-ChA-1 cells. A: [Ca²⁺]i, levels were determined by using a microfluorescent technique in Mz-ChA-1 cells previously loaded with the fluorescent Ca²⁺ indicator fluo-3 (1 μM for 10 min). The fluo-3 fluorescence was converted to [Ca²⁺]i levels by using a calibration kit from Molecular Probes. TUDCA (added at time 0) induced a 2-fold persistent increase in [Ca²⁺]i. Data are means ± SE of 3 experiments. *P < 0.05 vs. the corresponding basal values. B: TUDCA (200 μM for 90 min) increased total PKC-α protein expression in Mz-ChA-1 cells. Data are means ± SE of 23 experiments. *P < 0.05 vs. the corresponding basal values. C: immunoblots for PKC-α in a cytosol and membrane fraction isolated from Mz-ChA-1 cells treated with 0.2% BSA or TUDCA for 90 min. In Mz-ChA-1 cells treated with 0.2% BSA, the majority of PKC-α is found in the cytosol fraction. On the addition of TUDCA, PKC-α protein expression decreases significantly in the cytosol fraction. After TUDCA treatment, loss of PKC-α from the cytosol fraction was associated with an increase in PKC-α protein expression in the membrane fraction of Mz-ChA-1 cells. Data are means ± SE of 6 experiments. *P < 0.05 vs. the corresponding basal values. #P < 0.05 vs. the corresponding value of the cytosol fraction.
bands were visualized by using chemiluminescence (ECL Plus). The intensity of the bands was determined by scanning video densitometry using the ChemiImager 4000.

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 analysis of variance (ANOVA) if more than two groups were analyzed.

RESULTS

Time- and Concentration-Dependent Inhibition of the Growth of Mz-ChA-1 Cells by TUDCA

After 48 h of incubation, TUDCA significantly inhibited \([^{3}]H\) thymidine incorporation of Mz-ChA-1 cells at concentrations ranging from 2 to 200 μM (Fig. 1A). At the concentration of 200 μM, TUDCA significantly inhibited \([^{3}]H\) thymidine incorporation in Mz-ChA-1 cells from 24 to 96 h of the incubation period (Fig. 1B). The data show that TUDCA inhibits the growth of Mz-ChA-1 cells in a time- and concentration-dependent fashion. TUDCA (48 h at 200 μM) inhibited PCNA protein expression (an index of cell replication) (40) of Mz-ChA-1 cells (Fig. 1C). Consistent with the concept that PKC-α regulates TUDCA modulation of cholangiocarcinoma growth, TUDCA inhibition of PCNA protein expression of Mz-ChA-1 was blocked by BAPTA-AM and H7 (Fig. 1C). Trypan blue exclusion analysis showed that TUDCA did not increase the percentage of dead cells compared with controls.

TUDCA Increases \([Ca^{2+}]\) and PKC-α Protein Expression and Induces Membrane Translocation of PKC-α in Mz-ChA-1 cells

TUDCA (200 μM) caused a marked and sustained increase in \([Ca^{2+}]\), levels in Mz-ChA-1 cells (Fig. 2A). Previous studies from our laboratory (3) in cholangiocytes isolated from bile duct-ligated rats demonstrated that TUDCA mobilizes \(Ca^{2+}\) from intracellular stores rather than originating from extracellular stores. Further studies are needed to establish the source of mobilized calcium in cholangiocarcinoma cells.
Immunoblotting analysis shows that Mz-ChA-1 cells express the protein for the Ca\(^{2+}\)-dependent PKC-\(\alpha\) and that TUDCA increased total PKC-\(\alpha\) protein expression in Mz-ChA-1 cells (Fig. 2B). Figure 2C shows the subcellular distribution of PKC-\(\alpha\) in Mz-ChA-1 cells treated with 0.2% BSA or TUDCA for 90 min. In Mz-ChA-1 cells treated with 0.2% BSA, the majority of PKC-\(\alpha\) is found in the cytosol fraction (Fig. 2C); however, on addition of TUDCA, PKC-\(\alpha\) protein expression significantly decreases in the cytosol fraction (Fig. 2C). After TUDCA treatment, loss of PKC-\(\alpha\) from the cytosol fraction was associated with an increase in PKC-\(\alpha\) protein expression in the membrane fraction of Mz-ChA-1 cells (Fig. 2C). The magnitude of increases of total PKC-\(\alpha\) expression and membrane translocation of PKC-\(\alpha\) by TUDCA were similar to that we previously described by gastrin (27). The activation of PKC is due to increases in membrane-bound PKC-\(\alpha\) following TUDCA treatment. Figure 2C shows a significant increase in the membrane fraction for PKC-\(\alpha\) following TUDCA treatment. The increase in total PKC-\(\alpha\) is commonly seen for other activators of PKC (e.g., PMA) (24), which presumably provides more PKC-\(\alpha\) available for translocation.

