The Bile Acid Taurochenodeoxycholate Activates a Phosphatidylinositol 3-Kinase-dependent Survival Signaling Cascade*

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Liver injury during cholestasis reflects a balance between the effects of toxic and nontoxic bile acids. However, the critical distinction between a toxic and nontoxic bile acid remains subtle and unclear. For example, the glycine conjugate of chenodeoxycholate (GCDC) induces hepatocyte apoptosis, whereas the taurine conjugate (TCDC) does not. We hypothesized that the dissimilar cellular responses may reflect differential activation of a phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathway. In the bile acid-transporting McNtsp.24 rat hepatoma cell line, TCDC, but not GCDC, stimulated PI3K activity. Consistent with this observation, inhibition of PI3K rendered TCDC cytotoxic, and constitutive activation of PI3K rendered GCDC nontoxic. Both Akt and the atypical protein kinase C isoform (PKCζ) have been implicated in PI3K-dependent survival signaling. However, TCDC activated PKCζ, but not Akt. Moreover, inhibition of PKCζ converted TCDC into a cytotoxic agent, whereas overexpression of wild-type PKCζ blocked GCDC-induced apoptosis. We also demonstrate that TCDC activated nuclear factor κB (NF-κB) in a PI3K- and PKCζ-dependent manner. Moreover, inhibition of NF-κB by an IκB super-repressor rendered TCDC cytotoxic, suggesting that NF-κB is also necessary to prevent the cytotoxic effects of TCDC. Collectively, these data suggest that some hydrophobic bile acids such as TCDC activate PI3K-dependent survival pathways, which prevent their otherwise inherent toxicity.

Bile acids are hydrophobic, potentially cytotoxic compounds synthesized from cholesterol in the liver and secreted into the bile canaliculus, where they promote bile flow. In man, bile acids are conjugated to glycine or taurine, with the glycine conjugate predominating (1). The conjugation decreases their hydrophobicity and renders the molecules less cytotoxic at physiologic concentrations. However, hepatic accumulation of bile acids is a salient pathophysiologic consequence of cholestasis (a syndrome of bile flow impairment) due to the failure to secrete these compounds into the bile canalculus (2). Elevated concentrations of bile acids within the liver promote liver injury and the development of liver cirrhosis and liver failure. For example, children lacking the canalicular transport protein for bile acid secretion develop a progressive liver disease due to the inability to excrete bile acids from the hepatocyte (3). Numerous studies have now shown that bile acid concentrations that occur during cholestasis induce hepatocyte apoptosis, thus providing a cellular mechanism for bile acid-mediated liver injury (4). Not all bile acids are toxic, however, and minor changes in bile acid structure dramatically alter their potential cytotoxicity. For example, the glycine conjugate of chenodeoxycholate induces hepatocyte apoptosis in vitro, whereas the taurine conjugate does not (5). Previous concepts suggested that bile acid toxicity correlated with relative hydrophobicity, with hydrophobic bile acids being cytotoxic and hydrophilic bile acids being nontoxic. However, we could not establish a relationship between bile acid-induced apoptosis and relative hydrophobicity (5).

Recent studies demonstrated that bile acids activate cytoplasmic protein kinase cascades and function as ligands for the nuclear receptor farnesoid X receptor (6), suggesting that they may mediate their effects by altering cell signaling pathways. Indeed, the nontoxic bile acid taurocholate has been found to activate phosphatidylinositol 3-kinase (PI3K) (7), a potent activator of survival signals (8), raising the possibility that nontoxic, yet hydrophobic bile acids do not trigger apoptosis because they activate a PI3K-dependent survival signaling pathway.

