Hydrophobic bile acid apoptosis is regulated by sphingosine-1-phosphate receptor 2 in rat hepatocytes and human hepatocellular carcinoma cells

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Webster CR, Anwer MS. Hydrophobic bile acid apoptosis is regulated by sphingosine-1-phosphate receptor 2 in rat hepatocytes and human hepatocellular carcinoma cells. Am J Physiol Gastrointest Liver Physiol 310: G865–G873, 2016. First published March 17, 2016; doi:10.1152/ajpgi.00253.2015.—The hepatotoxic bile acid glycochenodeoxycholate (GCDC) modulates hepatocyte cell death through activation of JNK, Akt, and Erk. The nonhepatotoxic bile acid taurocholate activates Akt and Erk through the sphingosine-1-phosphate receptor 2 (S1PR2). The role of the S1PR2 in GCDC-mediated apoptosis and kinase activation is unknown. Studies were done in rat hepatocytes, HUH7 cells, and HUH7 cells stably transfected with rat Ntcp (HUH7-Ntcp). Cells were treated with GCDC and apoptosis was monitored morphologically by Hoechst staining and biochemically by immunoblotting for the active cleaved fragment of caspase 3. Kinase activation was determined by immunoblotting with phospho-specific antibodies. JTE-013, an inhibitor of S1PR2, significantly attenuated morphological evidence of GCDC-induced apoptosis and prevented caspase 3 cleavage in rat hepatocytes and HUH7-Ntcp cells. In hepatocytes, JTE-013 mildly suppressed, augmented, and had no effect on GCDC-induced JNK, Akt, and Erk phosphorylation, respectively. Similar results were seen in HUH7-Ntcp cells except for mild suppression of JNK and Erk phosphorylation. Knockdown of S1PR2 in HUH7-Ntcp augmented Akt, inhibited JNK, and had no effect on Erk phosphorylation. GCDC failed to induce apoptosis or kinase activation in HUH7 cells. In conclusion, S1PR2 inhibition attenuates GCDC-induced apoptosis and inhibits and augments GCDC-induced JNK and Akt phosphorylation, respectively. In addition, GCDC must enter hepatocytes to mediate cell death or activate kinases. These results suggest that S1PR2 activation is proapoptotic in GCDC-induced cell death but that this effect is not due to direct ligation of the S1PR2 by the bile acid.

Sphingosine-1-phosphate receptor 2 (S1PR2) is one of five different GPCRs in a family of receptors that bind sphingosine-1-phosphate (S1P) (21, 40). S1P is a bioactive sphingolipid produced by the enzymatic conversion of sphingomyelin to ceramide and sphingosine. Along with S1PR3, S1PR2 is the major S1P receptor subtype in hepatocytes (21, 44). The ubiquitously expressed S1PR2 is involved in cell survival and death in many cell types (40). Since bile acids control life and death in hepatocytes, it is tempting to speculate that bile acid cell fate signaling may involve S1PR2. In addition, there is ample evidence that bile acids are involved in sphingolipid metabolism and that bile acid apoptosis involves proapoptotic sphingomyelin signaling. Hepatotoxic bile acids increase ceramide in rat hepatocytes in vitro (3, 12) and in mice in vivo (28). In addition, they can increase S1P and sphingomyelinase levels in rat hepatocytes in vitro (21, 30). Genetic silencing or inhibition of sphingosine kinase (the enzyme responsible for the conversion of sphingosine to S1P) or knockdown of acidic sphingomyelinase inhibits hydrophobic bile acid-induced apoptosis in rat hepatocytes (9, 12, 36). Furthermore, S1PR2 knockout mice are resistant to the cholestatic effect of bile duct ligation (32) in which injury is primarily due to the accumulation of toxic bile acids. Thus S1PR2 may mediate the hepatotoxic effects of bile acids.

