Protein kinase Cδ protects against bile acid apoptosis by suppressing proapoptotic JNK and BIM pathways in human and rat hepatocytes

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Webster CR, Johnston AN, Anwer MS. Protein kinase Cδ protects against bile acid apoptosis by suppressing proapoptotic JNK and BIM pathways in human and rat hepatocytes. Am J Physiol Gastrointest Liver Physiol 307: G1207–G1215, 2014. First published October 30, 2014; doi:10.1152/ajpgi.00165.2014.—Retained bile acids, which are capable of inducing cell death, activate protein kinase Cδ (PKC-δ) in hepatocytes. In nonhepatic cells, both pro- and antiapoptotic effects of PKC-δ are described. The aim of this study was to determine the role of PKC-δ in glycochenodeoxycholate (GCDC)-induced apoptosis in rat hepatocytes and human HUH7-Na-taurocholate-cotransporting polypeptide (Ntcp) cells. Apoptosis was monitored morphologically by Hoechst staining and biochemically by immunoblotting for caspase 3 cleavage. The role of PKC-δ was evaluated with a PKC activator (phorbol myristate acetate, PMA) and PKC inhibitors (chelerythrine, H-7, or staurosporine). PKC-δ knockdown, and wild-type (WT) or constitutively active (CA) PKC-δ. PKC-δ activation was monitored by immunoblotting for PKC-δ Thr505 and Tyr311 phosphorylation or by membrane translocation. JNK and Akt phosphorylation and the amount of total bisindolylmaleimide (BIM) were determined by immunoblotting. GCDC induced the translocation of PKC-δ to the mitochondria and/or plasma membrane in rat hepatocytes and HUH7-Ntcp cells and increased PKC-δ phosphorylation on Thr505, but not on Tyr311, in HUH7-Ntcp cells. GCDC-induced apoptosis was attenuated by PMA and augmented by PKC inhibition in rat hepatocytes. In HUH7-Ntcp cells, transfection with CA or WT PKC-δ attenuated GCDC-induced apoptosis, whereas knockdown of PKC-δ increased GCDC-induced apoptosis. PKC-δ silencing increased GCDC-induced JNK phosphorylation, decreased GCDC-induced Akt phosphorylation, and increased expression of BIM. GCDC translocated BIM to the mitochondria in rat hepatocytes, and knockdown of BIM in HUH7-Ntcp cells decreased GCDC-induced apoptosis. Collectively, these results suggest that PKC-δ does not mediate GCDC-induced apoptosis in hepatocytes. Instead PKC-δ activation by GCDC stimulates a cytoprotective pathway that involves JNK inhibition, Akt activation, and downregulation of BIM.

Glycochenodeoxycholate; exchange protein activated by cAMP; cell death; HUH7; BCl2 proteins

Bile acids are important signaling molecules in the liver, which are involved in the regulation of bile formation, glucose and lipid metabolism, and cell fate (13). During cholestatic disorders, bile acids accumulate in the liver and fuel pathology that ultimately results in liver failure (13, 55). The importance of these toxic effects in humans is illustrated in progressive familial intrahepatic cholestasis, a genetic disorder of bile salt transport that is associated with accumulation of bile acids and progressive liver failure (26). In addition, recent studies highlight the important pathogenic role bile acid accumulation plays in the progression of nonalcoholic steatohepatitis in humans (7, 40). Despite the pathological importance of bile acid-induced hepatic injury, the mechanisms behind its toxic effects are not fully characterized, thus hampering development of therapeutic strategies to protect against their deleterious effects.

Glycochenodeoxycholate (GCDC), the predominant circulating bile acid in humans, paradoxically elicits prodeath as well as prosurvival signals in hepatocytes (19, 24, 46–48). Prodeath pathways converge on sustained phosphorylation of JNK. The role of JNK in hepatocyte death is supported by results that show that chemical or genetic inhibition of this kinase attenuates bile acid-induced apoptosis in vitro, and genetic silencing in vivo attenuates cell injury/fibrosis in bile duct-ligated rats (24, 29, 44). Bile acid-induced prosurvival signaling involves activation of phosphoinositide 3-kinase (PI3K) and Akt, as evidenced by studies that show that inhibition of either kinase results in increased cell death in response to GCDC (16, 48). Whether GCDC induces cell death or cell survival likely depends on which pathway is activated in a particular pathological setting. GCDC-induced apoptosis can be ameliorated by agents that either inhibit the prodeath pathway and/or activate the prosurvival pathway.