Downstream effectors of PI3K-dependent survival signals include Akt and the atypical protein kinase C (PKC) isoforms, especially PKCζ (9, 10). Akt, the cellular homolog of the viral oncoprotein v-Akt, suppresses apoptotic cell death in a number of cell types (11–13). One substrate for Akt is BAD, a pro-apoptotic member of the Bcl-2 family. Akt phosphorylates BAD, thereby blocking it from binding and inactivating Bcl-2 and Bcl-xL, two anti-apoptotic Bcl-2 family members (14). Another

1 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; NF-κB, nuclear factor κB; TCDC, taurochenodeoxycholate; GCDC, glycochenodeoxycholate; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; TNF, tumor necrosis factor.
substrate for Akt is the transcription factor NF-κB, a potent regulator of a number of anti-apoptotic gene products (15–17). Akt activates NF-κB by phosphorylating IκB kinase-α (18). Active IκB kinase-α then phosphorylates IκB, resulting in dissociation from NF-κB, allowing this transcription factor to enter the nucleus. PKCζ is another downstream effector of PI3K (19) and can also mediate activation of NF-κB (20, 21). Activation of NF-κB by PKCζ also involves IκB phosphorylation (22).

In this study, we demonstrate that the nontoxic bile acid taurochenodeoxycholate (TCDC) activates PI3K, whereas the toxic glycine conjugate (GCDC) does not activate this lipid kinase. Moreover, we show that genetic or pharmacologic inhibition of PI3K converts TCDC to a cytotoxic bile acid and that constitutive activation of PI3K blocks GCDC-induced apoptosis. PI3K mediated its anti-apoptotic effects by activating PKCζ and NF-κB. Correspondingly, inhibition of PKCζ and NF-κB converted TCDC to a potent inducer of apoptosis. Thus, the present data suggest that TCDC is not cytotoxic because it activates PI3K-dependent cell survival signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

The McNtcp.24 rat hepatoma cell line, which is stably transfected with the sodium taurocholate-cotransport polypeptide and undergoes bile acid-mediated apoptosis, was used for all experiments (23).

Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 10% bovine calf serum, 100,000 units/liter penicillin, 100 μg/liter streptomycin, 100 μg/liter gentamycin, and 200 μg/liter G418.

**Kinase Assays**

**PI3K Assay**—Cells were grown to 50–75% confluence in 60-mm dishes and made quiescent by culture in serum-free DMEM for 8–12 h. PI3K activity was measured using modifications of a published technique (24). After serum deprivation, cells were treated with diluent, 50 μM GCDC, or 50 μM TCDC for 15 min. Cells were lysed in ice-cold lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and 1% Nonidet P-40 and rocked for 20 min on ice. Clarified protein (1.5 mg/sample) was precipitated with 1 μl of protein A-Sepharose (Zymed Laboratories Inc., South San Francisco, CA) overnight at 4 °C. Immune complexes were precipitated by incubation with 60 μl of protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h. The immunoprecipitates were washed three times with buffer containing 137 mM NaCl, 20 mM Tris-HCl, 1 mM CaCl2, 1 mM MgCl2, 0.1 mM Na2VO4, and 1% Nonidet P-40 (pH 7.4); three times with buffer containing 0.1 M Tris-HCl, 5 mM LiCl, and 0.1 mM Na2VO4; and twice with buffer containing 150 mM NaCl, 5 mM Tris-HCl, 5 mM EDTA, and 0.1 mM Na2VO4. Assays were then performed in a reaction mixture containing 0.88 mM ATP, 100 mM MgCl2, 30 μCi of γ-32P-ATP, and 20 μg of phosphatidylinositol (Sigma) and incubated with agitation for 15 min at 37 °C. The reactions were stopped with 20 μl of 0.6 M HCl. The organic layer was extracted with 160 μl of CHCl3/CH2O/MeOH (1:1) and separated on a silica gel thin-layer chromatography plate (J. T. Baker Inc.). Thin-layer chromatography plates were developed in CHCl3/CH2O/H2O/NaOH (60:47:11:3.2) and dried. Radiolabeled phosphatidylinositol phosphates were visualized by autoradiography on X-Omat film (Eastman Kodak Co.).