Hepatotoxic bile acids produce hepatocellular apoptosis. Thus it is possible that bile acid-induced hepatocyte apoptosis may be mediated via S1PR2. However, the role of S1PR2 in bile acid-induced apoptosis is unclear. A recent study showed that chemical inhibition of S1PR2 had no effect on bile acid-induced apoptosis in rat hepatocytes (21). Another study (44) found that genetic silencing and chemical inhibition of S1PR2 blocked the activation of the known survival kinase Akt (6, 14, 32) by the conjugated bile acid taurocholate (TC) in rat hepatocytes. TC-induced activation of Akt was also diminished in S1PR2 knockout mice (44). If the S1PR2 couples bile acids to the activation of Akt then one would expect that S1PR2 activation would be antiapoptotic. However, it should be noted that TC is not considered a hepatotoxic bile acid and study of the role of S1PR2 in the activation of Akt by hepatotoxic bile acids has been rather limited. Thus the aim of our study was to further define the role of S1PR2 in GCDC-induced apoptosis as well as activation of both survival (Akt, Erk) and death kinases (JNK) in hepatocytes. The results of our study are consistent with a proapoptotic role of S1PR2.

MATERIALS AND METHODS

Reagents. Collagenase, Hoechst 33258, glycochenodeoxycholate (GCDC), deoxycholate (DCA), 1H-pyrazol-[3,4-b]pyridazin-6-yl]-1-[(13-dimethyl-4-(2-methylthyl)-4H-pyrrozolo[3,4-b]pyridin-6-yl]-4-(2,6-dichloro-4-pyridinyl)-sarcosamide (JTE-013), and all tissue culture reagents were pur-
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were plated at 5 × 10^5 cells/cm² on tissue culture dishes or coverslips coated with Type I rat tail collagen in Eagle's minimum essential 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 without supplements and, after an additional 3 h, apoptosis was initiated by the addition of 50 μM GCDC. In some experiments cells were pretreated with the 10 μM of JTE-013 30 min prior to the addition of GCDC. JTE-013 has been used to antagonize the hepatocytes S1PR2 receptors in several studies (18, 26, 27, 44). 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 approved by Tufts University institutional animal use and care committee.

HUH7-Ntcp cells, a human hepatoma cell line that stably overexpresses the rat bile salt transporter (Dr. Christian Rust, Munich, Germany; 39), were cultured in MEM supplemented with 10% fetal bovine serum, 100,000 U/l penicillin, 100 mg/l streptomycin, 1× 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 150 μM GCDC for 2 h. Modulators were added 30 min prior to GCDC.

Please note that two different concentrations of GCDC were used to produce apoptosis reliably in two different experimental models in this study, i.e., 50 μM GCDC in primary rat hepatocytes and 150 μM GCDC in HUH7-Ntcp cells. The concentrations used were based on previously published dose-response data in rat hepatocytes (22, 34) and HUH7-Ntcp7 cells (14). Previous studies by our laboratory (6, 15, 20, 50, 51) and others have used different concentrations to produce GCDC-induced apoptosis in rat hepatocytes (21, 22, 34, 36, 38, 43, 50) and Ntcp-transfected cell lines (7, 14, 15, 16, 39).

Assessment of apoptosis. Morphological evaluation of apoptotic cell death was conducted 2 h after the addition of GCDC to rat hepatocytes or HUH7-Ntcp cells, respectively as previously described (6, 15, 51, 120). Briefly, coverslips were stained with Hoechst 33258 and apoptosis was evaluated with fluorescent microscopy. Apoptotic cells were identified as those whose nucleus exhibited brightly staining condensed chromatin or nuclear fragmentation. Five hundred cells were counted by an observer blinded to the treatment conditions and the number of apoptotic cells was expressed as a % of the total number of cells counted. The presence of the p17/p19-kDa cleavage product of caspase 3 was used as a biochemical indicator of hepatocyte apoptosis. We monitored for GCDC-induced necrosis both through the morphological evaluation of Hoechst-stained cells as well as staining additional coverslips with Trypan blue prior to fixation. In all cases the amount of necrosis with bile acid treatment was compatible to that seen in control cultures and was less than 5% of the total cells counted. Cell lysates were prepared from rat hepatocytes or HUH7-Ntcp cells treated with GCDC for 2 h, respectively, in cell lysis buffer, 20 mM Tris, pH = 7.5, 150 mM NaCl, 1 mm EGTA, 1% Triton, 2 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM glycero-phosphate, 1 mM phenylmethylsulfonyl fluoride, 100 nM okadaic acid, 1 mM sodium orthovanadate, and 10 μg/ml of leupeptin, aprotinin, and pepstatin, separated on SDS-PAGE, and the proteins transferred to polyvinylidenefluoride membranes (PVDF). Immunoblotting was performed with a cleaved caspase 3 antibody and equal protein loading was verified by stripping and probing with an actin antibody.

