Sphingosine kinase-1 inhibition protects primary rat hepatocytes against bile salt-induced apoptosis

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1. Introduction

Sphingosine kinases (SphK1 and SphK2) are a distinct class of lipid kinases that are rapidly activated by a variety of extracellular and/or intracellular stimuli and are implicated in modulating cell survival and apoptosis [1]. Additionally, activation of SphKs results in activation of inflammatory processes, including generation of inflammatory cytokines and reactive oxygen species [2–4]. SphKs catalyze phosphorylation of sphingosine to generate sphingosine 1-phosphate (S1P) [5,6]. Interestingly, S1P has been reported to trigger both survival and apoptotic signals in different cell types [5,7–13]. S1P acts as a first messenger through the G protein coupled receptors (GPCRs), termed S1PR1, S1PR2, S1PR3, S1PR4, and S1PR5, which are differentially expressed in different tissues. The cell type-specific expression of S1P receptors (S1PRs), as well as their differential coupling to different G-proteins, provides a clue to the different functions of S1PR [13]. Additionally, S1P can act as a second messenger in various cell types by activating calcium channels and increasing intracellular [Ca2+] signaling and activation of S1PRs.

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Sphingosine kinases (SphKs) and their product sphingosine-1-phosphate (S1P) have been reported to regulate apoptosis and survival of liver cells. Cholestatic liver diseases are characterized by cytotoxic levels of bile salts inducing liver injury. It is unknown whether SphKs and/or S1P play a role in this pathogenic process. Here, we investigated the putative involvement of SphK1 and S1P in bile salt-induced cell death in hepatocytes. Primary rat hepatocytes were exposed to glycochenodeoxycholic acid (GCDCA) to induce apoptosis. GCDCA-exposed hepatocytes were co-treated with S1P, the SphK inhibitor Ski-II and/or specific antagonists of S1P receptors (S1PR1 and S1PR2). Apoptosis and necrosis were quantified. Ski-II significantly reduced GCDCA-induced apoptosis in hepatocytes (−70%, P < 0.05) without inducing necrosis. GCDCA increased the S1P levels in hepatocytes (P < 0.05). GCDCA induced [Ca2+] oscillations in hepatocytes and co-treatment with the [Ca2+] chelator BAPTA repressed GCDCA-induced apoptosis. Ski-II inhibited the GCDCA-induced intracellular [Ca2+] oscillations. Transcripts of all five S1P receptors were detected in hepatocytes, of which S1PR1 and S1PR2 appear most dominant. Inhibition of S1PR1, but not S1PR2, reduced GCDCA-induced apoptosis by 20%. Exogenous S1P also significantly reduced GCDCA-induced apoptosis (−50%, P < 0.05), however, in contrast to the GCDCA-induced (intracellular) SphK1 pathway, this was dependent on S1PR1 and not S1PR2. Our results indicate that SphK1 plays a pivotal role in mediating bile salt-induced apoptosis in hepatocytes by interfering with intracellular [Ca2+] signaling and activation of S1PR1.
injury is crucial for developing new therapeutic strategies to prevent hepatocyte apoptosis. We have previously shown that the sodium-taurocholate cotransporting polypeptide (NTCP) is crucial for conjugated bile salt-induced hepatocyte apoptosis [16], suggesting that bile salt-induced intracellular signaling pathways play important roles in modulating hepatocyte apoptosis. Additionally, it has been shown that bile salt-induced apoptosis is a mitochondria-dependent process which coincides with increased intracellular \([Ca^{2+}]\) levels [17]. Interestingly, it has been suggested that SphK2 inhibition improves mitochondrial function and hepatocyte survival after hepatic ischemia–reperfusion [18]. However, the role played by SphK1 in hepatocyte apoptosis is currently unclear. In the present study, we have investigated the role played by SphK1 in two models of bile salt- and cytokine-induced apoptosis in rat hepatocytes. We demonstrate that SphK1 plays a pivotal role in mediating bile salt-induced apoptosis in hepatocytes, but this kinase has no effect in cytokine-induced apoptosis. Additionally, we investigated the effect of SphK1 inhibition on bile salt-induced intracellular \([Ca^{2+}]\) mobilization.