**PKCζ Assay**—PKCζ activity was measured using modifications of a published technique (25). Briefly, cells were serum-deprived for 12 h and stimulated with diluent, 250 mM insulin, or 200 μM TCDC for 90 min. Cells were then washed twice with ice-cold phosphate-buffered saline and lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, and protease inhibitor mixture (Roche Diagnostics, Inc.) overnight at 4 °C, and the mixture was centrifuged (14,000 × g) for 15 min at 4 °C, and 1 mg of the cytosolic protein was incubated with 10 μl of anti-PKCζ antibodies (Santa Cruz Biotechnology) overnight at 4 °C. Immune complexes were precipitated with 100 μg of Protein A-Sepharose (Pharmacia, San Francisco, CA) overnight at 4 °C and then washed seven times with lysis buffer modified to contain 500 mM NaCl. Washed immunoprecipitates were incubated with 2 μg of myelin basic protein (Upstate Biotechnology, Inc.) and 10 μCi of [γ-32P]-ATP for 30 min at 37 °C in 35 mM Tris (pH 7.5), 10 mM MgCl2, 5 mM EGTA, 1 mM CaCl2, and 1 mM phenyl phosphate. Proteins were separated by SDS-polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose. Radiolabeled myelin basic protein was detected by autoradiography on BiomaxMR film (Kodak). At least three independent experiments of all PI3K and PKCζ assays were performed.

**Quantitation of Apoptosis**

Apoptosis was quantitated by assessing the characteristic nuclear changes of apoptosis using the DNA-binding dye 4,6-diamidino-2-phenylindole dihydrochloride and fluorescence microscopy (26).

**Plasmas and Transfection**

Plasmas for constitutively activated PI3K (pEF-BOSARI-ISH2-CAAX), dominant-negative PI3K (pEF-BOSARI-ap85), constitutively activated Akt (pCMV6-Myr-Akt-HA), and wild-type PKCζ (pDNA3HA-PKC) dominant-negative PKCζ (pDNA3HA-PKC-DN) and luciferase reporter plasmids p105 (conA-luc) and p106 (ε-conA-luc) have been previously described (27–29). The TK-RevGals-CMV plasmid was purchased from Promega (Madison, WI) and used to normalize for transfection efficiency in luciferase assays. GFP (pEGFP-N1) was purchased from CLONTECH (Palo Alto, CA). McNtcp.24 cells (1.5 × 10^5 cells/ml) were transiently transfected using LipofectAMINE (Life Technologies, Inc.) as described previously (30) and used 48 h after transfection.

**Electrophoretic Mobility Shift Assay (EMSA)**

Cells were stimulated with diluent (DMEM) or different concentrations of TCDC and GCDC. Nuclear protein extracts were then prepared using a modified lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and 1% Nonidet P-40 and rocked for 20 min on ice. Clarified protein (1.5 mg/sample) was incubated with 5 μl of anti-Pi3K p85 antibody (Upstate Biotechnology, Inc., Lake Placid, NY) overnight at 4 °C. Immune complexes were precipitated by incubation with 60 μl of protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h. The immunoprecipitates were washed three times with buffer containing 137 mM NaCl, 20 mM Tris-HCl, 1 mM CaCl2, 1 mM MgCl2, 0.1 mM Na2VO4, and 1% Nonidet P-40 (pH 7.4); three times with buffer containing 0.1 M Tris-HCl, 5 mM LiCl, and 0.1 mM Na2VO4; and twice with buffer containing 150 mM NaCl, 5 mM Tris-HCl, 5 mM EDTA, 1 mM Na2VO4, and 0.1 mM Na2VO4. Assays were then performed in a reaction mixture containing 0.88 mM ATP, 100 mM MgCl2, 30 μCi of γ-32P-ATP, and 20 μg of phosphatidylinositol (Sigma) and incubated with agitation for 15 min at 37 °C. The reactions were stopped with 20 μl of 0.6 M HCl. The organic layer was extracted with 160 μl of CHCl3/CH2O/MeOH (1:1) and separated on a silica gel thin-layer chromatography plate (J. T. Baker Inc.). Thin-layer chromatography plates were developed in CHCl3/CH2O/H2O/NaOH (60:47:11:3.2) and dried. Radiolabeled phosphatidylinositol phosphates were visualized by autoradiography on X-Omat film (Eastman Kodak Co.).

