Activation of CREB by TUDCA protects cholangiocytes from apoptosis induced by mTOR inhibition. Hepatology

ARTICLE *in* HEPATOLOGY · JUNE 2005

Impact Factor: 11.06 · DOI: 10.1002/hep.20697 · Source: PubMed

5 AUTHORS, INCLUDING:

Anne-Christine Piguet
Universität Bern
31 PUBLICATIONS **466** CITATIONS
SEE PROFILE

Jean-Francois Dufour
Universität Bern
294 PUBLICATIONS **5,212** CITATIONS
SEE PROFILE

Activation of CREB by Tauroursodeoxycholic Acid Protects Cholangiocytes From Apoptosis Induced by mTOR Inhibition

LiFu Wang,[1] Anne-Christine Piguet,[1] Karin Schmidt,[1] Thierry Tordjmann,[2] and Jean-François Dufour[1]

Tauroursodeoxycholic acid (TUDCA) is a cytoprotective bile acid frequently prescribed to patients with cholestatic diseases. Several mechanisms of action have been investigated, but the possibility that cyclic adenosine monophosphate responsive element binding protein (CREB), a transcription factor promoting cell survival, mediates TUDCA's protective effects has not been considered. We examined whether TUDCA activates CREB and whether this activation can protect biliary epithelial cells. Cholangiocytes were stressed by exposure to CCI-779, which inhibits signaling though the kinase mTOR (mammalian target of rapamycin), resulting in cell cycle arrest and apoptosis. Incubation of normal rat cholangiocytes (NRC) cells, with TUDCA resulted in phosphorylation of CREB (Western blotting analysis) and activation of CREB transcription activity (luciferase reporter assay). Inhibition of calcium signals and inhibition of protein kinase C prevented the TUDCA-induced activation of CREB. CCI-779 decreased the viability of rat cholangiocytes in a dose-dependent manner (MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay). TUDCA protected against CCI-779 cytotoxicity. A dominant negative form of CREB was stably transduced in NRC cells (NRC-M1). TUDCA protection was decreased in NRC-M1. While CCI-779 induced apoptosis in NRC cells as determined by caspase 3 activity, TUDCA attenuated CCI-779 –induced apoptosis, an effect absent in NRC-M1. Finally, CCI-779 blocked proliferation of both NRC and NRC-M1 (thymidine incorporation) and this was unaffected by TUDCA. In conclusion, TUDCA activates CREB in cholangiocytes, reducing the apoptotic effect of CCI-779. These findings suggest a novel cytoprotective mechanism for this bile acid. (HEPATOLOGY **2005;41:1241-1251.**)

Cell death is an important pathogenic feature of liver diseases,[1] in particular apoptosis in cases of biliary disorders.[2] Apoptosis does not affect only hepatocytes but also cholangiocytes.[3-5] Ursodeoxycholic acid (UDCA), which is conjugated with taurine *in vivo* to form tauroursodeoxycholic acid (TUDCA), is used to treat cholestatic disorders.[6] In hepatocytes, TUDCA increases the cytosolic free Ca^{2+} concentration[7] and translocates α-protein kinase C (α-PKC) isoform from the cytosol to the plasma membrane.[8] TUDCA activates p38 mitogen-activated protein kinase (MAPK) and inserts the bile salt export pump,[9] a substrate of PKC,[10] and the organic anion export pump Mrp2 into the canalicular membrane, increasing the bile flow.[11] TUDCA is not only choleretic, but also cytoprotective, and regulates the apoptotic threshold.[12,13] This hydrophilic bile acid protects hepatocytes from apoptosis caused by a variety of agents, such as hydrophobic bile acids,[14] ethanol, transforming growth factor-β1, and anti-Fas antibody.[15] Several mechanisms whereby UDCA confers protection in hepatocytes have been proposed, such as modulation of mitochondrial membrane integrity, replenishment of glutathione, and activation of transcription factors such as E2F-1,[16] glucocorticoid, and mineralocorticoid receptor.[17] The antiapoptotic effect of TUDCA in hepatocytes

Abbreviations: $[Ca^{2+}]_i$, cytosolic free calcium concentration; TUDCA, tauroursodeoxycholic acid; MAPK, mitogen-activated protein kinase; S6K1, S6 ribosomal protein kinase 1; CREB, cyclic adenosine monophosphate responsive element binding protein; cAMP, cyclic adenosine monophosphate; mTOR, mammalian target of rapamycin; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PMA, phorbol 12-myristate 13-acetate; NRC, normal rat cholangiocyte; SDS, sodium dodecyl sulfate; PKC, protein kinase C; EGTA, ethyleneglycoltetraacetic acid.

From the [1]Department of Clinical Pharmacology, University of Bern, Switzerland; and [2]INSERM U442, University Paris XI, Paris, France.

Received October 22, 2004; accepted March 4, 2005.

Supported by grants from the Swiss National Foundation 3100-100513, the "Büttner Stiftung," and the "Stiftung für die Leberkrankheiten" (J.-F.D.).

Address reprint requests to: Jean-François Dufour, Institute for Clinical Pharmacology, University of Bern, 35 Murtenstrasse, 3010 Bern, Switzerland. E-mail: jf.dufour@ikp.unibe.ch; fax: (41) 31-632-49-97

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20697

Potential conflict of interest: Nothing to report.

has been linked to the phosphorylation of MAPK via the stimulation of the epidermal growth factor receptor[18] as well as the activation of p38MAPK and ERK.[19] In contrast, the antiapoptotic effects of TUDCA in cholangiocytes have not been investigated.

Stimulation of cyclic adenosine monophosphate responsive element binding protein rescues cells from apoptosis.[20,21] Initially discovered for its ability to bind to cyclic adenosine monophosphate (cAMP)-responsive element, cAMP responsive element binding protein (CREB) is a transcription factor that integrates growth factors, Ca^{2+}, and cyclic AMP–induced signals.[22] Cyclic AMP-dependent protein kinase, Ca^{2+}/calmodulin-dependent protein kinase, p90RSK, Akt, and MSK1 are kinases able to phosphorylate CREB on serine 133.[22] Phosphorylated CREB binds to DNA as a dimer via a leucine zipper motif that recognizes cAMP-responsive elements and stimulates the transcription of genes. This results in the expression of numerous proteins,[22,23] some of them of particular interest in biliary physiology, such as cystic fibrosis transmembrane regulator,[24] somatostatin receptor type 2,[25] and the antiapoptotic factor Bcl-2.[21] Whether TUDCA can stimulate the phosphorylation of CREB in biliary epithelial cells is unknown.

Cellular stress and apoptosis can be induced experimentally by CCI-779, a prodrug ester of the immunosuppressor sirolimus.[26] Sirolimus forms a complex with the cytosolic protein FKBP-12, and this complex binds a serine/threonine kinase, the mammalian target of rapamycin (mTOR), inhibiting its function. The kinase mTOR phosphorylates p70 S6 ribosomal protein kinase (S6K1) and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Inactivation of S6K1 blocks the translation of mRNA species containing 5′terminal oligopyrimidine tracts, and hypophosphorylated 4E-BP1 associates with eukaryotic initiation factor 4E, preventing cap-dependent translation of mRNA. The availability of nutrients and growth factors regulate mTOR, ensuring that protein synthesis occurs when the supply of precursors amino acids is sufficient.[27,28] CCI-779 is currently tested as an antitumoral agent, because mTOR inhibition blocks cell cycle progression[29] and promotes apoptosis,[30] particularly in cells with mutations of *p53* or PTEN, suggesting tumor-selective effects.[31]