Determination of kinase phosphorylation. Rat hepatocytes or HUH7-Ntcp cells were treated with indicated bile acids for 60 min with and without prior treatment with JTE-013 (10 μM). Cells were lysed in ice-cold cell lysis buffer and equal amounts of protein were separated in SDS-PAGE, transferred to PVDF membranes, and probed with antibodies phospho-antibodies to Akt, JNK, p38, or ERK. Membranes were developed with chemiluminescence after incubation with a peroxidase-conjugated second antibody. Stripped membranes were reprobed with antibodies for the unphosphorylated form of the kinase or with actin or GAPDH to verify equal protein loading. Blots were digitalized and subjected to densitometric analysis.

Transfections. HUH7-Ntcp cells were transfected with siRNA by using Lipofectamine RNAiMAX according to the manufacturer’s instructions as previously described (20, 51). Briefly, the cell culture medium was changed to antibiotic-free regular medium, followed by addition of OptiMEM containing Lipofectamine RNAiMAX and scrambled control siRNA or siRNA to S1PR2 (20 nM) and incubated at 37°C for 48 h.

Bile acid uptake. The 30-min accumulation of radiolabeled bile acid, [3H]taurocholate (Perkin Elmer, Boston, MA), in hepatocyte cultures was determined as previously described (6).

Statistical evaluation. All results are expressed as means ± standard deviation of at least 3 separate experiments. Results were analyzed for statistical significance with Student’s t-test or one-way ANOVA. A P value of <0.05 was considered significant.

RESULTS

S1PR2 signaling is proapoptotic. The first series of studies were conducted to determine whether S1PR2 is involved in GCDC hepatocyte apoptosis. The role of S1PR2 was determined by inhibiting or knocking down the receptor. Rat hepatocytes and human HUH7-Ntcp cells were pretreated with JTE-013 and then the effect on GCDC-induced apoptosis was determined. The results show that the inhibitor decreased

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Fig. 1. Pharmacological inhibition of S1PR2 attenuates glycochenodeoxycholate (GCDC)-induced apoptosis in rat hepatocytes. Rat hepatocytes were treated sequentially with 10 μM JTE-013 then 50 μM of GCDC for 2 h. Apoptosis was quantified by morphological evaluation of Hoechst-stained cells. Whole cell lysates were prepared and immunoblotted for the p17/19-kDa active cleavage product of caspase 3 (CLV3). Equal protein loading was verified by stripping and immunoblotting for actin. *Significantly different from the amount of apoptosis in GCDC-treated hepatocytes.
morphological evidence of apoptosis by 60% in both rat hepatocytes and HUH7-Ntcp cells (Figs. 1 and 2A). In addition, JTE-013 reduced the amount of cleaved caspase 3 in both rat hepatocytes and HUH7-Ntcp cells (Figs. 1 and 2B). To substantiate our finding with the chemical inhibitor we used a siRNA targeted to the human S1PR2 to knock down the receptor in HUH7-Ntcp cells. After establishing that we had efficient knockdown of S1PR2 (90%) (Fig. 2C, inset) with the siRNA, we were able to demonstrate that silencing of S1PR2 reduced GCDC apoptosis by ~70% and decreased caspase 3 cleavage (Fig. 2, C and D). Knockdown of S1PR2 or treatment with JTE-013 alone had no effect on basal apoptosis. Note that cleavage products 17 and 19 kDa are detected by the antibody used, and depending on the resolution of the gel these products appear as one broad band (Figs. 1 and 2B) or two separate bands (Fig. 2D). Collectively these results suggest that S1PR2 mediates or is involved in GCDC-induced apoptosis consistent with studies showing that S1PR2 mediates other effects of hepatotoxic bile acids.