PKCs are critical regulators of cell proliferation and survival in many cells. (45). Although evidence suggests that PKCs are involved in GCDC-induced apoptosis (22, 27, 28, 35, 44), the exact mechanism whereby they modulate apoptosis remains unclear. Studies using broad-spectrum inhibitors/activators show that PKCs can both ameliorate and potentiate GCDC-induced apoptosis (23, 28, 29, 47). This paradox might be explained if different PKC isoforms are involved in prodeath and prosurvival effects of bile acids. The PKCs are a family of serine/threonine kinases that includes classical (α, β, and γ), novel (δ and ε), and atypical (ζ, λ, and η) isoforms (46). GCDC activates PKC-α, PKC-δ, PKC-ζ, and PKC-ε isoforms in hepatocytes (28, 39). In studies to date, bile acid-induced apoptosis can be attenuated by combined inhibition of PKC-α and -β (28), suggesting that these isoforms may be proapoptotic and attenuated by activation of PKC-ζ, suggesting that it may be antiapoptotic (47).

The role of PKC-δ in GCDC-induced apoptosis is controversial with studies showing that activation can be either pro- or antiapoptotic, depending on the stimulus (17, 36, 45, 57), a finding that has been reported in nonhepatic cells as well (10). With toxic agents, activation of PKC-δ is proapoptotic, whereas, in ischemic injury, activation is cytoprotective (17, 36, 45, 57). Whether PKC-δ is cytoprotective or proapoptotic in bile acid-induced toxicity is unknown. The aim of the present study was to determine the role of GCDC-induced activation of PKC-δ in bile acid-induced apoptosis. Our results
suggest that GCDC-induced activation of PKC-δ is part of a cytoprotective signaling pathway and that activation of this pathway leads to suppression of bisindolylmaleimide (BIM)- and JNK-mediated cell death pathways in hepatocytes.

MATERIALS AND METHODS

The broad-spectrum PKC inhibitors, chelerythrine, calphostin C, and H7, and streptavidin agarose were purchased from Calbiochem (San Diego, CA). Phorbol myristate acetate (PMA), collagenase, GCDC, and all tissue culture reagents were from Sigma Chemical (St. Louis, MO). 8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate sodium salt (2-Me-CPT-cAMP), a selective activator of cAMP guanine exchange factor, exchange protein activated by cAMP (EPAC), was from Axxora (Farmingdale, NY) (16). Antibodies to Akt (Ser473), JNK (Thr183/Tyr185), PKC-δ/H9254 (Thr505), PKC-δ/H9254 (Tyr311), BIM (Ser69), cleaved caspase 3, Akt, BIM, and Bax were from Cell Signaling Technology (Beverly, MA). Actin, total PKC-δ, and GADPH antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). E-cadherin and Bax A67 antibodies were purchased from BD Transduction Laboratories (San Jose, CA). Wild-type (WT) and constitutively active (CA) PKC-δ plasmids were purchased from Addgene (Cambridge, MA). siRNA against PKC-δ and BIM and negative-control siRNA were purchased from Qiagen (Valencia, CA). Lipofectamine 2000 and Lipofectamine RNAiMAX were obtained from Invitrogen (Carlsbad, CA). Sulfo-NHS-LC-Biotin was purchased from Pierce (Rockford, IL).

Cell culture and monitoring of apoptosis. Mouse or rat hepatocytes were prepared and cultured from Wistar rats (Charles River Laboratories) or PKC-δ/H9254/H11002/H11002 mice as described previously (16). Two breeding pairs of PKC-δ/H9254/H11002/H11002 mice bred into the C57BL/6 background, origi-

Fig. 1. Glycochenodeoxycholate (GCDC) activates PKC-δ. Rat hepatocytes (A–C) were treated with 50 μM GCDC (A–C) or 100 nM phorbol myristate acetate (PMA) (D) and then probed for total PKC-δ (A–C) or for equal loading protein loading with antibodies to cytochrome c oxidase (ACCO) (A) or E-cadherin (ECAD, C). The data in B represent the quantification of 3 representative immunoblots. HUH7-Na-taurocholate-cotransporting polypeptide (Ntcp) cells were treated with 250 μM GCDC (D–G) or 100 nM PMA (E–G) and then probed for total PKC (TPKC) (D), PKC-δ (Thr505) (E and F), or PKC-δ (Ty311) (G) in biotinylated plasma membrane fractions (D) or whole cell lysates (E–G). Equal protein loading was determined with E-cadherin (D) or TPKC-δ (F and G). *Significant increase over the amount of protein seen in control cells.