**Luciferase Reporter Gene Assay**

McNtcp.24 cells were cotransfected with 0.2 μg of TK-RevGals-CMV and 1.5 μg of either p105 or p106. Forty-eight hours later, the cells were cultured using serum-free DMEM for 18–24 h. Both firefly and Renilla luciferase activities were quantitated using the dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Background expression of luciferase, as determined in cells transfected with the p105 vector, was subtracted from p106 values.

**Adenoviral Infection**

The recombinant replication-deficient adenovirus Ad5 I-E8, containing an I-E8 protein in which serines 32 and 36 are mutated to alanine (generous gift of D. A. Brenner, University of North Carolina, Chapel Hill, NC), and Ad-ΔE1, an empty adenovirus for control experiments, were grown and purified by banding twice in CsCl gradients as described previously (32). For adenoviral infection, McNtcp.24 cells were grown to 50–75% confluence. The medium was replaced with DMEM containing 2% fetal bovine serum, and Ad5-I-E8 or Ad-ΔE1 viral stock solutions were added to each plate at a multiplicity of infection of 100. Culture dishes were rocked every 15 min for 2 h, and the culture medium was supplemented with 20% serum. Cells were then cultured for an additional 12–16 h before they were used for experiments.

**Immunoblot Analysis**

Immunoblot analysis of Akt was performed on McNtcp.24 whole cell lysates. Cells were lysed in 100 μl of SDS-polyacrylamide gel electrophoresis sample buffer. Proteins were then separated by SDS-polyacrylamide gel electrophoresis (12.5%) and transferred to nitrocellulose. The membrane was blocked with 5% nonfat dried milk in 20 mM Tris, 137 mM NaCl, and 0.05% Tween 20 (pH 7.0) for 60 min and then incubated overnight with a 1:1000 dilution of rabbit anti-Akt or rabbit anti-phospho-Akt(Ser473) antibody (New England Biolabs, Beverly, MA). After washing, membranes were incubated for 60 min with a 1:3000...
dilution of peroxidase-conjugated goat anti-rabbit IgG (New England Biolabs) and washed again. Bound antibody was visualized using chemiluminescent substrate (ECL, Amersham Pharmacia Biotech) and exposed to X-Omat film. Membranes were then stripped of antibodies in 100 mM mercaptoethanol, 62.5 mM Tris, and 2% SDS for 30 min at 50 °C and reblotted overnight with a 1:1000 dilution of goat anti-actin antibody (Santa Cruz Biotechnology) to demonstrate equal protein loading. Blots were washed as described above, incubated for 60 min with a 1:5000 dilution of peroxidase-conjugated swine anti-goat IgG (BIO SOURCE, Camarillo, CA), and visualized as described above. Antibodies against HA (Santa Cruz Biotechnology) were used in a 1:1000 dilution for immunoblots demonstrating expression of transfected Akt and wild-type and dominant-negative PKCζ, which all have an HA tag. Blots were washed as described above, incubated for 60 min with a 1:10000 dilution of peroxidase-conjugated goat anti-rabbit IgG (New England Biolabs) and washed again. Bound antibody was visualized using 1:5000 dilution of peroxidase-conjugated swine anti-goat IgG (BIO SOURCE), and visualized as described above. At least three independent experiments of Akt immunoblots and anti-HA immunoblots were performed.

Reagents

The PI3K inhibitors wortmannin and LY294002 were obtained from Calbiochem. The NF-κB consensus oligonucleotide was purchased from Promega. GCDC, TCDC, 4,6-diamidino-2-phenylindole dihydrochloride, and all other reagents were from Sigma.

RESULTS

Does PI3K Activity Modulate Bile Acid Cytotoxicity?—To ascertain a putative role for PI3K in modulating bile acid cytotoxicity, we first asked if toxic and nontoxic bile salts activate PI3K. Serum-deprived McNtcp.24 cells were stimulated with 50 μM TCDC, 50 μM GCDC, or diluent for 15 min. PI3K was immunoprecipitated, and the activity of the immunopurified protein was measured. The nontoxic bile acid TCDC readily activated PI3K, whereas no kinase activity was observed in quiescent cells (Fig. 1A). Wortmannin, a potent PI3K inhibitor (33), blocked the TCDC-induced stimulation of PI3K, demonstrating the specificity of the assay. In marked contrast, the cytotoxic glycine conjugate of chenodeoxycholate did not stimulate PI3K activity, demonstrating that PI3K is differentially activated by structurally similar, but not identical bile acids.