**S1PR2 and bile acid kinase activation.** Our finding that S1PR2 is involved in the apoptotic effect of GCDC raises the question whether this involvement is due to effects on pro- or antiapoptotic kinases. GCDC has a paradoxical effect on these kinases as this bile acid can simultaneously activate the death kinase JNK and the survival kinases Akt and Erk (15). It is likely that it is the relative balance between activation of these opposing kinases as well as modulation of specific kinase isoforms that controls the ultimate outcome after exposure to hepatotoxic bile

![Fig. 3](http://ajpgi.physiology.org/Downloaded from http://ajpgi.physiology.org/)

**Fig. 3.** Effect of pharmacological inhibition of S1PR2 on GCDC-induced JNK and Akt phosphorylation in rat hepatocytes. Rat hepatocytes were pretreated with 10 μM of JTE-013 for 30 min prior to treatment with GCDC for 1 h. Whole cell lysates were prepared and immunoblotted for active phosphorylated Akt(P) 

(A, Akt-P) or JNK(Ser183/Thr185) (B, JNK-P). Equal protein loaded was verified by reprobing with antibodies to actin or the total kinases (Akt-T or JNK-T). Representative blots are shown and the results of quantification of at least 3 separate experiments are shown. *Significantly different than that seen in the absence of JTE-013.
acids (15). If S1PR2 mediates its apoptotic effects via kinases, one might expect a relative increase in GCDC-induced activation of Akt and Erk and a relative decrease in the activation of JNK in the absence of S1PR2. To characterize the role of S1PR2 in GCDC-induced kinase activation we examined the effect of JTE-013 on GCDC-induced phosphorylation and hence activation of the Akt, Erk, and JNK. In our experiments we pretreated rat hepatocytes or HUH7-Ntcp cells with JTE-013 for 30 min prior to treatment with GCDC for 1 h and then analyzed for kinase phosphorylation by immunoblotting with phospho-specific antibodies. As we have previously reported (15, 20, 51), GCDC phosphorylates Akt, JNK, and Erk in rat hepatocytes (Fig. 3, A and B, and Fig. 5A) and HUH7-Ntcp cells (Fig. 4, A and B, and Fig. 5B). Pretreatment with JTE-013 significantly augmented Akt phosphorylation (Figs. 3A and 4A) in both rat hepatocytes and HUH7-Ntcp cells. The results on JNK phosphorylation were discordant in that JTE-013 caused a minor (14%) but insignificant inhibition of JNK activity in rat hepatocytes (Fig. 3B) whereas inhibition (35%) was significant in HUH7-Ntcp cells (Fig. 4B). Treatment with JTE-013 had no effect on GCDC-induced Erk phosphorylation in rat hepatocytes (Fig. 5A) but resulted in a mild suppression of GCDC-induced phosphorylation in HUH7-Ntcp cells (Fig. 5B). JTE-013 treatment had no effect on basal Akt, Erk, or JNK phosphorylation in rat hepatocytes (Figs. 3–5) and HUH-Ntcp cells or on the amount of total JNK, Erk, or Akt (Figs. 3–5). To substantiate the results with the chemical inhibitor, we knocked down S1PR2 with siRNA in HUH7-Ntcp cells and then treated with GCDC. Again we observed that knockdown of S1PR2 augmented Akt phosphorylation (Fig. 4C) and slightly decreased JNK phosphorylation (Fig. 4D). There was no effect on GCDC-induced Erk phosphorylation (Fig. 5C). Thus our studies show that, both in rat hepatocytes and in a human hepatocellular cell line, inhibition of S1PR2 partially attenuates GCDC-induced JNK phosphorylation and augments GCDC-induced Akt phosphorylation but has little to no effect on Erk phosphorylation. These results are consistent with the hypothesis that S1PR2 may mediate GCDC-induced apoptosis by modulating kinases (JNK and Akt) involved in apoptosis.