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nally derived by the laboratory of Dr. Keichi I. Nakayama (37), were a kind gift from Dr. Elizabeth Harrington (Providence VA Medical Center, Research Services, Providence, RI) (14). The mice were subsequently maintained and bred into the C57BL/6 background at the Cummings School of Veterinary Medicine Laboratory Animal facility. The absence of PKC-δ was confirmed by immunoblot analysis of ear tissues using a PKC-δ antibody.

Hepatocytes were plated on type I rat tail collagen in minimal Eagle’s medium (MEM) with l-glutamine, 100 nM insulin, and 10% heat-inactivated fetal calf serum and incubated at 37°C in a humidified atmosphere of 5% CO2 for 1 h. Medium was changed to MEM heat-inactivated fetal calf serum and incubated at 37°C in a humidified incubator.

Apoptosis was induced by adding GCDC (50 μM) for 2 h. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health, and all animal study protocols were submitted to and approved by an institutional animal use and care committee.

HUH7-na-taurocholate-cotransporting polypeptide (Ntcp) cells, a human hepatoma cell line that stably overexpresses the rat bile salt transporter (Dr. Christian Rust, Munich, Germany) (46), were cultured in Eagle’s MEM supplemented with 10% fetal bovine serum, 100,000 U/l penicillin, 100 μg/ml streptomycin, 1X nonessential amino acids, 1 mM pyruvate, and 0.5 μg/ml of genistein at 37°C in a 5% CO2-95% O2 air incubator. For experiments, cells were serum starved overnight and then treated with 200 μM GCDC for 4 h.

Apoptosis in hepatocytes or HUH7-Ntcp cells was detected morphologically by evaluation of Hoechst 33258-stained cells and biochemically by immunoblotting of whole cell lysates for the active 17-kDa cleavage fragment of caspase 3 as previously described (16).

Role of PKC. To determine the role of PKC in GCDC-induced apoptosis, cells were treated with PKC inhibitors, chelerythrine, calphostin C, or H7, or with the PKC activator, PMA, before the addition of GCDC. PKC-δ activation by GCDC was demonstrated by showing translocation of the kinase to the cell surface and/or to mitochondrial membranes. Mitochondria were prepared from hepato-

![Graphs and images](http://ajpgi.physiology.org/)

**Fig. 2.** PKC activation is cytoprotective. HUH7-Ntcp cells (A) or rat hepatocytes (B and C) were treated with 250 or 50 μM GCDC for 4 or 2 h, respectively. Some were pretreated with 100 nM PMA (A and B), 2.5 μM chelerythrine, 0.1 μM calphostin C, or 5 μM H7 (C). HUH7-Ntcp cells (D–F) were transfected with scrambled siRNA (SCRBi) or siRNA to PKC-δ (PKC-δsi) for 48 h (D) or with wild-type (WT) PKC-δ (WT), constitutively active (CA) PKC-δ, or empty vector (EV) (EV, EV) overnight (E and F) and cells treated with GCDC (250 μM). In E and F, C stands for control untreated controls and G for GCDC-treated cells. Apoptosis was monitored morphologically by Hoechst staining (A–F) or biochemically by detection of the cleavage product of caspase 3 (CLV3) (A, B, D–F). *Significantly different than the amount of apoptosis seen in untreated cells (A and B), GCDC-treated cells (C), or GCDC-treated cells transfected with a SCRBsi (D) or EV (E and F).
cytes by differential centrifugation or digitonin permeabilization as previously described (53). To determine PKC-δ translocation to the plasma membrane, a cell-surface biotinylation procedure was used as previously described (39). Briefly, after treatment, cell-surface proteins were biotinylated by exposure to sulfo-NHS-LC-Biotin followed by preparation of whole cell lysates that were then used to determine biotinylated and total PKC-δ by affinity purification and immunoblotting. To correct for loading variation, the blots were probed with E-cadherin. The phosphorylation of PKC-δ at Thr505 and Tyr311 was evaluated by immunoblot analysis of whole cell lysates using phospho-specific antibodies.

