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

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Tauroursodeoxycholate inhibits human cholangiocarcinoma growth via Ca\(^{2+}\)-, PKC-, and MAPK-dependent pathways. Am J Physiol Gastrointest Liver Physiol 286: G973–G982, 2004. First published December 30, 2003; 10.1152/ajpgi.00270.2003.—Tauroursodeoxycholate (TUDCA) is used for the treatment of cholangiopathies including primary sclerosing cholangitis, which is considered the primary risk factor for cholangiocarcinoma. The effect of TUDCA on cholangiocarcinoma growth is unknown. We evaluated the role of TUDCA in the regulation of growth of the cholangiocarcinoma cell line Mz-ChA-1. TUDCA inhibited the growth of Mz-ChA-1 cells in a concentration- and time-dependent manners. TUDCA inhibition of cholangiocarcinoma growth was blocked by BAPTA-AM, an intracellular Ca\(^{2+}\) chelator, and H7, a PKC-α inhibitor. TUDCA increased [Ca\(^{2+}\)]\(_i\) and membrane translocation of the Ca\(^{2+}\)-dependent PKC-α in Mz-ChA-1 cells. TUDCA inhibited the activity of MAPK, and this inhibitory effect of TUDCA was abrogated by BAPTA-AM and H7. TUDCA did not alter the activity of Raf-1 and B-Raf and the phosphorylation of MAPK p38 and JNK/stress-activated protein kinase. 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/stress-activated protein kinases. TUDCA may be important for the treatment of cholangiocarcinoma.

bile acids; bile ducts; cyclic adenosine monophosphate; cancer; mitosis

INTRAHEPATIC BILE DUCT epithelial cells (i.e., cholangiocytes) are the target cells in a number of chronic liver diseases including primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), graft-vs.-host disease, and cholangiocarcinoma (2). The growth of cholangiocarcinoma is modulated by a number of factors, including somatostatin (61), estrogens (57), adrenocortic- innervation (34), and gastrin (33). Due to our lack of understanding of the mechanisms of cholangiocarcinoma growth, there are no established measures to treat this neoplasm (2).

Bile acids regulate the growth of different epithelial cells (16, 18), including cholangiocytes (3). Bile acids have been implicated as tumor promoters (e.g., enhancing colonic epithelial cell proliferation) and are also associated with the development of tumors (16, 18, 42). On the other hand, other studies have shown that unconjugated hydrophobic bile acids do not enhance cell growth but rather have cytotoxic effects against various cell types (58), including cholangiocytes (10). The therapeutic bile acids ursodeoxycholate (UDCA) and its taurine conjugate tauroursodeoxycholate (TUDCA) have been effectively 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 cytoprotective effects (20, 63), preventing apoptosis (11) and choleretic effects on 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 phosphoinositide 3-kinase in normal colonic epithelial cells and colorectal cancer tissue (50). Furthermore, sodium tauro lithocholate reduces hepatocyte canicular secretion through activation of PKC-ε (13). In hepatocytes, glycochenodeoxycholate-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 tauroliothiocholate 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 Thr183 and Tyr185 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-η 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 activities, upstream regulators of MAPK? 5) Is TUDCA-inhibition of MAPK associated with changes in the phosphorylation of MAPK p42/44, p38, and JNK/stress-activated protein kinases (SAPKs)?

MATERIALS AND METHODS

Materials

Reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA; clone PC10); rabbit polyclonal antibody against Raf-1 (clone C-12); mouse monoclonal antibody against B-Raf (clone F-7); horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG; HRP-conjugated goat anti-mouse IgG; mouse monoclonal (IgG1) antibody p-JNK (clone G-7), which detects JNK1, JNK2, and JNK3 phosphorylated at Thr183 and Tyr185 of human origin; rabbit polyclonal antibody (clone FL), which was produced by immunization with full-length (amino acids 1–384) human JNK1 produced in Escherichia coli and reacts with JNK1, JNK2 p54, and JNK3; mouse monoclonal (IgG) antibody pp38 (clone D-8), which detects Tyr182-phosphorylated MAPK p38, Mxi2, and p38β; mouse monoclonal (IgG1) antibody p38 (clone A-12), which detects total MAPK p38 and p38β; rabbit monoclonal (IgG) antibody ERK1 (clone C-16), which detects the MAPK p44 and p42; rabbit monoclonal (IgG) antibody ERK2 (clone C-14), which detects the MAPK p44 and p42; and mouse monoclonal (IgG) antibody pERK (clone N-18), which detects phosphorylated MAPK p44 and p42; and rat Ca2+ -dependent PKC-α (rabbit IgG). [Methyl-3H]thymidine and [γ-32P]ATP were purchased from New England Nuclear Life Science Products, (Boston, MA). Raf-1 immunoprecipitation–kinase cascade assay kit, MAPK immunoprecipitation cascade assay kit (which detects MAPK p44/p42), Ras activation assay kit, protein A agarose beads, and protein G agarose beads were purchased from Upstate Biotechnology (Lake Placid, NY). Nitrocellulose membrane (0.2 μm) and Bio-Rad protein assay were purchased from Bio-Rad Laboratories (Hercules, CA).