\(\text{Mz-ChA-1 Cells Express Raf-1 and B-Raf: Effect of TUDCA on Raf-1, B-Raf, and MAPK Activities and on Phosphorylation of MAPK p42/44, p38, and JNK/SAPKs}\)

As shown in Fig. 3A, Mz-ChA-1 cells express the proteins for Raf-1 and B-Raf (74 and 95 kDa, respectively). Stimulation of Mz-ChA-1 cells with EGF (100 nM) for 24 h caused a significant \(P < 0.05\) increase in B-Raf (Fig. 3B) but not Raf-1 (results not shown) activity. EGF stimulation of B-Raf activity was not prevented by TUDCA after 24 h of incubation (Fig. 3B).

After serum starvation, stimulation of Mz-ChA-1 cells with EGF (100 nM) for 24 h significantly enhanced MAPK activity (i.e., ERK1/2) of these cells (Fig. 4A). EGF stimulation of MAPK activity of Mz-ChA-1 cells was inhibited by TUDCA (Fig. 4A). TUDCA inhibition of EGF-induced MAPK activity was blocked by pretreatment of Mz-ChA-1 cells with BAPTA-AM and H7 (Fig. 4A). Similar results (related to MAPK activity of ERK1/2) were obtained when Mz-ChA-1 cells were treated with TUDCA in the presence of serum (Fig. 4B). TUDCA inhibited basal MAPK activity of Mz-ChA-1 cells (Fig. 4B).

TUDCA (200 \(\mu\)M) inhibited the phosphorylation of MAPK p42/44 (expressed as ratio to total protein expression for p42/44), whose protein expression was similar to that of Mz-ChA-1 treated with BSA (Fig. 5A). Consistent with the concept that the Ca\(^{2+}\)-PKC pathway regulates cholangiocarcinoma growth by changes in MAPK activity, TUDCA inhibition of MAPK p42/44 phosphorylation was blocked by BAPTA-AM and H7 (Fig. 5A). TUDCA inhibition of cholangiocarcinoma growth was not associated with changes in the phosphorylation of MAPK p38 and JNK/SAPKs (expressed as ratio to total protein expression for p38 and JNK/SAPKs, respectively). Data are means ± SE of 7 experiments. \(\ast P < 0.05\) vs. the corresponding basal value.

\(\text{Fig. 4. A: effect of TUDCA (200 \(\mu\)M in the absence or presence of BAPTA-AM or H7) on EGF-induced MAPK activity in Mz-ChA-1 cells after 24 h of incubation time. Stimulation of Mz-ChA-1 cells with EGF for 24 h significantly enhanced MAPK activity of these cells. TUDCA inhibited EGF-induced increase in MAPK activity of Mz-ChA-1 cells. TUDCA inhibition of EGF-induced MAPK activity was blocked by BAPTA-AM and H7. Data are means ± SE of at least 3 experiments. \(\ast P < 0.05\) vs. the control value (stimulated with EGF). B: effect of TUDCA (200 \(\mu\)M) on basal MAPK activity in Mz-ChA-1 cells after 24 h of incubation time. TUDCA inhibited basal MAPK activity (i.e., ERK1/2) of Mz-ChA-1 cells. Data are means ± SE of at least 3 experiments. \(\ast P < 0.05\) vs. the corresponding basal value.}\)
giocarcinoma growth was not associated with changes in the phosphorylation of MAPK p38 and JNK/SAPKs (expressed as ratio to total protein expression for MAPK p38 and JNK/SAPKs, respectively) (Fig. 5, B and C). The data suggest that TUDCA inhibits Mz-ChA-1 growth through a signal transduction pathway involving MAPK p42/44 and PKC-α but independent from Raf proteins and MAPK p38 and JNK/SAPKs.