**Transfection of cells.** HUH7-Ntcp cells were transfected with an empty vector (EV) or hemagglutinin (HA)-tagged WT or CA PKC-δ constructs by using Lipofectamine 2000 according to the manufacturer's instructions. Briefly, culture medium was changed to OptiMEM containing Lipofectamine and EV, WT, or CA PKC-δ and incubated at 37°C for 24 h. The ratio of DNA and Lipofectamine was 1 μg DNA to 3 μl Lipofectamine. Expression of WT and CA PKC-δ in HUH7-Ntcp cells was confirmed by immunoblotting with anti-HA antibodies and/or total PKC-δ antibodies.

HUH7-Ntcp cells were transfected with scrambled siRNA or siRNA against PKC-δ by using Lipofectamine RNAiMAX according to the manufacturer's instructions. Briefly, the cell culture medium was changed to antibiotic-free regular media, followed by addition of OptiMEM containing Lipofectamine RNAiMAX and scrambled siRNA or siRNA PKC-δ (20 nM) and incubated at 37°C for 48 h.

**Role of BIM and Bax.** Mitochondria were prepared from rat hepatocytes treated with GCDC for 5, 15, 30, and 60 min and immunoblotted for total BIM. Equal protein loading was determined with antibodies to cytochrome c oxidase (Molecular Probes, Eugene, OR). The role of BIM in GCDC-induced apoptosis was evaluated after knockdown with siRNA to BIM (20 nM) as described above. Bax activation was determined by immunoprecipitation of whole cell lysates (250 μg) prepared from control and GCDC-treated cells with a conformation-specific antibody, Bax A67, according to manufacturer's instructions. The amount of activated Bax was determined by immunoblotting with a conformation-neutral antibody.

**Data analysis.** Results were expressed as means ± SD and represented the results of at least three separate experiments. Data were analyzed by paired t-test. P < 0.05 was considered statistically significant.

**RESULTS**

**GCDC translocates PKC-δ to the plasma membrane and to mitochondria.** Activation of novel and classical PKC isoforms involves translocation to a variety of cellular membranes (4, 45). Previous studies have shown that GCDC translocates PKC-δ to a mixed-membrane compartment in rat hepatocytes (27). As our previous data suggest that bile acid-induced apoptosis proceeds primarily through an intrinsic mitochondrial mediated pathway (24, 53), we looked at translocation of PKC-δ to the mitochondria as well as the plasma membrane. In rat hepatocytes, GCDC treatment resulted in a two- and four-fold increase in the amount of PKC-δ in the mitochondrial membranes at 5 and 15 min, respectively, which decreases to baseline by 30 min (Fig. 1B). In both rat hepatocytes and HUH7-Ntcp cells, GCDC treatment resulted in twofold increase in PKC-δ in the plasma membrane (Fig. 1, C and D). Because activation of PKC-δ also involves phosphorylation, we examined phosphorylation of the molecule at two critical sites: Thr505 in the activation loop and Tyr311 in the hinge region. The former is necessary for PKC-δ activation, whereas the latter has been implicated in proapoptotic signaling (6, 10). We show that GCDC increases phosphorylation at Thr505 site (Fig. 1, E and F) but not on Tyr311 (Fig. 1G). These results suggest that GCDC activates PKC-δ by phosphorylating Thr505 and that GCDC does not activate the Tyr311 proapoptotic form of PKC-δ.

**Role of PKC/PKC-δ in GCDC-induced apoptosis.** Global activation of PKC with PMA protects rat hepatocytes and HUH7-Ntcp cells from GCDC-induced apoptosis (Fig. 2, A and B). Conversely, global PKC inhibition with calphostin C, H7, or chelerythrine augmented GCDC-induced apoptosis in (Fig. 2C). These results indicate that overall broad-spectrum PKC activation is cytoprotective against GCDC-induced apoptosis. Next we determined the role of PKC-δ in GCDC-induced hepatocyte apoptosis. Although rotterlin is often used as a selective pharmacological PKC-δ inhibitor, it has multiple nonspecific effects, including uncoupling of mitochondrial ox-
Protein kinase Cδ and bile acid-induced apoptosis

The proapoptotic effect of GCDC is mediated via inhibition of proapoptotic or activation of antiapoptotic pathways. The effect of inhibition of PKC-δ was determined. When PKC-δ was silenced in HUH7-Ntcp cells with siRNA, GCDC-induced activation of JNK was augmented 1.8-fold (Fig. 3D), whereas GCDC-induced activation of Akt was decreased almost in half (Fig. 3B). These results are consistent with the hypothesis that the proapoptotic effect of GCDC-induced activation of PKC-δ involves both suppression of a proapoptotic pathway as well as augmentation of an antiapoptotic pathway.