The aims of this study were to determine whether TUDCA phosphorylates and activates CREB in cholangiocytes, to test the antiproliferative and apoptotic effects of CCI-779 in these cells, and to investigate whether TUDCA-induced CREB activation could rescue cholangiocytes exposed to CCI-779.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 medium, fetal bovine serum, forskolin, nonessential amino acids, chemically defined lipids, trypsin inhibitor, dexamethasone, bovine pituitary extract, 3,3′,5-triiodo-L-thyronine, epithelial growth factor, penicillin/streptomycin, gentamicin, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and the antibody against actin were from Sigma Chemical (Buchs, Switzerland). Rat tail collagen I–coated flask and plates were from BD Biosciences (Basel, Switzerland). TUDCA, taurochenodeoxycholic acid, phorbol 12-myristate 13-acetate (PMA), BAPTA/AM, KN-62, calphostin C, Gö 6983, chelerythrine, PD98050, and SB203580, as well as the protein kinase activity C assay, were from Calbiochem (Lucerne, Switzerland). CCI-779 was provided by Wyeth Ayerst (Wyeth AHP, Zug, Switzerland). Fura2 in acetoxy-methylated form, pluronic acid, and rhodamine-conjugated phalloidin were obtained from Molecular Probes (Eugene, OR). Restriction enzymes and the high-fidelity polymerase chain reaction (PCR) system were from Roche Diagnostics (Rotkreutz, Switzerland). The primers were obtained from Microsynth (Balgach, Switzerland). Antibodies against phospho-CREB, CREB, 4E-BP1, Bcl-2, Bcl-xL, and Bax were from Cell Signaling (Allschwill, Switzerland), against c-$FLIP_L$ from Chemicon (Lucerne, Switzerland). The secondary anti-rabbit antibody was from Pierce (Lausanne, Switzerland). [^{3}H]-thymidine was from Amersham Biosciences (Otelfingen, Switzerland). Path-Detect CRE cis-reporting system was from Stratagene (Amsterdam, the Netherlands). Luciferase assay system was from Promega (Wallisellen, Switzerland).

Cell Culture. The normal rat cholangiocyte (NRC) cell line was kindly provided by N. LaRusso (Mayo Clinic, Rochester, MN), and the 293T cells by D. Schuemperli (University of Bern). NRC cells were grown on rat tail collagen I–coated flasks or plates in DMEM-Ham's F-12 medium with serum and supplements as described by Vroman and La Russo.[32] Cells reached confluence in 72 hours under incubation at 37°C in the presence of 5% CO_2. Before stimulation, the cells were kept for 24 hours in DMEM-Ham's F-12 medium with nonessential amino acids, chemically defined lipids, MEM vitamins solution, trypsin inhibitors, and 0.393 μg/mL dexamethasone and without serum. To produce lentiviruses, 293T cells were grown in DMEM with 10% fetal bovine serum at 37°C in the presence of 5% CO_2.

Plasmid Construction. RSV CREB, RSV CREB M1 (provided by M. Montminy, La Jolla, CA),[33] and pCRE-Luc (Stratagene) were used to clone CREB, CREB M1, and

CRE-Luc reporter genes into lentivector pWPT-GFP (provided by D. Trono, University of Geneva), respectively. Briefly, the genes of CREB and CREB M1 were amplified by high-fidelity polymerase chain reaction (Roche Diagnostics) with the primers of 5′-GCGGATCCGAATTCATGACCATGGACTCTGGA-3′ (forward) and 5′-GGCTCTAGAGTCGACTTAATCTGACTTGTGG CAGT-3′ (reverse). The fragments cut by *Bam*HI and *Sal*I were inserted into pWPT-GFP digested *Bam*HI and *Sal*I to replace the GFP gene under the control of the EF-1 promoter. For generation of CRE-Luc reporter in lentivector, the fragment including CRE enhancer, TATA box, and luciferase gene amplified with the primer of 5′-ACGGCTCGAGCATGTCTGGATCCAAGCT-3′ (forward) and 5′-ACGGCTCGAGCGTCA TCGCTGAATACA-3′ (reverse) was digested by *Xho*I and inserted into pWPT-GFP digested with *Xho*I to remove the GFP gene and the EF-1 promoter. The plasmids were confirmed by sequencing.

Retroviral Transduction of NRC Cells. A lentivirus vector with a conditional packaging system was provided by D. Trono (University of Geneva) to obtain efficient gene transduction.[34] Lentiviruses were produced by transient transfection into 293T cells (80% confluence) of 5 μg pMD.G, 10 μg pCMVδ8.91, and 15 μg CREB-pWPT, or 15 μg CREB M1-pWPT, or 15 μg CRE-LUC-pWPT with calcium phosphate precipitation. After 36 hours, virus-containing supernatants were harvested, filtered through 0.45-μm filters. The viruses were concentrated by ultracentrifugation (26,000*g*, 90 minutes) and used to transduce NRC cells (80% confluence) supplemented with 8 μg/mL polybrene for 4 hours before changing the medium.

Western Blotting Analysis. At the end of stimulation time, cells were scraped and sonicated in a lysis buffer containing 15.6 mmol/L Tris-HCl (pH 6.8), 0.5% sodium dodecyl sulfate (SDS), 0.18 mol/L mercaptoethanol, 2% glycerol, protease inhibitor cocktail Complete Mini from Roche, and 1 mmol/L phenylmethyl sulfonyl fluoride. Whole-cell lysates were separated by electrophoresis through a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked in TBST buffer containing 10 mmol/L Tris-HCl (pH 7.6), 0.1 mol/L NaCl, 0.1% Tween 20 with 5% bovine serum albumin and nonfat dry milk for 2 hours, and then incubated in TBST with 5% BSA containing anti-phospho CREB 1:1000 for 2 hours at room temperature. The membrane was washed in TBST, and then incubated with secondary anti-rabbit IgG-coupled HRP antibody 1:60,000 (Pierce). After 60 minutes, blots were washed with TBST, and visualized by enhanced chemiluminescence (Perkin Elmer, Zaventen, Belgium). The membrane was stripped in 0.1 mol/L Glycin (pH 2.5) for 1 hour, washed in TBST for 15 minutes each and then re-probed by anti-CREB 1:1,000 and then antiactin 1:1,000 with the same procedure. The other primary antibodies were used at a 1:1,000 dilution.

Cis-CREB Reporter Assay. Cells with stable retroviral transduction of CRE-Luc were seeded in 12-well plates and grown to 80% confluence. After initial incubation with specific antagonists when required at 37°C for 1 hour, cells were stimulated with TUDCA for an additional 5 hours. Cells were washed once with 1× phosphate-buffered saline (PBS), scraped in 100 μL 1× lysis buffer, and centrifuged 12,000*g* for 2 minutes at 4°C. Luciferase activity was read immediately in a luminometer after mixing 20 μL supernatant of cell lysate and 100 μL luciferase assay substrate (Promega). The protein concentration of cell lysate was measured according to Lowry to normalize luciferase activity.[35]

Cyclic Adenosine Monophosphate Measurement. NRC cells were grown in 24-well plates to 80% confluence before being exposed to TUDCA. Intracellular cAMP was measured according to the procedure described in the kit of the cAMP Biotrak Enzyme Immunoassay (EIA) system (Amersham Biosciences).

MTT Assay. Cells were seeded in 96-well plates at density of 4×10^3/well. After 24 hours, cells were treated with CCI-779 or TUDCA at given concentrations for 48 hours. At the end of experiment, 12.5 μL 5 mg/mL [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) was added to each well, followed by incubation at 37°C for 4 hours. This assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals that are impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The medium was carefully aspirated, and 100 μL isopropanol containing 0.04 N HCl was then added to each well to dissolve blue formazan crystals formed in the live cells. Optical density was measured with a spectrometer at 570 nm.

[^{3}H] Thymidine Incorporation. Cells were seeded in 24-well plates at a density of 2×10^4/well. After 24 hours, cells were treated with CCI-779 or TUDCA at given concentrations for 48 hours. [^{3}H] Thymidine (Amersham Biosciences) was added with 0.1 μCi per well 4 hours before collecting the samples. Cells were washed with 1× PBS, fixed with ice-cold 5% trichloroacetic acid at 4°C and lysed in 250 μL of 1 N NaOH/0.1% SDS. The reaction was stopped with 250 μL of 1 N HCl, and samples were collected and mixed with 4.5 mL liquid of scintillation. The [^{3}H] thymidine incorporation was determined after analysis in a scintillation counter.