Because the nontoxic bile acid TCDC activated PI3K and the cytotoxic bile acid GCDC did not, we next tested the possibility that PI3K activity might be protective in this setting. McNtcp.24 cells were pretreated with wortmannin or LY294002 and then incubated with 50 μM TCDC for 4 h. Neither PI3K inhibitor alone caused cell death. Additionally, as a single agent, TCDC also did not kill cells. However, TCDC effectively induced apoptosis in cells pretreated with the PI3K inhibitors (Fig. 1B). To further confirm that PI3K activity protected the cells from TCDC-induced cell death, we transiently expressed dominant-negative PI3K and treated the cells with TCDC (Fig. 1C). Consistent with the results seen with the pharmacologic inhibitors, genetic blockade of PI3K activation also sensitized the cells to TCDC-induced apoptosis. These findings predicted that constitutive activation of PI3K would prevent normally toxic bile salts from killing cells. We tested this possibility by expressing constitutively active PI3K, which effectively blocked apoptosis induced by the cytotoxic bile salt GCDC (Fig. 1D). Collectively, these results demonstrate that bile acid-induced PI3K activation can modulate bile acid cytotoxicity. Additionally, they suggest that TCDC is inherently toxic; however, its cytotoxicity is blocked by activation of a PI3K-dependent survival signal.

Which PI3K-activated Effectors Mediate Cell Survival following TCDC Stimulation?—Both Akt and PKCζ are downstream PI3K effectors that, in some settings, prevent apoptosis. Therefore, we next investigated if these two kinases are involved in downstream signaling of PI3K-mediated survival signaling.

Akt activation was investigated by immunoblot analysis in whole cell lysates using a phospho-specific anti-Akt antibody to identify active Akt (34). Although Akt was expressed in the McNtcp.24 cells, activated Akt was detected only in cells stimulated with insulin (a positive control), but not in those stimulated with TCDC (Fig. 2A). To demonstrate that Akt does not participate in preventing bile acid-induced cell death, we overexpressed a constitutively active form of Akt in McNtcp.24 cells. Overexpression of active Akt did not decrease GCDC-induced apoptosis compared with control transfected cells; however, staurosporine-induced apoptosis was reduced in cells overexpressing Akt (Fig. 2B). These results suggest that TCDC-mediated PI3K survival signals are unlikely to involve Akt activation.

We next determined if PKCζ, another downstream effector of PI3K, participates in mediating TCDC-induced activation of cell survival signaling. Analogous to the experimental approach used above for PI3K, McNtcp.24 cells were treated with TCDC or insulin, and PKCζ activity was measured. As demonstrated in Fig. 3A, TCDC activated PKCζ in a wortmannin-dependent manner, suggesting that PKCζ activation requires a PI3K-generated signal. This TCDC-induced activation of PKCζ was concentration-dependent (Fig. 3B). We next asked whether PKCζ is also required for the survival response. To demonstrate that we could manipulate PKCζ activity, McNtcp.24 cells were transfected with empty vector, dominant-negative mu-
Dependent experiments. Correspondingly, expression of dominant-negative PKCζ means that PKCζ, or wild-type PKCζ. Transfection of dominant-negative PKCζ markedly reduced PKCζ activity, whereas overexpression of wild-type PKCζ increased total PKCζ activity (Fig. 3C). We then used this dominant-negative PKCζ construct to assess whether PKCζ participates in the survival response. Correspondingly, expression of dominant-negative PKCζ increased TCDC-induced apoptosis—4-fold compared with cells transfected with an empty plasmid (Fig. 3D). To further assess the possible protective effect of PKCζ in bile acid-induced apoptosis, McNtcp.24 cells were transfected with either a control vector or wild-type PKCζ or with GFP and empty vector. Cells were then treated with 50 μM GCDC or 1 μM staurosporine (STP) for 4 h, and apoptosis was quantitated in GFP-expressing cells. The inset shows a representative immunoblot that demonstrates expression of transfected Akt. Results are the means ± S.D. of several fields (each field >300 cells) from three independent experiments.