BIM is necessary for GCDC-induced apoptosis. In keratinocytes, epidermal growth factor attenuates keratinocyte apoptosis by a PKC-δ-mediated pathway that blocks activation of the proapoptotic Bcl-2 homology domain 3 protein, BIM (42). BIM is known to play a vital role in mitochondria-mediated hepatocyte cell death in response to fatty acids, viral infection, and acetaminophen and transforming growth factor-β expression.

**Fig. 4.** Bisindolylmaleimide (BIM) regulates GCDC apoptosis. Rat hepatocytes were treated with GCDC (50 μM) (A, B, D, and E), 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′-5′-cyclic monophosphate sodium salt (CPT-2-Me-cAMP) (E), or both (D) followed by preparation of mitochondrial fractions (A, B, and D) or whole cell lysates (E), which were immunoblotted with antibodies to BIMEL (A, B, and D) or phospho-BIM(Ser69) (E). HUH7-Ntcp cells (C and F) were transfected with control siRNA (SCRBsi) or siRNA to BIM (BIMsi) followed by treatment with GCDC (250 μM) for 4 h. Apoptosis (C) was monitored by Hoechst staining and CLV3 and BIM knockdown and expression with antibodies to BIMEL (A, B, and D) or phospho-BIM(Ser69) (E). HUH7-Ntcp cells (G and H) were transfected with scrambled siRNA (SCRBsi) or siRNA to PKC-δ (PKC-δsi) for 48 h. The cells were treated with 250 μM GCDC for 3 h and then used to prepare mitochondrial fractions by digitonin permeabilization (G) or subjected to immunoprecipitation with an antibody that recognizes the active form of Bax, Bax A67 (H). The immunoprecipitation products and mitochondria lysates were then immunoblotted for total Bax. Equal protein was verified with GADPH or cytochrome c oxidase.

*Significantly different from the amount of total (A, B, D, and F) or phospho (E) BIM in control (B, D, E, and F) or GCDC-untransformed (C) cells. #Significantly different from GCDC-treated (D).
It is known that GCDC mediates intrinsic mitochondrial apoptosis in hepatocytes and that it can translocate the proapoptotic BCL-2 protein, Bax, to the mitochondrial membrane (12, 18, 53). Once in the membrane, BIM facilitates Bax-mediated apoptosis by promoting integration and oligomerization of Bax in the mitochondrial membrane. Direct evidence of a role for BIM in GCDC-induced apoptosis in hepatocytes, however, is lacking.

We, therefore, sought to determine whether BIM is necessary for GCDC-induced apoptosis in hepatocytes. First we show that GCDC translocates the major form of BIM, BIMEL, to the mitochondria. Within 5 min of exposure to GCDC, the amount of BIMEL in the mitochondria increases by 40% (Fig. 4, A and B). To determine whether BIMEL was necessary for GCDC-induced apoptosis, HUH7-Ntcp cells were transfected with siRNA to BIM. After BIM knockdown, transfected cells were more resistant to GCDC-induced apoptosis compared with cells transfected with scrambled siRNA (Fig. 4C). These results provide evidence that GCDC-induced mitochondrial translocation of BIMEL contributes to GCDC-induced apoptosis.

To determine whether modulation of BIM might be a common method by which cytoprotective agents modulate GCDC apoptosis, we evaluated the role of BIMEL in the survival effect of cAMP. Our previous studies have shown that cAMP activates PKC-δ in hepatocytes and that cAMP-mediated activation of EPAC protects against GCDC-induced apoptosis (16, 49). We now show that treatment of rat hepatocytes with a cell-permeable EPAC activator, 2-Me-CPT-cAMP, prevents GCDC-induced mitochondrial translocation of BIMEL and induces phosphorylation BIM on Ser69 (Fig. 4, D and E). Phosphorylation on Ser69 targets BIM for ubiquitination and degradation (25). Consistent with this, treatment of hepatocytes with 2-Me-CPT-cAMP for 24 h significantly decreased total BIMEL by 78% ± 22% compared with the level in control hepatocytes (P = 0.03). These results suggest that cAMP inhibits GCDC-induced apoptosis by inhibiting mitochondrial translocation of BIMEL, an effect possibly mediated by enhancing BIM phosphorylation and its subsequent degradation.