DISCUSSION

The study shows that TUDCA inhibits the growth of the human cholangiocarcinoma cell line Mz-ChA-1. TUDCA inhibition of growth occurs in a dose- (2–200 μM) and time- (24–96 h) dependent fashion. We also show that TUDCA increased [Ca^{2+}], and PKC-α protein expression and PKC-α membrane translocation and that TUDCA inhibition of cholangiocarcinoma growth was partially blocked by the [Ca^{2+}]-chelator BAPTA-AM and the PKC-α inhibitor H7. Furthermore, TUDCA inhibited MAPK activity, and the inhibitory effects of TUDCA on EGF-stimulated MAPK activity were partially blocked by BAPTA-AM and H7. TUDCA inhibited phosphorylation of MAPK p42/44 but not p38 and JNK/SAPKs. Consistent with the concept that the Ca^{2+}-PKC pathway regulates cholangiocarcinoma growth by changes in MAPK activity, TUDCA inhibition of phosphorylation of MAPK p42/44 was blocked by BAPTA-AM and H7. TUDCA did not inhibit Raf-1 or B-Raf activities. The data suggest that TUDCA inhibition of MAPK and Mz-ChA-1 growth occurs by activation of Ca^{2+}-dependent PKC-α upstream to MAPK but that the inhibition occurs independent of the Raf proteins.

PKC-α regulates a variety of cellular responses, including proliferation and apoptosis, through the activation (by phosphorylation) of Ras-Raf-MEK-MAPK (39, 66). Whereas in most cells PKC, activated by hormones and/or growth factors, mediates the stimulation of cell growth, in other cell types it exerts a negative control (5). The differential pattern of PKC in the modulation of cell growth may be due to the varying expressions of different isoforms of the PKC superfamily (47). For example, in the crypt-villus axis, PKC-α mediates inhibition of growth of intestinal epithelial cells (46). The activation of PKC-α decreases cell growth and tumorigenicity of intestinal cell lines (8). In the liver, PKC-α has been shown to play an important role in the regulation of cholangiocyte proliferation (1, 3, 26, 27, 33). For example, with activation of PKC-α (by gastrin) we found inhibition of cAMP levels and cell replication in cholangiocytes from bile duct-ligated rats (26, 27) and cholangiocarcinoma cell lines (33). Whereas activation of PKC-α (by taurocholate and tauroliothiolcholate) leads to activation of cholangiocyte proliferation of normal cholangiocytes (3), PKC-α membrane translocation (by UDCA and TUDCA) induces inhibition of cholangiocyte proliferation from bile duct-ligated rats (1). Phorbol 12,13-dibutyrate (a PKC activator) (6) also inhibits the proliferation of Mz-ChA-1 cells (33). The different cross-talk between Ca^{2+}-dependent PKC and cAMP (which leads to stimulatory or inhibitory effects on MAPK and cell proliferation) (22, 65) is due to the type of receptor [gastrin (26, 27)] or transporter (Na^{+}-dependent bile acid transporter) up- or downregulated (1, 3), which differentially activates different PKC isoforms (1, 3, 26, 27), thus leading to activation or inhibition of cholangiocyte proliferation. These interactions may result in a different cross-talk between intracellular Ca^{2+}-PKC and specific adenylate cyclase isoforms, leading to inhibition or stimulation of adenylate cyclase and therefore of cAMP, MAPK, and cell proliferation.