Cytosolic Ca^{2+} Measurements. NRC cells were dye-

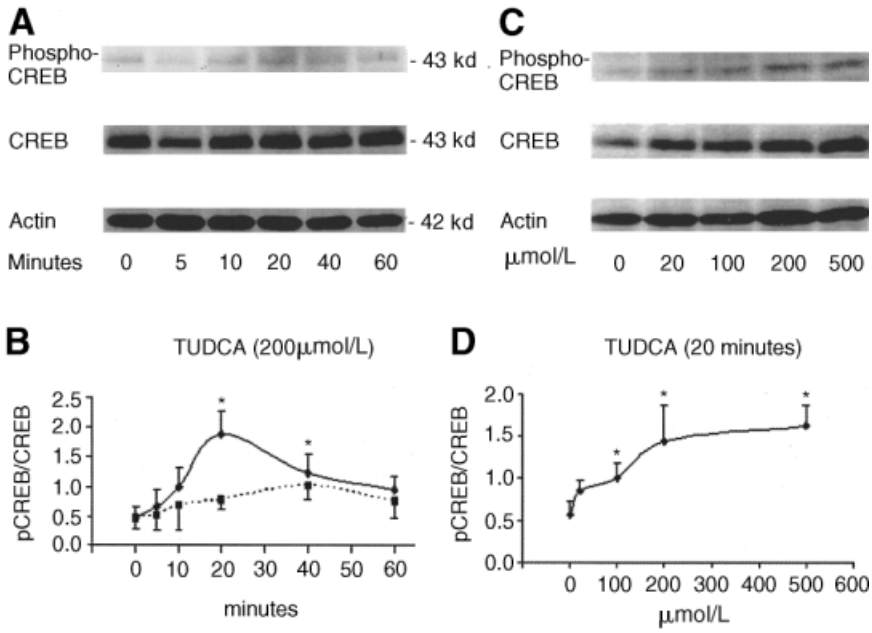

Fig. 1. TUDCA induces the phosphorylation of CREB in NRC cells. (A) Cells were incubated with 200 μmol/L TUDCA for the indicated times. The top panel shows a representative immunoblot of phosphorylated CREB. The middle panel is the same membrane re-probed after stripping with an anti-CREB antibody; the lower panel, with an antiactin antibody. (B) Quantification of the phosphorylation of CREB in the minutes after exposure of NRC cells to 200 μmol/L TUDCA (n = 6). This time course reveals that TUDCA-induced phosphorylation peaked after 20 minutes of incubation (*$P <$ .05 in comparison to 0 minutes). The dashed line represents the time course with 200 μmol/L taurochenodeoxycholic acid (n = 3), which shows that exposure to this bile acid does not lead to the phosphorylation of CREB. (C) Cells were incubated with increasing concentrations of TUDCA for 20 minutes. The top panel shows a representative immunoblot of phosphorylated CREB. The middle panel is the same membrane re-probed after stripping with an anti-CREB antibody and the lower panel with an antiactin antibody. (D) Quantification of the phosphorylation of CREB showed a dose-dependent effect of TUDCA (n = 4-6). Phosphorylation was statistically significant with 100 μmol/L TUDCA, and the ratio pCREB/CREB increased with the concentration of TUDCA (*$P <$ .05 in comparison to incubation without TUDCA). TUDCA, tauroursodeoxycholic acid; NRC, normal rat cholangiocyte; CREB, cyclic AMP responsive elemental binding protein.

loaded by incubation with fura2,[36] then placed on the thermostated stage of a Zeiss Axiovert 35 epifluorescence microscope and superfused with continually renewed modified Eagle's medium. Perfusing solutions (saline containing 10 mmol/L HEPES, 116 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.8 mmol/L $MgCl_2$, 0.96 mmol/L NaH_2PO_4, 5 mmol/L $NaHCO_3$, and glucose 1 g/L, pH 7.4, and agonists) converged on the chamber by inlet tubes, at 34°C.[37] The excitation light was supplied by a high-pressure xenon arc lamp (75-watt), and the excitation wavelengths were selected by 340- and 380-nm filters (10-nm bandwidth) mounted in a processor-controlled rotating filter wheel (Sutter, Novato, CA) between the ultraviolet lamp and the microscope. Fluorescence images were collected by a low-light level ISIT camera (Lhesa, France), digitized, and integrated in real time by an image processor (Metafluor, Princeton, NJ).

Protein Kinase C Activity Assay. NRC cells were grown in 6-well plates to 80% confluence and then placed in minimal essential medium. After 24 hours, cells were pretreated with PKC inhibitors for 15 minutes and treated for 10 minutes with 100 nmol/L PMA. Cells were collected and sonicated. Lysates were centrifuged for 8 minutes at 140,000*g* (TL-100 Ultracentrifuge, Beckmann Instruments, Palo Alto, CA). The assay was carried out twice in triplicate, following the Protein Kinase C Assay Kit instructions. Optical density was measured with a microplate-reader (Sunrise, Tecon, Austria).

Caspase 3 Activity Assay. Cells were seeded in 96-well plates at a density of 4×10^3 cells/well. After 24 hours, cells were treated with CCI-779 with or without TUDCA for the times indicated. The supernatant of the cells was removed, and the cell layer was washed once with PBS. Ten microliters of lysis buffer was added in each well. After 1 hour on ice, the activity of caspase 3 was measured by adding 200 μL of the reaction mix (fluorimetric Caspase 3 Assay kit, Sigma Chemical) and by determining the fluorescence 3 hours later in a microplate fluorometer (excitation, 360 nm; emission, 460 nm).

Flow Cytometry. After 24 hours of incubation, cells were harvested with trypsin and labeled with fluorescein isothiocyanate–conjugated Annexin V and propidium iodide (MBL, LabForce, Nunnigen, Switzerland). Annexin V binds with high affinity to phosphatidylserine, a phospholipid that becomes exposed at the cell surface after induction of apoptosis. Propidium iodide is a marker of

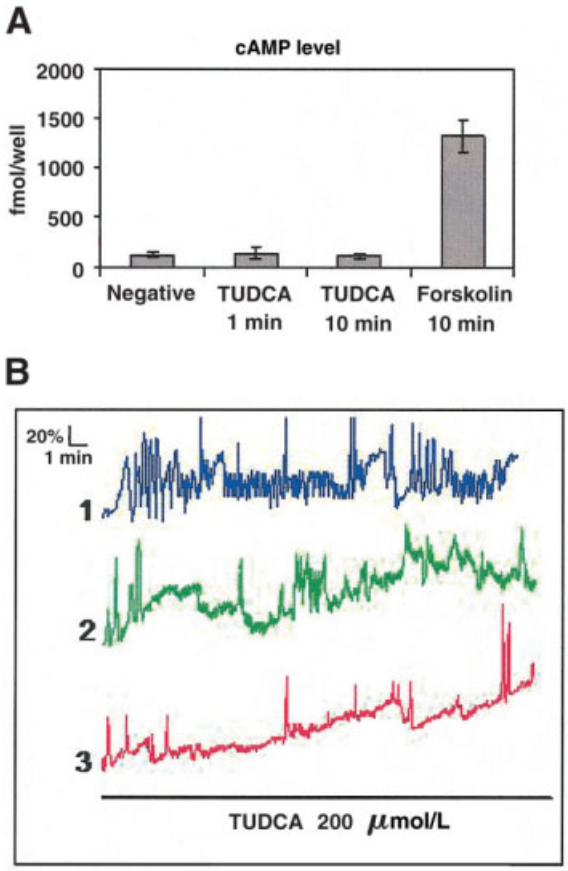

Fig. 2. TUDCA stimulates Ca^{2+} signals and not cAMP levels in NRC cells. (A) Cells were incubated with 200 μmol/L TUDCA or 10 μmol/L forskolin. Cyclic AMP content was determined by enzyme immunoassay. Forskolin stimulated the production of cAMP, but TUDCA did not (n = 3 in triplicates). (B) TUDCA-induced Ca^{2+} signals in Fura2-loaded NRC cells. The figure shows three traces showing the response of intracellular Ca^{2+} in 3 representative cells out of those obtained with 63 cells in 4 experiments. Cells were stimulated with 200 μmol/L TUDCA; time is indicated by the horizontal bar. $[Ca^{2+}]_i$ is expressed as Fura2 fluorescence ratio. The baseline was the same for the three traces, which are vertically spaced for clarity. Scale is shown in the left upper corner. TUDCA, tauroursodeoxycholic acid; cAMP, cyclic adenosine monophosphate; NRC, normal rat cholangiocyte.

necrotic cells because this substance only enters the cell on loss of membrane integrity. A Becton Dickinson BD FacScan flow cytometer was used to quantify Annexin V–positive propidium iodide–negative apoptotic cells.