Bile acid must enter hepatocytes to induce apoptosis and activate kinases. The above results suggest that S1PR2 is prodeath in GCDC apoptosis. One obvious mechanism may be that GCDC directly activates S1PR2 at the plasma membrane. For this mechanism to be valid, GCDC-induced apoptosis should not require the bile acid to enter hepatocytes. However, previous studies have demonstrated that conjugated bile acids must enter hepatocytes to cause apoptosis (41). In these studies, GCDC failed to induce caspase 3 activation in primary rat hepatocytes (Figs. 3–5) and HUH-Ntcp cells or on the amount of total JNK, Erk, or Akt (Figs. 3–5). To substantiate the results with the chemical inhibitor, we knocked down S1PR2 with siRNA in HUH7-Ntcp cells and then treated with GCDC. Again we observed that knockdown of S1PR2 augmented Akt phosphorylation (Fig. 4C) and slightly decreased JNK phosphorylation (Fig. 4D). There was no effect on GCDC-induced Erk phosphorylation (Fig. 5C). Thus our studies show that, both in rat hepatocytes and in a human hepatocellular cell line, inhibition of S1PR2 partially attenuates GCDC-induced JNK phosphorylation and augments GCDC-induced Akt phosphorylation but has little to no effect on Erk phosphorylation. These results are consistent with the hypothesis that S1PR2 may mediate GCDC-induced apoptosis by modulating kinases (JNK and Akt) involved in apoptosis.

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hepatocytes cultured for 72 h, a time at which expression of Ntcp is lost (37). In addition, GCDC also failed to cause apoptosis in a wild-type HepG2, a human hepatoblastoma cell line, which does not express NTCP but did induce apoptosis in HepG2 cells transfected with rat Ntcp. We have extended these findings in the present study by demonstrating that wild-type HUH7 cells lacking Ntcp are resistant to apoptosis with GCDC (Fig. 6A). However, whether GCDC needs to enter hepatocytes to activate kinases has not been determined. To answer this question we examined whether GCDC could phosphorylate Akt, Erk, and JNK in HUH7 wild-type cells. We have already reported that GCDC can activate these kinases in HUH7-Ntcp cells (15, 20, 51). In wild-type HUH7 cells GCDC fails to phosphorylate JNK, Akt, and Erk, but the membrane-soluble bile acid deoxycholate induces phosphorylation of all three kinases in wild-type HUH7 cells (Fig. 6B). On the contrary, TC, which can activate Akt and Erk through the S1PR2 in rodent hepatocytes, is capable of mediating phosphorylation of Erk, JNK, and Akt in a JTE-013-dependent manner (Fig. 6C). These results suggest that TC-induced kinase activation proceeds through S1PR2, but GCDC must enter cells not only to induce apoptosis but also to initiate kinase phosphorylation. Thus GCDC-induced apoptosis and activation of kinases do not result from direct activation of S1PR2 by GCDC. Instead, S1PR2 may play a permissive role in GCDC apoptosis (see DISCUSSION).

**Bile acid uptake.** To ensure that chemical inhibitors did not interfere with bile acid uptake, we examined the 30-min accumulation of radiolabeled TC in rat hepatocytes and HUH7-Ntcp cells with and without pretreatment with JTE-013. The results show that JTE-013 did not significantly inhibit TC uptake in hepatocytes and that HUH7-Ntcp cells pretreated with JTE-013 and HUH7 wild-type cells did not accumulate TC (Fig. 7).

**DISCUSSION**

The objective of our study was to clarify the role of the S1PR2 in GCDC-mediated cell death and kinase activation in rat and human hepatocytes. In the present work, we show that chemical inhibition and knockdown of S1PR2 in rat hepatocytes and human HUH7-Ntcp cells attenuates GCDC-induced apoptosis while slightly diminishing phosphorylation of the death kinase JNK and augmenting phosphorylation of the survival kinase Akt. In addition we demonstrate that GCDC must enter hepatocytes not only to induce apoptosis but also to trigger phosphorylation of several intracellular kinases including JNK, Akt, and ERK.