FIG. 2. Akt is not involved in TCDC-mediated survival signaling. A, McNtcp.24 cells were pretreated with diluent (DMEM) or 250 nM wortmannin (WORT) for 15 min. Cells were then stimulated with 50 μM TCDC or 300 nM insulin for 15 min. Equivalent amounts of protein were sequentially immunoblotted with anti-phospho-Akt, anti-Akt, and anti-actin antibodies. A representative blot is shown. B, McNtcp.24 cells were cotransfected with GFP and constitutively active Akt or with GFP and empty vector. Cells were then treated with 50 μM GCDC or 1 μM staurosporine (STP) for 4 h, and apoptosis was quantitated in GFP-expressing cells. The inset shows a representative immunoblot with anti-HA antibody demonstrating expression of transfected Akt. A representative autoradiogram and the densitometry of several independent experiments are shown. A representative assay is shown. B, PKCζ assays were performed after immunoprecipitation of the kinase derived from McNtcp.24 cells treated for 90 min with diluent (DMEM) or TCDC at the indicated concentrations. A representative autoradiogram and the densitometry of several independent experiments are shown. C, McNtcp.24 cells were transfected with empty vector (control) or dominant-negative (DN) or wild-type (WT) PKCζ. After 48 h, PKCζ was immunoprecipitated and incubated with myelin basic protein (MBP) and γ[32P]ATP. A representative autoradiogram is shown. D, McNtcp.24 cells were cotransfected with GFP and dominant-negative PKCζ or with GFP and empty vector and treated with 50 μM TCDC for 4 h. Apoptosis was quantitated in GFP-expressing cells. The inset shows a representative immunoblot with anti-HA antibody demonstrating expression of transfected wild-type PKCζ. The results of D and E are the means ± S.D. of several fields (each field >300 cells) from three independent experiments.

FIG. 3. PKCζ mediates TCDC-induced PI3K survival signals. A, serum-deprived McNtcp.24 cells were pretreated with diluent (DMEM) or 250 nM wortmannin (WORT) and then stimulated with 200 μM TCDC or 250 nM insulin for 90 min. PKCζ was immunoprecipitated and incubated with myelin basic protein (MBP) and γ[32P]ATP. A representative assay is shown. B, PKCζ assays were performed after immunoprecipitation of the kinase derived from McNtcp.24 cells treated for 90 min with diluent (DMEM) or TCDC at the indicated concentrations. A representative autoradiogram and the densitometry of several independent experiments are shown. C, McNtcp.24 cells were cotransfected with wild-type vector (control) or dominant-negative (DN) or wild-type (WT) PKCζ. After 48 h, PKCζ was immunoprecipitated and incubated with myelin basic protein. D, McNtcp.24 cells were cotransfected with GFP and dominant-negative PKCζ or with GFP and empty vector and treated with 50 μM GCDC. Apoptosis was quantitated after 4 h of treatment in GFP-expressing cells. The inset shows a representative immunoblot with anti-HA antibody demonstrating expression of transfected wild-type PKCζ. The results of D and E are the means ± S.D. of several fields (each field >300 cells) from three independent experiments.

Next, we determined if TCDC-induced NF-κB activation requires PI3K activity. McNtcp.24 cells were treated with 200 μM TCDC in the absence or presence of the PI3K inhibitors LY294002 and wortmannin (Fig. 5C). Both of these structurally dissimilar PI3K inhibitors markedly reduced NF-κB acti-
vation by TCDC, demonstrating that TCDC activates NF-κB by a mechanism dependent upon and downstream of PI3K activity.