Statistics. Results are expressed as mean ± standard deviation. Mann-Whitney *U* test or, when specified, Kruskal-Wallis ANOVA test were applied. A *P* value of .05 or less was considered statistically significant.

Results

Western blotting analysis with antibodies specific for CREB and for phosphorylated CREB at serine 133 showed that normal rat cholangiocytes express CREB and that incubation with the bile acid TUDCA leads to phosphorylation of CREB (Fig. 1A). This phosphorylation peaked after 20 minutes of exposure to 200 μmol/L TUDCA (Fig. 1B). Exposure to taurochenodeoxycholic acid did not stimulate the phosphorylation of CREB (Fig. 1B). Phosphorylation of CREB was significant with 100 μmol/L TUDCA and increased with higher concentrations (Fig. 1C-D).

To determine which messengers are involved in the phosphorylation of CREB in response to TUDCA, we measured cAMP and cytosolic Ca^{2+}. As expected, forskolin increased in a sustained manner the level of cAMP in NRC cells. In contrast, TUDCA did not increase the level of cAMP in NRC cells (Fig. 2A). Experiments with fluorescent videomicroscopy in fura2-loaded NRC cells showed that 200 μmol/L TUDCA elicited an increase in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in approximately 50% of the cells (Fig. 2B). In most of the TUDCA-responding cells, $[Ca^{2+}]_i$ oscillations were observed, although latencies and oscillation frequencies were heterogeneous in a given microscope field, or between different experiments (n = 4). In a minority of cells (10%-20%), TUDCA induced large increases in $[Ca^{2+}]_i$ without the typical oscillating pattern (see trace 3, Fig. 2B).

Phosphorylation experiments with inhibitors showed that chelation of the intracellular Ca^{2+} with BAPTA/AM but not of the extracellular Ca^{2+} with ethyleneglycoltetraacetic acid (EGTA) prevented TUDCA-induced CREB phosphorylation. KN-62, which inhibits Ca^{2+}/calmodulin-dependent protein kinase, also blocked the phosphorylation of CREB by TUDCA. Different inhibitors of PKC prevented the phosphorylation of CREB by TUDCA. Calphostin C impairs the redistribution of PKC to the plasma membrane, whereas chlelerythrine and Gö 6983 inhibit the catalytic domain of the kinase. In NRC cells, 2 μmol/L chelerythrine and 2 μmol/L Gö 6983 inhibited PMA-induced PKC activity by 66% and 77%, respectively (Table 1). Inhibition of MAPKK by PD98050 and inhibition of p38MAPK by SB203580 did not decrease significantly the phosphorlyation of CREB in response to TUDCA (Fig. 3B).

Experiments with a reporter gene transduced in NRC cells, the luciferase gene under the control of CRE elements, showed that TUDCA-induced phosphorylation

Table 1. Protein Kinase C Activity

Compound	Optical Density
PMA	0.127 ± 0.013
PMA + chelerythrine	0.043 ± 0.006
PMA + Gö 6983	0.029 ± 0.001

NOTE. Results are given as mean ± standard deviation.

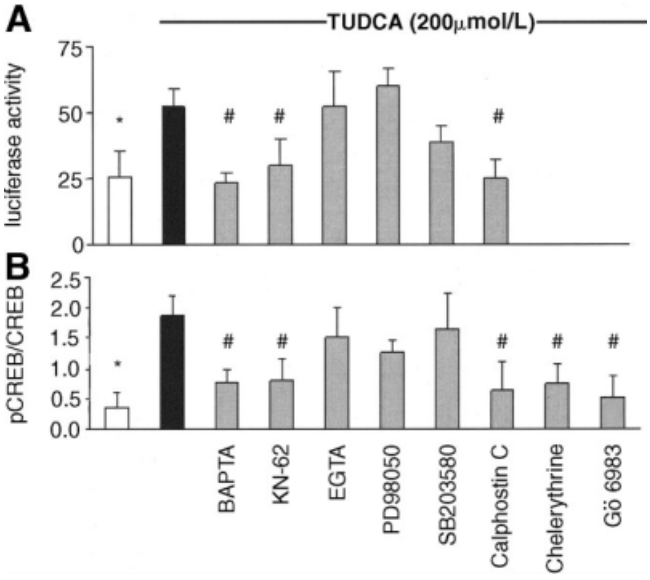

Fig. 3. Intracellular Ca^{2+} signaling and PKC mediate the phosphorylation and the transactivation of CREB. (A) TUDCA stimulates the transcription activity of CREB in NRC cells. NRC cells transduced with cis-CREB reporter system were incubated for 5 hours with 200 μmol/L TUDCA. TUDCA significantly increased the transcription activity of CREB ($n = 6$, $^*P < .004$). This stimulation was prevented by the presence of 50 μmol/L BAPTA/AM, which blocks cytosolic calcium signals, 1 μmol/L KN-62, which inhibits Ca^{2+}/calmodulin-dependent protein kinase, and 0.1 μmol/L calphostin C, which inhibits PKC ($n = 3$, $\#P < .05$), but not significantly in the presence of 0.5 mmol/L EGTA, which chelates extracellular calcium; 10 μmol/L PD98059, which inhibits MAPKK; and 10 μmol/L SB203580, which inhibits p38MAPK. (B) TUDCA-induced phosphorylation of CREB ($^*P < .04$) is significantly reduced in presence of 50 μmol/L BPTA/AM, 1 μmol/L KN-62 as well as 0.1 μmol/L calphostin C, 2 μmol/L chelerythrine, and 2 μmol/L Gö 6983, three inhibitors of PKC ($n = 3$, $\#P < .05$), but not significantly in the presence of 0.5 mmol/L EGTA, 10 μmol/L PD98059, and 10 μmol/L SB203580. CREB, cyclic adenosine monophosphate responsive element binding protein; TUDCA, tauroursodeoxycholic acid; NRC, normal rat cholangiocyte.

of CREB activates its transcriptional activity in cholangiocytes (Fig. 3A). In the presence of BAPTA/AM, which blocks cytosolic Ca^{2+} signals, the stimulation by TUDCA of the transcription activity of CREB was completely blocked. The presence of EGTA, which chelates the extracellular Ca^{2+}, had no significant effect. Addition of KN-62 resulted in a similar inhibition to that with the addition of BAPTA/AM, and calphostin C decreased also significantly the transactivation of CREB by TUDCA. Inhibition of MAPKK by PD98050 and inhibition of p38MAPK by SB203580 did not decrease significantly the TUDCA-induced transcription activity of CREB in line with the phosphorylation experiments. Taken together, these results suggest that Ca^{2+} signals and PKC are necessary for the activation of CREB by TUDCA.

To delineate the role of CREB activation, the CREB dominant negative M1, whose serine 133 has been mutated for an alanine, was transduced by using a lentivirus vector in NRC cells (NRC-M1 cells). In NRC-M1 cells, the extent of phosphorylation of CREB in response to forskolin as well as to TUDCA was significantly reduced and remained at levels observed in the absence of stimulation (Fig. 4). As a control, NRC cells were transduced with a lentivirus vector constructed with the sequence of wild-type CREB (NRC-Control).