Hydrophobic bile acids are important toxins that accumulate in hepatocytes during cholestatic disease leading to apoptosis, necrosis, and the generation of inflammatory responses (1, 5, 49, 52). Accumulating evidence suggest that sphingolipid me-
metabolism plays an important role in bile acid-mediated hepatocyte injury (33). Sphingolipids can be generated by de novo synthesis or by the action of sphingomyelinase on membrane sphingomyelin. This leads to generation of ceramide, which can be further acted upon to yield sphingosine. Sphingosine in turn can be phosphorylated by sphingosine kinase to generate S1P. S1P can be exported out of cells presumably by ABC transporter family members and can then bind to its receptors on the same or neighboring cells (S1PR) to stimulate G protein-regulated signaling pathways (40). Several of these sphingolipid intermediates have a role in bile acid-induced apoptosis. Hepatotoxic bile acids can increase intracellular ceramide (3, 28) and S1P levels in hepatocytes (21). Feeding bile acids to mice increases sphingosine kinase activity (30), and increased serum bile acids due to bile duct ligation in mice drive increases in S1PR2 expression (17). Furthermore, inhibition of bile acid-induced ceramide or S1P generation is protective against bile acid apoptosis (3, 12, 21, 36).

In our present studies pharmacological or genetic inhibition of S1PR2 protects human hepatocellular carcinoma and rat hepatocytes from GCDC-induced apoptosis, suggesting that activation of S1PR2 is proapoptotic. A recent paper in rat hepatocytes showed no effect of S1PR2 inhibition on bile acid-induced apoptosis; instead apoptosis was dependent on activation of S1PR1 (21). Reasons for the discordance between these results and ours are not readily apparent. In both studies, rat hepatocytes were isolated from Wistar rats and plated under similar conditions. Possible explanations might include different sources for the chemical inhibitor JTE-013 or variations in the time of day experiments were done since there is evidence that circadian rhythms can influence cell signaling (47). In the present studies it is noteworthy that we were able to substantiate our data in rat hepatocytes by showing that both chemical inhibition and genetic silencing of S1PR2 attenuated GCDC-induced apoptosis in human HUH7-Ntcp cells. Furthermore, in other models of hepatocyte cell injury, blocking S1PR2 is hepatoprotective. For example, inhibition of S1PR2 increases survival in mice treated with CCl4 and improves mitochondria function.

Fig. 7. Bile acid uptake in hepatocytes. Rat hepatocytes, HUH7-Ntcp, and wild-type HUH7 cells were incubated with 50 μM taurocholate (TC) in the presence of trace amounts of [3H]TC and the amount of radioactive TC taken up at 30 min determined. Results in the presence of JTE-013 (30 min pretreatment with 10 μM) are expressed as a percentage of the amount of uptake in either control rat hepatocytes or control HUH7-Ntcp cell.
and survival in mouse models of ischemia/reperfusion models (18, 42).

Since previous studies have established that GCDC-induced apoptosis in hepatocytes is in part controlled by modulation of kinase activation (2), we determined whether the cytoprotective action of S1PR2 inhibition in bile acid apoptosis was accompanied by modulation of GCDC-induced kinase phosphorylation. Activation of JNK is associated with hepatocyte cell death, whereas Akt and Erk are associated with survival (2, 6, 12, 15, 21, 32, 36, 41, 51). In these studies we show that blockage of S1PR2 receptor either pharmacologically or by RNA silencing in rat hepatocytes and human hepatocellular carcinoma cells results in an a decrease in JNK phosphorylation and an increase in Akt phosphorylation but is without effect on Erk phosphorylation. The changes in JNK and Akt phosphorylation status would be expected to promote hepatocyte survival, which is what we see with S1PR2 inhibition.

Our results showing that GCDC-induced Akt phosphorylation increases after S1PR2 inhibition differ from those previously described (44). In these latter experiments inhibition of S1PR2 with JTE-013 or silencing of S1PR2 with shRNA in rat hepatocytes was associated with inhibition of Akt phosphorylation induced by the nontoxic bile acid TC. Similarly in hepatocytes isolated from S1PR2 knockout mice, TC-induced phosphorylation of Akt was diminished. These results might be explained by the fact that TC is a relatively nontoxic bile acid and might be expected to modulate kinases differently than the hepatotoxic bile acid GCDC. Another possible explanation for the discordant results is that our studies used hepatocyte cultures maintained in minimal media 4 h after isolation whereas the studies of Studer et al. (44) were done in rat hepatocytes cultures maintained in media supplemented with dexamethasone and thyroxine for 24 h after isolation. It is known that the hepatocyte apoptotic behavior changes with time in culture (48) and that both hormone supplements have effects on hepatocyte signaling (10, 29, 31, 45, 46). Support for the idea that divergent culture conditions may explain the divergent results come from the observation that in the Studer et al. study hepatotoxic bile acids failed to even increase Akt phosphorylation (44).