2. Materials and methods

2.1. Animals

Specified pathogen-free male Wistar rats (220–250 g) were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). Animals were kept under standard laboratory conditions with free access to standard laboratory chow and water. All experiments were performed in accordance with the guidelines of the local Committee for Care and Use of laboratory animals.

2.2. Experimental design

Hepatocytes were isolated and cultured in William’s E medium in a humidified incubator at 37 °C and 5% CO₂ as described before [19]. Experiments were started after the attachment period of 4 h. In order to induce apoptosis, monolayers of cultured primary hepatocytes were exposed to 50 μM GCDCA (Sigma-Aldrich, St Louis, MO, USA) for 4 h or 20 ng/ml recombinant murine TNFα (R&D Systems, Abingdon, U K) for 16 h. Where indicated, hepatocytes were treated with 10 μmol/L S1P (Sigma-Aldrich) prior to the exposure to the apoptotic stimulus. The following specific inhibitors were used: Sk-2 (Tocris Biosciences, Bristol, UK) inhibitor of SphK1 (1–10 μM), VPC23019 (Tocris Biosciences) antagonist of S1PR1 (5 or 10 μM), JTE-013 (Tocris Biosciences) inhibitor of S1PR2 (5 or 10 μM), U0126 (Promega, Madison, USA) inhibitor of ERK1/2 (10 μM), SB203580 (VWR, Amsterdam, The Netherlands) inhibitor of p38 MAPK (10 μM), LY 294002 (VWR) inhibitor of PI3 kinase (50 μM), calphtoin-C and bisindolylmaleimide I (BSM-I) (VWR) inhibitors of protein kinase-C (1 μM), and actinomycin-D (Roche Diagnostics, Almere, The Netherlands) transcriptional inhibitor (200 ng/ml). All inhibitors and receptor antagonists were added to the cultured hepatocytes 30 min prior to the apoptotic stimulus unless stated otherwise. The intracellular \([Ca^{2+}]\) chelator, BAPTA (25 μM, Sigma-Aldrich), was added 30 min prior to GCDCA. Hepatocytes cultured in HBSS (containing Ca²⁺/Mg²⁺) instead of William’s E medium were used as an internal control in these experiments. All experiments were performed in triplicate and each experiment was repeated at least three times using hepatocytes from different rats. Cells were harvested at the indicated time points as described previously [16].

2.3. Apoptosis assay

Caspase-3 activity was measured as described previously [16]. The arbitrary fluorescence unit (AFU) was corrected for total protein content. Protein concentration was determined using a commercially available kit (Bio-Rad, Veenendaal, The Netherlands).

2.4. Quantification of lipids

After incubation the cells were immediately scraped, centrifuged and re-suspended in methanol to stop any enzymatic activity. Equal volumes of the re-suspended pellet were processed for either sphingolipid or phospholipid determination.

For the extraction of sphingolipids a mixture of iso-propanol: water:ethyl acetate in the ratio 30:10:60 was added to the cell pellet. After sonication and centrifugation the supernatant was transferred to another tube, the pellet was re-extracted and the supernatants were combined and dried. The extracted sphingolipids were analyzed by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) on a PE-Scieix API 3000 triple quadrupole mass spectrometer equipped with a turbo ionspray source as described previously [20,21]. HPLC separation was performed as described previously [22], with the following changes: an Alltima C-18 column (2.1 × 150 mm, 5 μm; Grace Davison Discovery Sciences) was used at a flow rate of 200 μl/min. N₂ was used as the nebulizing gas and drying gas for the turbo ionspray source. The ion spray needle was held at 5500 V; the orifice temperature was set to 500 °C. N₂ was used to collisionally induce dissociations in Q2. Multiple reaction monitoring scans were acquired by setting Q1 and Q3 to pass the precursor and product ions of the most abundant sphingolipid molecular species. MRM transitions were optimized for each individual component (C-17SOP: 366.2/250.4; C-17SAP: 368.2/270.4; C-18SOP: 380.2/264.4; C-18SAP: 382.2/274.4; C-17SO: 286.2/238.1; C-17SA: 288.2/240.1; C-18SO: 300.2/252.3; C18SA: 302.2/254.2). Quantitation was achieved by spiking the samples before extraction with sphingosine (d17:1), sphinganine (d17:0), sphingosine-1-phosphate (d17:1), and sphinganine-1-phosphate (d17:0) (Avanti Polar Lipids).