To address the effect of the activation of CREB by TUDCA on cholangiocyte survival, NRC cells were exposed to CCI-779. CCI-779 acts as an inhibitor of mTOR, reducing the phosphorylation of 4E-BP1 (Fig. 5A). Incubation with CCI-779 was cytotoxic in a dose-dependent manner for NRC and NRC-M1 cells as determined by the MTT assay (Fig. 5B). This assay measures the ability of a mitochondrial dehydrogenase enzyme from viable cells to form coloured formazan crystals. The MTT assay is therefore directly proportional to the number of surviving cells. TUDCA (Fig. 5C) but not taurochenodeoxycholic acid (data not shown) decreased the cytotoxicity of CCI-779 in a dose-dependent manner in NRC cells. Importantly, this protective effect was lost in NRC-M1 cells, expressing the CREB dominant negative, and present in the NRC-Control cells (Fig. 5C). In contrast, TUDCA at concentrations up to 500 μmol/L was

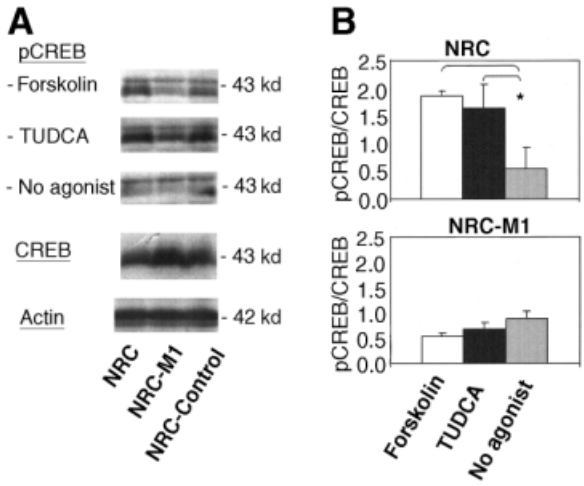

Fig. 4. TUDCA-induced phosphorylation of CREB is absent in NRC cells expressing the dominant negative M1. The cDNA for the dominant negative M1 or for the wild-type CREB were inserted in the lentivirus vector. The lentiviruses were produced in 293T cells, and NRC cells were transduced with them, providing a cell line expressing the dominant negative M1 (NRC-M1) and a control cell line expressing wild-type CREB (NRC-Control). (A) Western blot analysis of CREB and phosphorylated CREB after 20 minutes of incubation with 10 μmol/L forskolin, 200 μmol/L TUDCA, and no agonist. (B) Densitometric determination of the ratio pCREB/CREB ($n = 3$). Forskolin and TUDCA increased significantly the ratio pCREB/CREB in NRC cells (Kruskal-Wallis test, $^*P < .05$), but not in NRC-M1 cells (lower panel). CREB, cyclic adenosine monophosphate responsive element binding protein; TUDCA, tauroursodeoxycholic acid; NRC, normal rat cholangiocyte.

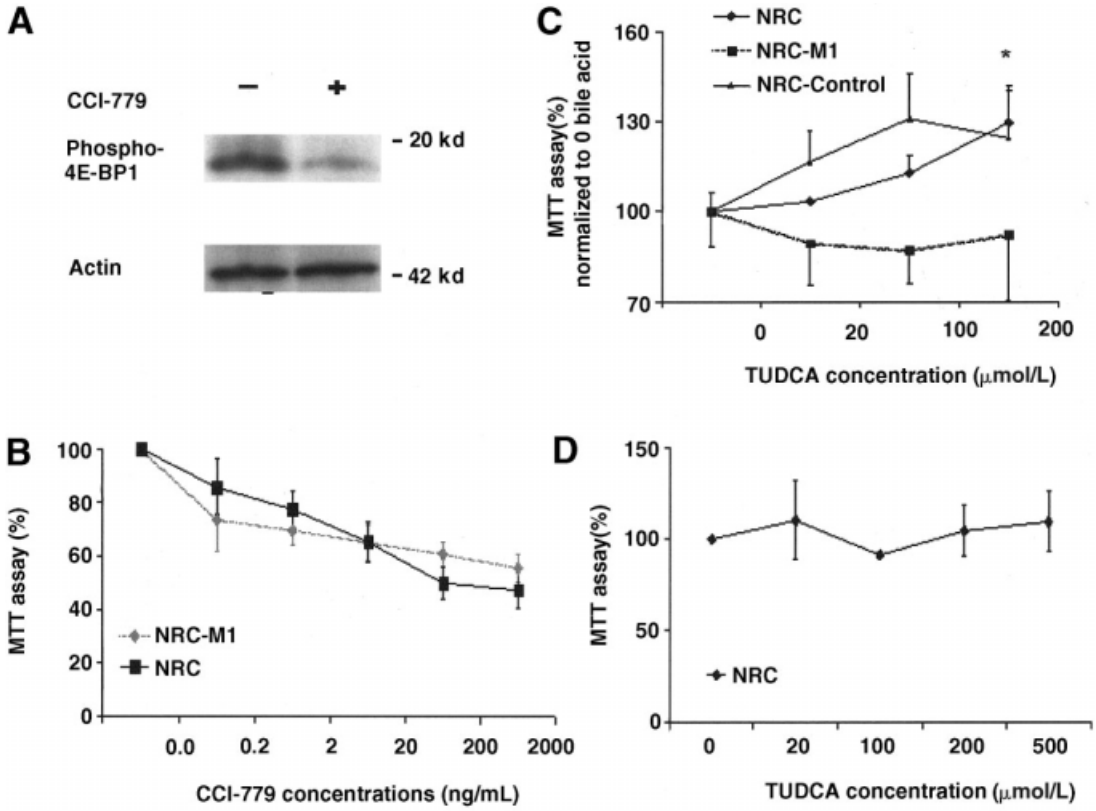

Fig. 5. TUDCA protects against the cytotoxicity of CCI-779 in NRC cells. (A) Western blotting analysis of the phosphorylated form of 4E-BP1, showing a decreased level of phosphorylation in the presence of the mTOR inhibitor CCI-779. (B) NRC cells and NRC-M1 cells were exposed to increasing concentrations of CCI-779 (0.2-2,000 ng/mL). After 48 hours MTT was added, and 4 hours later the formation of formazan was measured by optical density (n = 3 in triplicates). The results suggest that CCI-779 is cytotoxic in a comparable dose-dependent manner for the 2 cell lines. (C) TUDCA decreases the cytotoxicity of CCI-779 in NRC cells but not in NRC-M1 cells. Cells were exposed to CCI-779 0.2 μg/mL in the presence of different concentrations of TUDCA. After 48 hours, the cytotoxicity was assessed by the MTT assay. TUDCA decreased in a dose-dependent manner the cytotoxic effect of CCI-779 in NRC cells and in NRC-Control cells. This protective effect of TUDCA was not observed in the cell line expressing M1, a mutated CREB lacking the serine 133 (n = 4 in triplicates; Kruskal-Wallis $*P < .03$). (D) NRC cells were exposed to increasing concentrations of TUDCA (20-500 μmol/L). After 48 hours MTT was added, and 4 hours later the formation of formazan was measured by optical density (n = 3 in triplicates, mean ± standard deviation). TUDCA was not cytotoxic. TUDCA, tauroursodeoxycholic acid; NRC, normal rat cholangiocyte; CREB, cyclic adenosine monophosphate responsive element binding protein.

not cytotoxic (Fig. 5D). As shown in Fig. 6, the effect of TUDCA was not attributable to a reduction of the antiproliferative effect of CCI-779. This mTOR inhibitor significantly decreased thymidine incorporation in NRC, NRC-M1, and NRC-Control cells independently of the presence of TUDCA (Fig. 6).

Incubation With CCI-779–Induced Apoptosis in NRC Cells. The number of apoptotic cells detected by flow cytometry increased significantly with CCI-779, and this effect was attenuated by TUDCA (Fig. 7A). CCI-779 increased significantly the activity of the caspase 3 in NRC cells, with a peak at 6 hours (Fig. 7B). This apoptotic effect observed in NRC, NRC-M1, and NRC-Control cells was reduced in the presence of 100 μmol/L TUDCA and further in the presence of 200 μmol/L TUDCA in NRC and in NRC-Control cells, but not in NRC-M1 cells (Fig. 7C). The antiapoptotic effect of TUDCA was partially reversed when intracellular Ca^{2+} signals were chelated by BAPTA, suggesting a role for this messenger in mediating the protective effect of TUDCA (Fig. 7D). The expression of *Bcl-2*, *Bcl-xL*, and *Bax* was not affected by the presence of 200 μmol/L TUDCA in NRC cells (Fig. 8A). Real-time quantitative PCR experiments could not detect changes in the abundance of *Bcl-2* mRNA in presence of TUDCA (data not shown). The expression of c-$FLIP_L$ in NRC cells nearly doubles in the hours after TUDCA exposure (Fig. 8B), but this was not statistically significant.