To confirm that TCDC transcriptionally activates NF-κB, we transfected McNtcp.24 cells with a luciferase reporter construct and stimulated the cells with the bile acids TCDC and GCDC. To normalize for transfection efficiency, a control Renilla luciferase construct was cotransfected into the cells. Consistent with the EMSA results, TCDC, but not GCDC, increased expression of the luciferase construct (Fig. 5E). Moreover, the TCDC-induced transcriptional response was blocked by LY294002, whereas LY294002 did not reduce luciferase activity in control cells (data not shown). Thus, both the EMSA and luciferase assays demonstrated that TCDC activated NF-κB in a PI3K-dependent manner.

PKCζ mediates NF-κB activation in several cell types. To determine the role of PKCζ in NF-κB activation in our model, we performed NF-κB reporter gene assays in cells that were transfected with wild-type or dominant-negative PKCζ and stimulated with diluent or 100 μM TCDC. As shown in Fig. 5F, luciferase activity was markedly increased in TCDC-stimulated cells expressing wild-type PKCζ, whereas only a minimal increase was observed in cells expressing dominant-negative PKCζ. These data strongly suggest that PKCζ regulates NF-κB activation in the proposed TCDC-induced survival signaling cascade.

**Does TCDC-induced NF-κB Activation Participate in the Survival Response?**—Unless NF-κB is inhibited, TNF-α-induced apoptosis usually does not occur in many cell types because the simultaneous activation of NF-κB blocks this cell death signaling pathway. To determine the role of NF-κB in TCDC-induced survival signaling, McNtcp.24 cells were transfected with an adenovirus that expresses the super-repressor of IkB (Ad5-IkB) or an empty control virus (Ad-ΔE1). Ad5-IkB contains an IkB that cannot be phosphorylated because serines 32 and 36 have been mutated to alanines (32). At a multiplicity of infection of 100, nearly all cells were infected under these conditions as confirmed by demonstrating a 95% rate of apoptosis in transfected cells treated with 28 ng/ml TNF-α for 4 h (data not shown). Control experiments also showed that Ad5-IkB effectively blocked TNF-α-induced activation of NF-κB as demonstrated by EMSA, whereas Ad-ΔE1 had no effect on NF-κB activation compared with uninfected cells (data not shown). Following infection with Ad5-IkB, TCDC-induced apoptosis increased ~8-fold compared with cells infected with the empty virus (Fig. 6), thus demonstrating that NF-κB plays a key role in the transduction of a TCDC-activated survival signal.

**DISCUSSION**

In this study, we have demonstrated that the nontoxic bile acid TCDC, but not the toxic bile salt GCDC, activated PI3K and initiated an anti-apoptotic signaling cascade in hepatocytes. Consistent with this observation, inhibition of PI3K transformed TCDC into a cytotoxic agent. Our data also suggest that the PI3K-dependent survival signal is mediated by the atypical PKC isofrom PKCζ, but not by the protein kinase Akt, which is implicated in many PI3K-dependent survival signaling pathways. We also showed that TCDC activated NF-κB in a PKCζ-dependent manner and that NF-κB was a key participant in the anti-apoptotic response. Thus, these data demonstrate that some bile acids prevent their inherent cytotoxicity by simultaneously activating intrinsic cell survival signals. These observations are relevant to liver injury in cholestasis and suggest that liver injury can be attenuated by activation of PI3K-dependent survival pathways.
Accumulating evidence suggests that bile acids modulate signal transduction pathways in hepatocytes (36). For example, signaling pathways affected by bile acids include protein kinase A, protein kinase C, and calcium-dependent signal transduction cascades (36). In addition, the bile acid taurocholate was recently identified as an activator of PI3K. However, these studies did not address the impact of PI3K activation on hepatocyte survival (7). The present studies demonstrate that a nontoxic bile acid selectively activates PI3K-dependent survival signals in hepatocytes. However, the mechanisms by which bile acids activate PI3K remain to be elucidated. Misra et al. (7) could not identify a direct effect of bile acids on PI3K activity, suggesting an indirect mechanism for activation. Bile acids likely stimulate PI3K activity by facilitating its association with receptor complexes known to activate this lipid kinase.