In addition, PKC has been shown to mediate the effects of bile acids on a number of epithelia, including cholangiocytes (1, 3, 13, 32, 44). In our study, TUDCA inhibition of MAPK activities and cholangiocyte PCNA protein expression was abrogated by the presence of the [Ca^{2+}]-chelator (BAPTA-AM) (27) and a Ca^{2+}-dependent PKC-α inhibitor (H7) (27), compounds that were previously used to ascertain the role of the Ca^{2+}-dependent PKC-α in the regulation of cholangiocyte functions (27). These findings support the idea that TUDCA inhibition of MAPK and cholangiocarcinoma growth is PKC-α dependent. Although phosphorylation of MAPK by PKC-α may induce the activation of MAPK (66), in this study and our previous study involving gastrin inhibition of Mz-ChA-1 cholangiocarcinoma growth (33) PKC-α inhibits MAPK. In support of our findings, recent studies have shown that the activation of PKC pathway is not always associated with activation of MAPK (43). For example, PKC-α inhibits UV-induced activation of caspase-3 in normal human keratinocytes by inhibition of p38 MAPK pathway (43). Cross-talk between the PKC-α pathway and other not yet identified pathways may also be involved in TUDCA inhibition of cholangiocarcinoma growth. TUDCA may also inhibit cholangiocarcinoma growth by reducing cyclooxygenase-2 (COX-2) since COX-2 overexpression in cholangiocarcinoma (59) may promote growth, and endogenous bile acids have been shown to alter gene expression of COX-2 by a PKC-dependent mechanism (67).

Bile acid modulation of MAPK has been shown to alter bile secretion (38), cell proliferation (49), and apoptosis (29). Although bile acids have been shown to modify cell growth and MAPK through phosphorylation of the EGFR receptor (53), the subsequent bile acid-induced changes in MAPK (in contrast to this study) were dependent on the activity of Ras and Raf. Thus it is unlikely that TUDCA inhibits growth by altering EGFR receptor in Mz-ChA-1 cells. In other studies (38), TUDCA enhances MAPK-dependent bile secretion in hepatocytes by increasing phosphoinositol 3-kinase, which leads to Ras-dependent activation of ERK1/2. Because the TUDCA stimulation of MAPK in hepatocytes was independent of PKC (38), TUDCA inhibition of cholangiocarcinoma MAPK may be due to the ability of TUDCA to increase PKC-α in this cell line.

Cholangiocarcinoma is a liver neoplasm arising from intrahepatic bile duct and the extrahepatic bile ducts (62). Cholangiocarcinoma exhibits a poor prognosis, and surgical resection is virtually the only measure for the curative treatment, although other attempts, including radiotherapy (23) and photodynamic therapy (56), to relieve biliary obstruction due to unresectable tumors have been demonstrated successfully as an adjuvant therapy following surgery or as palliative therapy. However, to date, some gastrointestinal hormones and neurotropes have been reported to be effective in the modulation of the growth of cholangiocarcinoma (21, 27, 61). Somatostatin, for example, prevents the growth of human cholangiocarcinoma cells implanted in athymic mice through somatostatin receptors (61). Also, as we have previously shown, gastrin inhibits the growth of human cholangiocarcinoma cell lines through inositol 1,4,5-trisphosphate- and PKC-α-dependent
pathways (33). α2-Adrenergic receptor stimulation also inhibits the growth via cAMP-PKA-Raf-MAPK-dependent pathways (34). Because in humans, cholestatic liver diseases are important risk factors for cholangiocarcinoma, the finding that TUDCA is effective in improving clinical and histological features of cholestatic liver diseases, including PBC and PSC (2, 52), has important pathophysiological relevance. Our findings suggest that the use of TUDCA in these clinical settings may not only improve clinical outcomes of cholestatic liver diseases by slowing the development of these diseases but has the potential to prevent the initiation or the development of cholangiocarcinoma.

GRANTS

This work was supported by a grant award to G. LeSage and G. Alpini from Scott & White Hospital and The Texas A&M University System, by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant DK-54208 (to G. LeSage), and by a Veterans Affairs Merit Award and NIDDK Grant DK-58411 (to G. Alpini).

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