Discussion

The results show that (1) TUDCA activates CREB in biliary epithelial cells in a Ca^{2+}- and PKC-dependent manner; (2) CCI-779 has cytotoxic effects on biliary epithelial cells; and (3) CREB activation by TUDCA attenuates the cytotoxic effects of CCI-779.

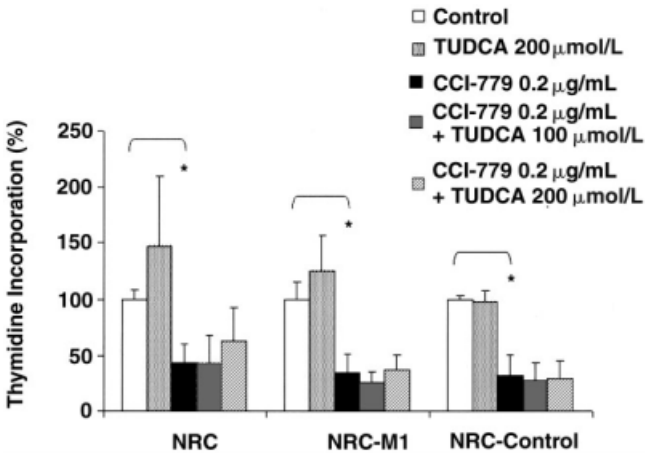

Fig. 6. CCI-779 inhibits NRC cell proliferation. CCI-779 blocks NRC cell proliferation. Incorporation of tritiated thymidine was reduced by 60% in NRC cells incubated with 0.2 μg/mL CCI-779. The same was observed in cells expressing the dominant negative M1 (NRC-M1) and in cells expressing the wild-type CREB (NRC-Control) (n = 3 in triplicates). The difference of thymidine incorporation of cells exposed to CCI-779 with cells not exposed to CCI-779 was significant (Mann-Whitney *U* test, $*P < .01$). This effect was not influenced by incubation with TUDCA. CREB, cyclic adenosine monophosphate responsive element binding protein; TUDCA, tauroursodeoxycholic acid; NRC, normal rat cholangiocyte.

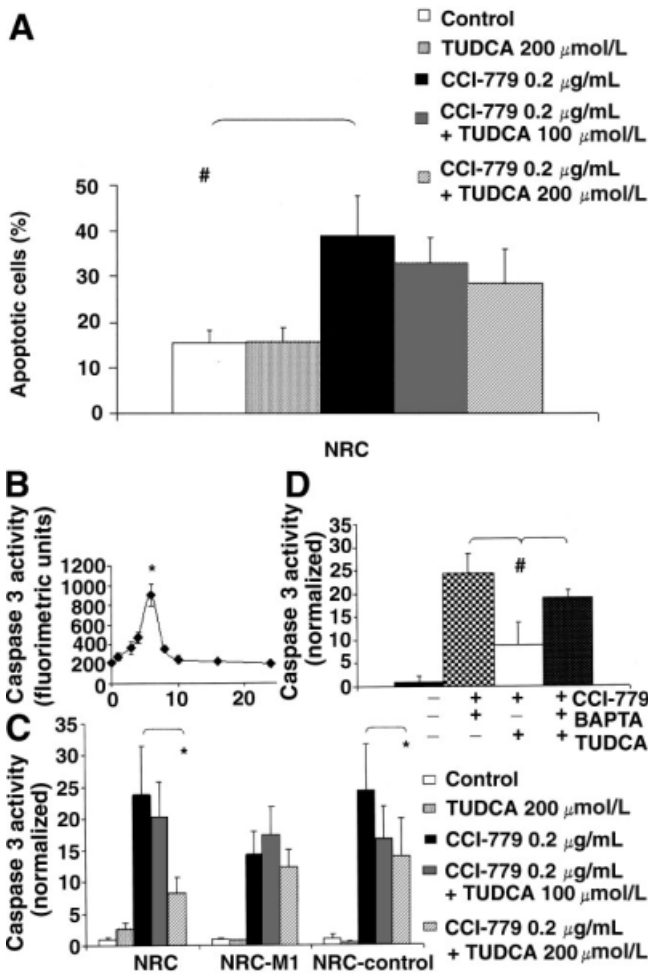

Fig. 7. TUDCA attenuates CCI-779–induced activation of caspase 3 in NRC cells, and this effect is lost with M1 and in the presence of BAPTA. (A) CCI-779 induces apoptosis in NRC cells. Determination of the number of apoptotic cells by flow cytometry found a significant increase after exposure to CCI-779 and a dose-dependent amelioration in presence of TUDCA (n = 3 in triplicates, $\#P < .05$). (B) Time course of the caspase 3 activity in NRC cells incubated for different times with CCI-779 0.2 μg/mL. Incubation with this drug activates the caspase 3 with a peak of activity after 6 hours (n = 3 in triplicates, $*P < .01$). (C) The activity of the caspase 3 was determined in NRC cells after 6 hours of incubation with and without 0.2 μg/mL CCI-779 and 100 or 200 μmol/L TUDCA (n = 3 in triplicates). In NRC cells, CCI-779 significantly increased the activity of the caspase 3. TUDCA attenuated this effect slightly at 100 μmol/L and significantly at 200 μmol/L ($*P < .05$). In NRC cells transduced with the dominant negative M1 (NRC-M1), CCI-779 increased the caspase 3 activity, and this effect could not be rescued by TUDCA. In NRC-Control cells, CCI-779 induced apoptosis and TUDCA decreased the activation of the caspase 3 ($*P < .05$). (D) Incubation with 50 μmol/L BAPTA/AM, which chelates intracellular calcium signals, partially reversed the protective effect of 200 μmol/L TUDCA. Comparison of NRC cells exposed to CCI-779 and BAPTA/AM to NRC cells exposed to CCI-779 and TUDCA and to cells exposed to the combination of the 3 showed a significant effect of TUDCA (Kruskal-Wallis $\#P < .05$). TUDCA, tauroursodeoxycholic acid; NRC, normal rat cholangiocyte.

TUDCA is actively secreted into the canalicular bile, and downstream it may affect the functions of the biliary epithelial cells. TUDCA has been shown to stimulate the secretion of adenosine triphosphate in these cells.[38] The possibility that TUDCA regulates a transcription factor in the cells lining the biliary tree has not been considered. We found that TUDCA phosphorylates and transactivates CREB in biliary epithelial cells. Various intracellular signaling pathways can activate CREB. Originally described as a target of the cAMP/PKA pathway , CREB has been found to be activated by Ca^{2+} signals and Ca^{2+}/calmodulin-dependent protein kinase[39] and to be phosphorylated by kinases of the MAPK pathway.[22] The activation of CREB in biliary epithelial cells in response to TUDCA is unlikely to be mediated by the cAMP/PKA pathway, because, in agreement with Housset and coworkers,[40] we did not observe an increase of the level of cAMP in cholangiocytes exposed to TUDCA. Our results imply that the activation of CREB in biliary epithelial cells in response to TUDCA involves Ca^{2+} signaling. This bile acid has been reported to increase the cytosolic calcium concentration in the biliary Mz-ChA-1 cell line.[41,42] We observed that TUDCA elicits Ca^{2+} signals in NRC cells and that inhibition of these signals with BAPTA/AM significantly reduced the stimulation of CREB by TUDCA. Chelation of the extracellular Ca^{2+} with EGTA did not inhibit the activation of CREB by TUDCA, suggesting release of Ca^{2+} from the endoplasmic reticulum through the inositol triphosphate receptors, the intracellular Ca^{2+} channels expressed in cholangiocytes.[43] Moreover, the inhibition of the Ca^{2+}/calmodulin-dependent protein kinase prevented CREB