PI3K is implicated as an activator of a variety of anti-apoptotic signaling effectors, including Akt and the atypical isoforms of PKC (PKCζ and PKCλ/ι) (9, 37). We could not find evidence for Akt activation in cells treated with TCDC. This was surprising, as Akt is activated by the PI3K product phosphatidylinositol 3,4,5-trisphosphate (38). Perhaps, bile acids alter the lipid-binding site of Akt, its cellular distribution, or other events necessary for Akt activation by phosphatidylinositol 3,4,5-trisphosphate. Our data did, however, implicate PKCζ as a critical component of a PI3K-induced signaling cascade. This interpretation is supported by the observation that the PI3K inhibitor wortmannin blocked TCDC-induced activation of PKCζ. Furthermore, a dominant-negative PKCζ mutant blocked the PI3K-dependent survival pathway and transformed TCDC to a cytotoxic bile acid. Others have shown that PKCζ activation resulted in NF-κB-dependent transcriptional activity (39), and we confirmed this observation in our model. Two mechanisms have been proposed for PKCζ regulation of NF-κB activity. PKCζ may activate IκB kinase-β, the kinase that phosphorylates IκBα, resulting in the dissociation of NF-κB from its inhibitor and subsequent nuclear translocation of the transcriptionally active subunits (22). Alternatively, PKCζ activation may also act in concert to promote phosphorylation of the RelA subunit of NF-κB, leading to enhanced transcriptional activity (39). The two mechanisms may also act in concert to promote NF-κB transcriptional activity. Collectively, our studies implicate PKCζ as a likely link between TCDC-induced PI3K activation and NF-κB activation in bile acid-treated hepatocytes.

We have previously shown that bile salt cytotoxicity both in vivo and in vitro is mediated by the death receptor Fas (40, 41). Toxic bile acids induce Fas oligomerization and activate caspase-8, resulting in apoptosis (40). A recent study demonstrated that enhanced PI3K activity inhibits Fas-mediated apoptosis (42). Thus, bile acid-induced Fas activation appears to be inhibited by the simultaneous activation of a kinase-dependent, anti-apoptotic signaling pathway that blocks bile acid cytotoxicity. We identified the transcription factor NF-κB as one of the downstream targets of the TCDC-stimulated PI3K activity. NF-κB regulates expression of a large number of potential anti-apoptotic genes, including cIAP-1, XIAP, and IEX-IL (15–17). Interestingly, cIAP-1 inhibits apoptosis by suppressing activation of an apical caspase, most likely caspase-8 (15). Thus, NF-κB may suppress bile acid-mediated Fas/caspase-8 activation by up-regulating cIAP-1 expression.

Our observations of TCDC stimulation of both pro- and anti-apoptotic signaling processes are reminiscent of signaling by TNF receptor-1 (35). Although TNF receptor-1 signaling has been shown to activate PI3K (18), we observed TCDC-mediated activation of NF-κB by EMSA in mouse hepatocytes obtained from TNF receptor-1 knockout animals (data not shown). These data exclude a role for TNF receptor-1 in TCDC-associated activation of NF-κB.

The observations presented in this study have significant implications for human liver diseases. Hepatic retention of toxic bile acids is thought to play a key role in liver injury during cholestasis (43) and is, in part, caused by hepatocyte apoptosis (44). Our data suggest that some bile acids attenuate their inherent cytotoxic effects by activating a PI3K-dependent survival signal that is mediated by PKCζ and NF-κB. This concept suggests that the net effect of bile acids in mediating liver injury reflects a balance between pro- and anti-apoptotic processes. Thus, factors that positively modulate the PI3K-dependent survival signaling cascade may attenuate liver injury. Administration of modified bile acids that activate survival pathways might represent a rational therapy for cholestatic liver injury. Such pharmacologic agents could also be useful in other liver diseases associated with Fas-mediated liver injury (e.g. viral hepatitis and alcohol-associated liver disease). Because bile acids can be administered orally and taken up by the liver with a high first pass clearance, these concepts deserve further investigation.

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