To determine the phospholipid content the lipids were extracted from the cell pellet [23] and the phosphorus content, as a measure for the phospholipid content, was determined using a phosphate assay [24].

2.5. Quantitative PCR

RNA isolation, reverse transcription and quantitative real-time PCR (qPCR) were performed as described previously [25] and mRNA expression levels were calculated relative to the housekeeping gene 18S. Primers and probes are listed in Table 1.

2.6. Determination of intracellular \([Ca^{2+}]\) changes in hepatocytes

Freshly isolated hepatocytes were loaded with the Ca²⁺-probe Fura2 (3 μM, Molecular Probes, USA) and Ca²⁺ imaging experiments were performed 1 h after plating as described previously [26].

Table 1

| Sequence of primers and probes used for quantitative PCR analysis. |
|--------------------------|--------------------------|
| 18S | Sense | 5'-CGGTACCACTCACTCAAGCA-3' |
|  | Antisense | 5'-CAATAGGCGGCTCAAGAA-3' |
|  | Probe | 5'-GACCTAGGATCCCTGAAA-3' |
| S1P+1 | Sense | 5'-CCCGGACGCCGTCGTC-3' |
|  | Antisense | 5'-CGCAAGCACCTCCTGTT-3' |
|  | Probe | 5'-GACCTAGGATCCCTGAAA-3' |
| S1P+2 | Sense | 5'-GGGGGACGCGCGTCCC-3' |
|  | Antisense | 5'-TTTGTCTTTGCTCTGTT-3' |
|  | Probe | 5'-GACCTAGGATCCCTGAAA-3' |
| S1P+3 | Sense | 5'-CGCGGAGCAGAGGTCG-3' |
|  | Antisense | 5'-CAGCGATAGAGAGTGG-3' |
|  | Probe | 5'-GACCTAGGATCCCTGAAA-3' |
| S1P+4 | Sense | 5'-TGGGACGCGCGTCGTT-3' |
|  | Antisense | 5'-CCCGGACGCCGACCCCCT-3' |
|  | Probe | 5'-GACCTAGGATCCCTGAAA-3' |
| S1P+5 | Sense | 5'-CCCGGACGCCGACCCCCT-3' |
|  | Antisense | 5'-CCCGGACGCCGACCCCCT-3' |
|  | Probe | 5'-GACCTAGGATCCCTGAAA-3' |
2.7. Statistical analysis

Results are presented as the mean of at least 3 independent experiments ± SD. Two way ANOVA was used to determine significance of differences between experimental groups. A P-value of less than 0.05 (P < 0.05) was considered statistically significant.

3. Results

3.1. Sphingosine kinase-1 inhibition protects hepatocytes against GCDCA-induced apoptosis, but has no effect on TNFα/ActD-induced apoptosis

GCDCA (at 50 μM) and TNFα/ActD induce caspase-3 activity in primary rat hepatocytes that peaks after 4 h- and 16 h-exposure, respectively [16]. Therefore, the effect of Ski II, a selective inhibitor of SphK1 [27], on GCDCA- and TNFα/ActD-induced caspase-3 activity was investigated at these time points. Ski II significantly inhibited the GCDCA-induced caspase-3 activity up to 70% (P < 0.05), in a concentration-dependent manner (Fig. 1A). Exposure of primary rat hepatocytes to Ski II (1–10 μM) alone did not induce caspase-3 activity, nor did it reduce the caspase-3 activity in TNFα/ActD-treated rat hepatocytes (Fig. 1A and B). Since no toxic effects of Ski II were observed at the highest concentration used, a concentration of 10 μM was used for all subsequent experiments. These data show that inhibition of SphK1 specifically protects primary rat hepatocytes against GCDCA-induced apoptosis indicating that SphK1 activity is pro-apoptotic in these cells.