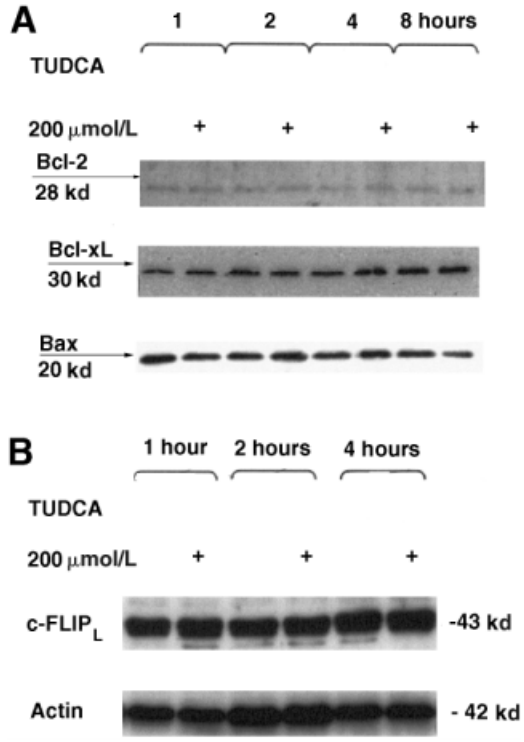

Fig. 8. Expression of *Bcl-2*, *Bcl-xL*, *Bax,* and c-FLIP$_L$ in NRC cells exposed to UDCA. (A) Western blotting analysis of the level of expression of *Bcl-2*, *Bcl-xL*, and *Bax* in NRC cells incubated for the indicated times with and without 200 μmol/L TUDCA. (B) Incubation of NRC cells with 200 μmol/L TUDCA did not significantly increase the level of expression of c-FLIP$_L$ as determined by Western blot analysis. NRC, normal rat cholangiocyte; TUDCA, tauroursodeoxycholic acid.

activation by TUDCA. Because in hepatocytes TUDCA activates PKC and PKC mediates the stimulation of Ca^{2+}-dependent exocytosis by TUDCA,[8] we tested whether PKC could be involved. Inhibition of PKC by calphostin C, chelerythrine, and Gö 6983 prevented CREB activation by TUDCA. These results suggest that TUDCA engages Ca^{2+} signals and PKC to activate CREB in cholangiocytes. Despite the fact that inhibition of MAPKK and p38MAPK did not block TUDCA-induced CREB phosphorylation and transcription activation, our findings do not exclude the possibility that other pathways than Ca^{2+} signals and PKC stimulate CREB in cholangiocytes.

To investigate whether activation of CREB in biliary epithelial cells by TUDCA is cytoprotective, we tested the effect of CCI-779, a chemotherapeutic agent currently being evaluated in clinical trials.[26] By impairing mTOR signaling, CCI-779 decreases the phosphorylation state of p70 S6 kinase and 4E-BP1, inhibiting the translation of specific mRNAs. This blocks cell cycle progression and induces apoptosis. We found that CCI-779 is cytotoxic for NRC cells, inhibits the proliferation of NRC cells, activates caspase 3, and induces apoptosis. As expected for nontumoral cells, the effect on apoptosis was less marked than that on proliferation.[31] The cytotoxicity of CCI-779 is reduced in presence of TUDCA, but not of taurochenodeoxycholic acid. Moreover, this effect is lost in NRC cells expressing a CREB with a mutated serine 133 (NRC-M1). TUDCA did not influence the antiproliferative effect of CCI-779 but did partially reverse the apoptotic effect of this mTOR inhibitor. In an analogous manner, insulin-like growth factor I has been reported to prevent sirolimus-induced apoptosis in a rhabdomyosarcoma cell line.[44] Insulin-like growth factor I–mediated protection against sirolimus-induced apoptosis was mediated neither by the *Ras-Erk*1-*Erk*2 pathway nor by the phosphatidylinositol 3′-kinase-Akt pathway,[44] implying the involvement of other mechanisms. We found that the dominant negative M1 reduced the protective effect of TUDCA against the cytotoxicity and the apoptotic effects of CCI-779, suggesting that they are mediated by CREB. S6K1 has been reported to have the capacity to phosphorylate CREB, suggesting that CREB may play a role in the apoptosis resulting from mTOR inhibition.[45] Qiao et al. found that expression in hepatocytes of the same dominant negative M1 enhances deoxycholic acid–induced apoptosis.[46] This could be attributable to the upregulation of antiapoptotic proteins by CREB.

The *Bcl*-2 promoter possesses an active cAMP-responsive element site, and CREB-induced expression of *Bcl*-2 rescues immature B cells from apoptosis.[21] However, we could not detect a change in the level of expression of *Bcl*-2 in NRC cells in response to TUDCA. This could be due to the cell type–specific transcription effects of CREB.[47] Other factors, Bcl-xL and Bax, were not affected. We observed an insignificant increase in the level of expression of c-FLIP$_L$, an antiapoptotic factor modulating death-receptor function[48] and bile acid–mediated apoptosis at the level of p38MAPK.[49] Dent and coworkers found a decreased expression of this factor in primary rodent hepatocytes infected with M1 plasmid adenovirus.[46] Other factors such as protein phosphatase 5 or apoptosis signal–regulating kinase 1 might be involved in the antiapoptotic effect of CREB in response to mTOR inhibition by CCI-779.[50]

In summary, TUDCA activates the transcription factor CREB in cholangiocytes and attenuates by this mechanism the cytotoxicity of the mTOR inhibitor CCI-779. The phosphorylation of CREB by TUDCA is mediated by Ca^{2+} signals and PKC. This provides a novel mechanism of action for TUDCA in biliary epithelial cells.

Acknowledgment: The authors thank Prof. D. Trono (University of Geneva) for the lentivirus vector and the packaging system, Prof. M. Montminy (Salk Institute for

Biological Studies) for the cDNA of the dominant negative M1. The NRC cells were a gift of Prof. N. LaRusso (Mayo Clinic), and the 293T cells were provided by Prof. D. Schuemperli (University of Bern). The authors are indebted to Prof. D. Schuemperli and Prof. R. Friis (University of Bern) for invaluable advice.