We have corroborated previous findings showing that GCDC must enter hepatocytes to cause apoptosis (20, 41). In addition, we further demonstrate that GCDC-induced activation of several kinases, JNK, Erk, and Akt, relies on the presence of the bile salt transporter Ntcp. This suggests either that GCDC has to enter the cell to modulate intracellular kinase phosphorylation or that binding to Ntcp is necessary to induce kinase phosphorylation. We also substantiate previous findings in rodent hepatocytes and cholangiocytes (27, 44) showing that the hydrophilic and nontoxic bile acid TC activates Akt and Erk kinases in an S1PR2-dependent manner by demonstrating that TC exposure results in JTE-013-dependent phosphorylation of Akt, JNK, and Erk in wild-type HUH7 cells that lack the bile salt transporter. Precisely how intracellular GCDC induces kinase activation has not been fully characterized although there is evidence that some bile acids may act through intramembrane perturbations (19), inhibition of phosphatases (35), stimulating movement of receptors to the cell surface (Fas) (3, 35, 36), and/or transactivation of tyrosine kinase receptors (13, 19) through activation of Src (11) or the generation of reactive oxygen species (35, 36).

Collectively accumulating evidence suggests a model for the role of S1PR2 in GCDC-induced apoptosis. Because GCDC must enter hepatocytes to induce apoptosis, this model does not involve direct binding of the bile acids to the receptor. Instead we propose that GCDC enters the hepatocyte and stimulates ceramide synthesis, which activates the proapoptotic death kinase JNK, leading to apoptosis. GCDC also leads to activation of sphingosine kinase and then generation of S1P. The S1P is transported out of the cell by an ABC transporter and binds to S1PR2, leading to activation of Gαi and inhibition of cAMP production (40). Since our previous studies provide strong evidence that intracellular cAMP is cytoprotective (6, 20), S1P ligation of the S1PR2 leading to a decrease in intracellular cAMP is expected to be proapoptotic (Fig. 8).

In conclusion, we provide evidence that induction of apoptosis by GCDC is facilitated by S1PR2 ligation and that GCDC must enter hepatocytes to induce both apoptosis and kinase phosphorylation. Because bile acids not only are important regulators of cell death/survival but also control glucose and lipid metabolism (4, 25), characterization of how they signal in cells has broad implications. Separate studies showing that S1PR2 is involved in bile acid-induced proliferation, growth, and invasion of cholangiocarcinoma cells (27), as well as in the activation of endoplasmic reticulum stress in mouse models of fatty liver (24) and the proliferation of hepatic myofibroblasts and induction of hepatic fibrosis (18), substantiate the importance of S1PR2 signaling in hepatocyte health and indicate that additional studies to characterize the role of its signaling pathways in bile acid physiology are needed.

Fig. 8. Putative interaction between glycochenodeoxycholate (GCDC) and sphingolipid signaling. GCDC enters hepatocytes via NTCP, the sodium-cotransporting peptide. Once within the cytoplasm GCDC increases ceramide levels, which by themselves have been linked to activation of the death receptor JNK and subsequent apoptosis. GCDC also activates sphingosine kinase, which leads to the generation of sphingosine-1-phosphate (S1P) (shown in Ref. 18). S1P is transported out of the hepatocyte by an ABC transporter (ABC) and binds to the S1PR2. Since S1PR2 can be coupled to Gi, G proteins that inhibit adenylyl cyclase, we hypothesize that S1P ligation of S1PR2 leads to a decrease in intracellular cAMP. This in turns decreases the amount of activated exchange protein activated by cAMP (EPAC), which in turns leads to decreased Akt phosphorylation. Since Akt phosphorylation is antia apoptotic, decreased activation of this kinase would permit GCDC to induce additional apoptosis.
BILE ACID SIGNALING THROUGH S1PR2

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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

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