3.2. GCDCA increases the intracellular level of S1P in rat hepatocytes

To investigate whether GCDCA-induced SphK1 activation increases the intracellular S1P (the product of SphK1) level, we measured the levels of S1P and sphingosine (the precursor of S1P) at several time points after the addition of GCDCA to hepatocytes. Our data indicated that GCDCA (at 50 μM) significantly increased the level of S1P in primary rat hepatocytes which peaked at 10 min-exposure (P < 0.05; Fig. 1C). In addition, we observed a decrease in the sphingosine levels at the same time point after the addition of GCDCA (although the difference in the level of sphingosine was not statistically significant; Fig. 1D). These data strongly indicate that GCDCA-induced activation of SphK1 increases the intracellular S1P level.

3.3. The anti-apoptotic effect of SphK1 inhibition is independent of the activation of ERK1/2, p38 MAP kinase, PI3-kinase, protein kinase C and gene transcription

To investigate whether specific kinase pathways are involved in the anti-apoptotic effects of Ski II, GCDCA-exposed rat hepatocytes were co-treated with inhibitors of MAPK, PI3K or PKC. These inhibitors alone did not induce caspase-3 activity in rat hepatocytes (data not shown) nor did they induce caspase-3 activity in hepatocytes co-treated with Ski II (Fig. 2). Importantly, the protective effect of Ski II against GCDCA-induced caspase-3 activation was not abolished by inhibition of ERK1/2, p38 MAPK, PI3K and PKC (Fig. 2), suggesting that the anti-apoptotic effect of Ski II is essentially independent of the activation of these kinase pathways. These inhibitors at the concentrations...
tested here were effective in our positive control experiments [19]. In addition, we studied the role of gene transcription in the protective effect of Ski II against GCDCA-induced apoptosis. Actinomycin D (200 ng/ml) is an inhibitor of gene transcription and sensitizes hepatocytes to TNFα-induced apoptosis, but has no effect on GCDCA-induced caspase-3 activity in rat hepatocytes [16] and our data). In addition, ActD treatment did not abolish the protective effect of Ski II against GCDCA-induced caspase-3 activation (Fig. 2). This finding suggests that adaptive gene regulation is not required for the anti-apoptotic effect of Ski II against GCDCA-induced apoptosis.

3.4. GCDCA-induced apoptosis is dependent on mobilization of intracellular [Ca2+] and SphK1 inhibition blunts GCDCA-induced [Ca2+] oscillations in rat hepatocytes