References

1. Jaeschke H, Gujral JS, Bajt ML. Apoptosis and necrosis in liver disease. Liver Int 2004;24:85-89.
2. Celli A, Que FG. Dysregulation of apoptosis in the cholangiopathies and cholangiocarcinoma. Semin Liver Dis 1998;18:177-185.
3. LeSage EG, Alvaro D, Benedetti A, Glaser S, Marucci L, Baiocchi L, et al. Cholinergic system modulates growth, apoptosis, and secretion of cholangiocytes from bile duct-ligated rats. Gastroenterology 1999;117: 191-199.
4. Alpini G, Ueno Y, Tadlock L, Glaser SS, LeSage G, Francis H, et al. Increased susceptibility of cholangiocytes to tumor necrosis factor-alpha cytotoxicity after bile duct ligation. Am J Physiol Cell Physiol 2003;285: C183–194.
5. Tinmouth J, Lee M, Wanless IR, Tsui FW, Inman R, Heathcote EJ. Apoptosis of biliary epithelial cells in primary biliary cirrhosis and primary sclerosing cholangitis. Liver 2002;22:228-234.
6. Paumgartner G, Beuers U. Mechanisms of action and therapeutic efficacy of ursodeoxycholic acid in cholestatic liver disease. Clin Liver Dis 2004;8: 67-81, vi.
7. Beuers U, Nathanson MH, Boyer JL. Effects of tauroursodeoxycholic acid on cytosolic Ca^{2+} signals in isolated rat hepatocytes. Gastroenterology 1993;104:604-612.
8. Beuers U. Effects of bile acids on hepatocellular signaling and secretion. Yale J Biol Med 1997;70:341-346.
9. Kurz AK, Graf D, Schmitt M, Vom Dahl S, Haussinger D. Tauroursodesoxycholate-induced choleresis involves p38(MAPK) activation and translocation of the bile salt export pump in rats. Gastroenterology 2001;121: 407-419.
10. Noe J, Hagenbuch B, Meier PJ, St-Pierre MV. Characterization of the mouse bile salt export pump overexpressed in the baculovirus system. HEPATOLOGY 2001;33:1223-1231.
11. Beuers U, Bilzer M, Chittattu A, Kullak-Ublick GA, Keppler D, Paumgartner G, et al. Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase C-dependent mechanisms in cholestatic rat liver. HEPATOLOGY 2001;33:1206-1216.
12. Guicciardi ME, Gores GJ. Is ursodeoxycholate an antiapoptotic drug? HEPATOLOGY 1998;28:1721-1723.
13. Rodrigues CM, Steer CJ. The therapeutic effects of ursodeoxycholic acid as an anti-apoptotic agent. Expert Opin Invest Drugs 2001;10:1243-1253.
14. Benz C, Angermuller S, Otto G, Sauer P, Stremmel W, Stiehl A. Effect of tauroursodeoxycholic acid on bile acid-induced apoptosis in primary human hepatocytes. Eur J Clin Invest 2000;30:203-209.
15. Rodrigues CMP, Fan G, Ma X, Kren BT, Steer CJ. A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. J Clin Invest 1998;101:2790-2799.
16. Sola S, Ma X, Castro RE, Kren BT, Steer CJ, Rodrigues CM. Ursodeoxycholic acid modulates E2F-1 and p53 expression through a caspase-independent mechanism in transforming growth factor beta1-induced apoptosis of rat hepatocytes. J Biol Chem 2003;278:48831-48838.
17. Sola S, Castro RE, Kren BT, Steer CJ, Rodrigues CM. Modulation of nuclear steroid receptors by ursodeoxycholic acid inhibits TGF-beta1-induced E2F-1/p53-mediated apoptosis of rat hepatocytes. Biochemistry 2004;43:8429-8438.
18. Qiao L, Yacoub A, Studer E, Gupta S, Pei XY, Grant S, et al. Inhibition of the MAPK and PI3K pathways enhances UDCA-induced apoptosis in primary rodent hepatocytes. HEPATOLOGY 2002;35:779-789.
19. Schoemaker MH, Conde de la Rosa L, Buist-Homan M, Vrenken TE, Havinga R, Poelstra K, et al. Tauroursodeoxycholic acid protects rat hepatocytes from bile acid-induced apoptosis via activation of survival pathways. HEPATOLOGY 2004;39:1563-1573.
20. Persengiev SP, Green MR. The role of ATF/CREB family members in cell growth, survival and apoptosis. Apoptosis 2003;8:225-228.
21. Wilson BE, Mochon E, Boxer LM. Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. Mol Cell Biol 1996;16:5546-5556.
22. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2001;2:599-609.
23. Conkright MD, Guzman E, Flechner L, Su AI, Hogenesch JB, Montminy M. Genome-wide analysis of CREB target genes reveals a core promoter requirement for cAMP responsiveness. Mol Cell 2003;11:1101-1108.
24. Matthews RP, McKnight GS. Characterization of the cAMP response element of the cystic fibrosis transmembrane conductance regulator gene promoter. J Biol Chem 1996;271:31869-31877.
25. Woltje M, Kraus J, Hollt V. Identification of a functional 3',5'-cyclic adenosine monophosphate response element within the second promoter of the mouse somatostatin receptor type 2 gene. FEBS Lett 1998;440:107-110.
26. Elit L. CCI-779 Wyeth. Curr Opin Investig Drugs 2002;3:1249-1253.
27. Jacinto E, Hall MN. Tor signalling in bugs, brain and brawn. Nat Rev Mol Cell Biol 2003;4:117-126.
28. Shamji AF, Nghiem P, Schreiber SL. Integration of growth factor and nutrient signaling: implications for cancer biology. Mol Cell 2003;12:271-280.
29. Fingar DC, Richardson CJ, Tee AR, Cheatham L, Tsou C, Blenis J. mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. Mol Cell Biol 2004;24:200-216.
30. Huang S, Bjornsti MA, Houghton PJ. Rapamycins: mechanism of action and cellular resistance. Cancer Biol Ther 2003;2:222-232.
31. Neshat MS, Mellinghoff IK, Tran C, Stiles B, Thomas G, Petersen R, et al. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc Natl Acad Sci U S A 2001;98:10314-10319.
32. Vroman B, La Russo NF. Development and characterization of polarized primary cultures of rat intrahepatic bile duct epithelial cells. Lab Invest 1996;74:303-313.
33. Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 1989;59: 675-680.
34. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol 1998;72:8463-8471.
35. Lowry O, Rosebrough N, Farr L, Randall R. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-275.
36. Combettes L, Berthon B, Claret M. Caffeine inhibits cytosolic calcium oscillations induced by noradrenaline and vasopressin in rat hepatocytes. Biochem J 1994;301:737-744.
37. Tordjmann T, Berthon B, Jacquemin E, Clair C, Stelly N, Guillon G, et al. Receptor-oriented intercellular calcium waves evoked by vasopressin in rat hepatocytes. EMBO J 1998;17:4695-4703.
38. Nathanson MH, Burgstahler AD, Masyuk A, Larusso NF. Stimulation of ATP secretion in the liver by therapeutic bile acids. Biochem J 2001;358: 1-5.
39. Sheng M, Thompson MA, Greenberg ME. CREB: a $Ca^{(2+)}$-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 1991;252:1427-1430.
40. Chignard N, Mergey M, Veissiere D, Poupon R, Capeau J, Parc R, et al. Bile salts potentiate adenylyl cyclase activity and cAMP-regulated secretion in human gallbladder epithelium. Am J Physiol Gastrointest Liver Physiol 2003;284:G205–G212.
41. Shimokura GH, McGill JM, Schlenker T, Fitz JG. Ursodeoxycholate increases cytosolic calcium concentration and activates Cl- currents in a biliary cell line. Gastroenterology 1995;109:965-972.

42. Alpini G, Kanno N, Phinizy JL, Glaser S, Francis H, Taffetani S, et al. Tauroursodeoxycholate inhibits human cholangiocarcinoma growth via Ca^{2+}-, PKC-, and MAPK-dependent pathways. Am J Physiol Gastrointest Liver Physiol 2004;286:G973–G982.
43. Hirata K, Dufour JF, Shibao K, Knickelbein R, AF ON, Bode HP, et al. Regulation of $Ca(^{2+})$ signaling in rat bile duct epithelia by inositol 1,4,5-trisphosphate receptor isoforms. HEPATOLOGY 2002;36:284-296.
44. Thimmaiah KN, Easton J, Huang S, Veverka KA, Germain GS, Harwood FC, et al. Insulin-like growth factor I-mediated protection from rapamycin-induced apoptosis is independent of Ras-Erk1-Erk2 and phosphatidylinositol 3′-kinase-Akt signaling pathways. Cancer Res 2003;63:364-374.
45. de Groot RP, Ballou LM, Sassone-Corsi P. Positive regulation of the cAMP-responsive activator CREM by the p70 S6 kinase: an alternative route to mitogen-induced gene expression. Cell 1994;79:81-91.
46. Qiao L, Han SI, Fang Y, Park JS, Gupta S, Gilfor D, et al. Bile acid regulation of C/EBPbeta, CREB, and c-Jun function, via the extracellular signal-regulated kinase and c-Jun NH2-terminal kinase pathways, modulates the apoptotic response of hepatocytes. Mol Cell Biol 2003;23:3052-3066.
47. Cha-Molstad H, Keller DM, Yochum GS, Impey S, Goodman RH. Cell-type-specific binding of the transcription factor CREB to the cAMP-response element. Proc Natl Acad Sci U S A 2004;101:13572-13577.
48. Tschopp J, Irmler M, Thome M. Inhibition of fas death signals by FLIPs. Curr Opin Immunol 1998;10:552-558.
49. Grambihler A, Higuchi H, Bronk SF, Gores GJ. cFLIP-L inhibits p38 MAPK activation: an additional anti-apoptotic mechanism in bile acid-mediated apoptosis. J Biol Chem 2003;278:26831-26837.
50. Huang S, Shu L, Easton J, Harwood FC, Germain GS, Ichijo H, et al. Inhibition of mammalian target of rapamycin activates apoptosis signal-regulating kinase 1 signaling by suppressing protein phosphatase 5 activity. J Biol Chem 2004;279:36490-36496.