To determine whether modulation of BIM might be involved in the cytoprotective effect of PKC-δ, we determined the effect of silencing PKC-δ on BIMEL expression. HUH7-Ntcp cells transfected with PKC-δ siRNA had an almost twofold increase in the expression of total BIMEL (Fig. 4F), suggesting that PKC-δ might influence GCDC-induced apoptosis by modulating BIMEL expression.

Because we have previously shown that GCDC induces translocation of the final death effector, Bax, to the mitochondria (24, 53), the next set of experiments was done to determine whether there was a link between PKC-δ and Bax activation. Activation of Bax, which is facilitated by BIM, requires both translocation to the mitochondria and induction of a conformational change, leading to oligomerization within the membrane. We examined the effect of PKC-δ knockdown on Bax translocation and activation. Silencing of PKC-δ in HUH7-Ntcp cells increases the amount of Bax in the mitochondria in control and GCDC-treated cells (Fig. 4G). We used an antibody that recognizes the active conformation of Bax in the mitochondria (A67) for immunoprecipitation of Bax. Silencing of PKC-δ in HUH7-Ntcp cells was increased in the amount of activated BAX after GCDC treatment (Fig. 4H). These results suggest that silencing of PKC-δ is associated with increased movement and activation of BAX in the mitochondria and suggest that this may contribute to the potentiation of GCDC-induced apoptosis seen in the absence of PKC-δ.

PKC-δ-/- mice have increased sensitivity to GCDC-induced apoptosis. Hepatocytes isolated from control and PKC-δ-null mice were treated with GCDC (100 μM), and the amount of apoptosis was determined morphologically and biochemically after 4 h. Hepatocytes from PKC-δ-null mice had a sixfold increase in the amount of GCDC-induced apoptosis compared with the amount seen in control hepatocytes (Fig. 5, A and B). These data are similar to that we observed in HUH7-Ntcp cells when PKC-δ is silenced (Fig. 2D).

**DISCUSSION**

Bile acids are important signaling molecules in cholestatic liver disorders, where they can elicit both prodeath and prosurvival signaling. The mechanisms whereby bile acids act in such an ambiguous manner are not fully understood but are of growing interest, as drugs targeting bile acid receptors are being developed to treat a variety of hepatobiliary and nonhepatobiliary disorders (23, 40). Although it has been known for many years that bile acids activate PKC (27, 38), the role of PKC isoforms in bile acid cell fate signaling has not been well characterized. As PKC-δ has been implicated in cell fate in nonhepatic cells (6, 10), our aim was to investigate whether this kinase could be involved in bile acid-induced apoptosis. The results of the present study suggest that activation of PKC-δ by GCDC is cytoprotective in rat hepatocytes and human hepatoma cells. This conclusion is supported by the following evidence: 1) GCDC activates PKC-δ; 2) broad-
spectrum pharmacological inhibition of PKCs or specific genetic inhibition of PKC-δ (with siRNA in HUH7-Ntcp or in PKC-δ-null mice) augments GCDC-induced apoptosis; and 3) broad-spectrum activation of PKCs or specific activation of PKC-δ ameliorates bile acid-induced apoptosis. These studies also provide initial mechanistic insight into how PKC-δ may confer a protective advantage. These potential prosurvival actions include 1) suppression of GCDC-induced JNK activation, 2) decreased expression of BIM, and 3) increased GCDC-induced activation of Akt signaling.

Despite the knowledge for many years that bile acids activate PKC in hepatocytes (4, 27, 35), information on their role in bile acid signaling is limited. What data are available are often contradictory, implicating bile acid-induced PKC activation in both cholestasis and cholestasis, as well as both cell death and survival. In general, PKC activation by more hydrophobic bile acids, such as GCDC, tauroliothocholate, and taurochenodoxycholate, is associated with cholestasis and cell death (9, 15, 32, 50), whereas PKC activation by more hydrophilic bile acids, such as tauroursodeoxycholate and taurocholate, is associated with cholestasis and survival (3, 8, 24, 56). These divergent effects could be explained by differential activation of PKC isoforms by the two groups of bile acids. There is some support for this hypothesis in the literature. For instance, previous studies have shown that tauroursodeoxycholate activates PKC-α to induce cholestasis, whereas taurochenodoxycholate activates PKC-ε to induce cholestasis (4, 8, 9). In addition, PKC-δ activation is involved in cAMP-induced membrane translocation of NTCP and multidrug resistance-associated protein 2 (4, 49). Previous studies have suggested that hydrophobic bile acid activation of classical PKCs or PKC-ζ (27, 46) is proapoptotic, whereas the results of the present study show that GCDC activation of PKC-δ is cytoprotective.