Cell Line Culture

Mz-ChA-1 cells (human gallbladder in origin) (36) were a gift from Dr. Fitz (University of Colorado, Denver, CO). We have previously used this cell line to evaluate the effect of gastrin and the α1-adrenergic receptor agonist UK-14304 on cholangiocarcinoma growth (33). Cells were maintained at 37°C in a 5% CO2 incubator with the conditioned culture medium (CCM) composed of CMRL Medium-1066 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin and streptomycin, and 2 mM l-glutamine.

Effect of TUDCA on the Growth of Mz-ChA-1 Cells

Measurement of [3H]thymidine incorporation. After trypsinization, Mz-ChA-1 cells were suspended in CCM at 5 × 104 cells/ml, and 200 μl of the cell suspension were seeded into flat-bottomed 96-well plates. 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 PCNA protein expression. Mz-ChA-1 cells (1.5 × 104) 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 2) TUDCA (200 μM) in the absence or presence of BAPTA-AM (a chelator of [Ca2+ ]i); 5 μM (33, 34) or H7 (a PKC-α inhibitor; 2 μM) (33) for 48 h. After the selected treatment, cells were washed twice with ice-cold PBS and then lysed 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 phenylmethylsulfon- nyl 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 1x 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-PCNA antibody diluted to 1:200 as the primary antibody overnight 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+ ], 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+ ]; levels were determined by a microfluorescent technique (1, 30) in Mz-ChA-1 cells previously loaded with the fluorescent Ca2+ indicator flou-3 (1 μM for 10 min). The flou-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 1:1,000) 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 ChemiImager 4000. The effects of TUDCA on [Ca²⁺], and PKC-α protein expression were performed at different incubation times with TUDCA (15 min for Ca²⁺ and 90 min for PKC), because we anticipated that the Ca²⁺-dependent activation of PKC-α protein expression would occur at a later time than the increase in [Ca²⁺]. 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 period (15 min for Ca²⁺ 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 Ca²⁺ levels and the expression and membrane translocation of Ca²⁺-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 antibodies (B-Raf primary antibodies and the corresponding secondary antibodies). Additionally, the expression of Raf-1 and B-Raf in Mz-ChA-1 cells was evaluated by immunoblotting for PCNA expression except for the use of mouse anti-PKC-α (diluted to 1:1,000) 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 ChemiImager 4000. The effects of TUDCA on [Ca²⁺], and PKC-α protein expression were performed at different incubation times with TUDCA (15 min for Ca²⁺ and 90 min for PKC), because we anticipated that the Ca²⁺-dependent activation of PKC-α protein expression would occur at a later time than the increase in [Ca²⁺]. 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 period (15 min for Ca²⁺ 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 Ca²⁺ levels and the expression and membrane translocation of Ca²⁺-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).

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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 × 10^6) 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 system. The cell samples were treated according to the manufacturer’s protocols (Upstate Biotechnology) of the Raf-1 immunoprecipitation kinase cascade assay kit and MAPK immunoprecipitation cascade assay kit, with the difference that in the B-raf assay goat anti-B-Raf antibodies (clone C-19) instead of anti-Raf-1 were used. Raf-1, B-Raf, and MAPK activities were evaluated with the phosphorylated MBP kinase cascade assay kit, with the difference that in the B-raf assay goat anti-B-Raf antibodies and the corresponding secondary antibodies. After washes, phosphorylation of MAPK p42/44, p38, and JNK/SAPKs in Mz-ChA-1 cells was evaluated by immunoblotting (25, 27, 34) using the selected primary antibodies and the corresponding secondary antibodies. After washes,

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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 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 employing 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.

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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 [3H]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 [3H]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 [Ca2+]i 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 [Ca2+]i 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 Ca2+ 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.

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 p44/p42, 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 cholano-
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, 46). 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 tauroliotholate) 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 EGF 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 EGF 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 neupeptides 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 inostiol 1,4,5-trisphosphate- and PKC-α-dependent
pathways (33). α2-Adrenergic receptor stimulation also inhibits the growth via cAMP-PAK-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.

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REFERENCES

8. Baumgartner U, Scholmerich J, Sellige M, Reinhardt M, Ruf G, and Farthmann EH. Different protective effects of tauroursodeoxycholate, ursodeoxycholate, and 25-methyl-ursodeoxycholate against tauro- 