Consistent with our observation are studies that show that PKC-δ activation ameliorates hepatocyte damage attributable to ischemia and serum deprivation (52, 57). PKC-δ activation, however, has also been reported to mediate hepatocyte cell death (17, 21, 35). Emerging evidence suggests that distinct inciting agents differentially influence PKC-δ activity and substrate specificity. These differential effects can be controlled by tyrosine phosphorylation and caspase-mediated cleavage of PKC-δ, as well as by the presence of different PKC-δ splice variants (6, 10). Tyrosine phosphorylation at the 311 position has been correlated with the proapoptotic activity of PKC-δ. The inability of GCDC to phosphorylate PKC-δ at Tyr311 in the present study might explain, in part, why activation of PKC-δ by GCDC is not cytotoxic.

Sustained JNK activation is a prodeath signal in bile acid-induced apoptosis in hepatocytes (24, 44), whereas activation of PI3K/Akt is prosurvival (24, 53). Our studies show that genetic silencing of PKC-δ results in an increase in GCDC-induced phosphorylation of JNK while at the same time decreasing GCDC phosphorylation of Akt. These opposing effects would both promote hepatocyte cell death, and indeed PKC-δ knockdown is associated with enhanced GCDC-induced apoptosis. The mechanisms by which PKC-δ maintains Akt phosphorylation and inhibits JNK phosphorylation in hepatocytes remain to be clarified. Previous studies have suggested a link between Akt phosphorylation and PKC-δ. For example, in hepatocytes, genetic silencing of PKC-δ decreases insulin-stimulated Akt phosphorylation (11), whereas work in nonhepatic cells has established the presence of PKC-δ pathways leading to Akt activation (20, 33).

Bile acids, such as GCDC, are well-known mitochondrial toxins that induce intrinsic mitochondrial-mediated apoptosis. These bile acids translocate Bax, the final effector proapoptotic BCl-2 protein, to the mitochondria, where it can induce permeabilization of the outer mitochondrial membrane (12, 18, 53). We now present several lines of evidence suggesting that activation of Bax could be mediated by GCDC modulation of BIM. This evidence includes the observations that 1) GCDC translocates BIM to the mitochondria; 2) genetic silencing of BIM attenuates GCDC apoptosis; 3) silencing of cytoprotective PKC-δ signaling augments BIM expression; and 4) the prosurvival protein, EPAC, prevents GCDC-induced translocation of BIM to the mitochondria and phosphorylates BIM, leading to decreased protein expression. Several studies have already demonstrated an important role for BIM in intrinsically mediated mitochondrial apoptosis in vitro in hepatocytes. In addition, a recent study reported increased expression of BIM in vivo in the liver of human patients with primary biliary cirrhosis verifying the importance of this proapoptotic protein in a clinical setting (31). Developing targeted strategies to downregulate BIM may represent a therapeutic tool to decrease cholestatic liver injury. Such targeted therapies are currently being incorporated into chemotherapy protocols because up-regulation of BIM in tumors is associated with chemotherapy resistance (2).

In conclusion, our studies present evidence that GCDC-induced activation of PKC-δ triggers downstream events that promote cell survival. These downstream events include suppression of prodeath JNK and BIM signaling and upregulation of the survival kinase, Akt. Better understanding of the survival signaling controlled by bile acid will be crucial to designing future bile acid-targeted therapies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: C.R.W. and M.S.A. conception and design of research; C.R.W. and A.J. performed experiments; C.R.W. and A.J. analyzed data; C.R.W. and M.S.A. interpreted results of experiments; C.R.W. prepared figures; C.R.W. drafted manuscript; C.R.W., A.J., and M.S.A. edited and revised manuscript; C.R.W., A.J., and M.S.A. approved final version of manuscript.

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