It was previously shown that GCDCA-induced apoptosis coincides with intracellular [Ca2+] mobilization from the ER, caspase-12 activation and mitochondrial dysfunction resulting in leakage of cytchrome c [17], suggesting that intracellular [Ca2+] mobilization contributes to GCDCA-induced apoptosis. We investigated the effect of BAPTA (25 μM), a chelator of intracellular [Ca2+], on apoptosis in GCDCA-exposed hepatocytes. BAPTA did not increase caspase-3 activity in hepatocytes when added alone (Fig. 3A). Importantly, the GCDCA-induced caspase-3 activity was significantly reduced in BAPTA loaded hepatocytes (−50%, P < 0.05), confirming that the mobilization of intracellular [Ca2+] pools plays an important role in GCDCA-induced apoptotic signaling. The same results were obtained when hepatocytes cultured in HBSS (containing Ca2+/Mg2+), serving as controls for experiments performed in Williams’ E medium, were analyzed (Fig. 3A). We investigated the pattern of intracellular [Ca2+] mobilization in GCDCA-exposed fura2-loaded hepatocytes and the effect of SphK1 inhibition on this intracellular [Ca2+] mobilization. Our data showed that GCDCA (at 300 μM) in the presence of extracellular [Ca2+], induces [Ca2+] oscillations in rat hepatocytes starting about 2–8 min after the addition of GCDCA (Fig. 3B). Incubation with 300 μM GCDCA did not induce necrosis in hepatocytes up to at least 1 h (data not shown). We did not observe [Ca2+] oscillations with concentration below 300 μM GCDCA, likely due to the experimental limitations. Interestingly, the [Ca2+] oscillations induced by GCDCA were not coordinately induced in hepatocytes nor were they homogenously induced in all cells at the same time (Fig. 3B) unlike [Ca2+] oscillations induced by angiotensin II (Section 3.5). Importantly, these GCDCA-induced [Ca2+] oscillations were blunted or less frequent in the presence of Ski II (10 μM) in 65% of hepatocytes, which showed [Ca2+] oscillations induced by GCDCA (Fig. 3C), suggesting that GCDCA-induced [Ca2+] oscillations in these hepatocytes are mediated via S1P produced by SphK1. When added alone, Ski II did not induce calcium oscillations (data not shown). In addition, Ski II had the same inhibitory effect on tauroliothiocholic-sulfate (TLCS)–induced [Ca2+] oscillations in hepatocytes (Supplementary data), suggesting that the inhibitory effect of Ski II on cholestatic bile salt-induced [Ca2+] oscillations is not limited to GCDCA.

3.5. S1PR2-dependent signaling contributes to the pro-apoptotic effect of GCDCA while exogenous S1P protects hepatocytes against GCDCA-induced apoptosis via interaction with S1PR2

S1P acts as a first messenger via its receptors, S1PR1-S5 [13]. We detected significant mRNA expression levels of all S1P receptors in rat hepatocytes (Fig. 4A). S1PR1 and S1PR2 were expressed more abundantly compared to the other, which is in accordance with a previous report [28]. We investigated the effect of inhibiting S1PR1 and S1PR2 on GCDCA-induced apoptosis using specific antagonists of S1PR1 (VPC23019, at 5 and 10 μM) and S1PR2 (JTE-013, at 5 and 10 μM), respectively. None of these S1P receptor antagonists induced caspase-3 activation when added to hepatocytes alone (Fig. 4B). The apoptotic effect of GCDCA was reduced significantly (−20%, P < 0.05) in the presence of S1PR1 antagonist, indicating that S1PR1-dependent signaling contributes to the pro-apoptotic effect of GCDCA (Fig. 4B). By contrast, the S1PR2 antagonist did not significantly inhibit GCDCA-induced caspase-3 activity, suggesting that GCDCA-induced apoptosis is not mediated via S1PR2-dependent signaling. In fact, hepatocytes may become even more sensitive to the toxic effect of GCDCA in the presence of the S1PR2 antagonist (Fig. 4B).

Endogenous S1P can induce intracellular signaling pathways independent of S1P receptors and/or via interaction with S1P receptors while exogenous S1P interacts with S1P receptors. Therefore, different functions can be expected from endogenous S1P and exogenous S1P [8,13,29]. Consequently, we also investigated the effect of exogenous S1P (10 μM) on GCDCA-induced apoptosis in hepatocytes. The concentration of exogenous S1P (100 nM–10 μM) that causes various responses in cells is comparable with the plasma and tissue concentrations of S1P in vivo [29–32]. When added alone, S1P (10 μM) did not induce caspase-3 activation in rat hepatocytes, but significantly inhibited GCDCA-induced caspase-3 activity (−50%, P < 0.05; Fig. 4C). Interestingly, the protective effect of exogenous S1P against GCDCA-induced apoptosis was reversed...
in hepatocytes co-treated with the S1PR2 antagonist (JTE-013, 10 μM), but not in hepatocytes co-treated with the S1PR3 antagonist (VP23019, 10 μM; Fig. 4C). Exogenously added S1P (10 μM) did not induce \([Ca^{2+}]\) oscillations in rat hepatocytes (Fig. 4D) and prevented GCDCA-induced \([Ca^{2+}]\) oscillations as those could subsequently be induced by the positive control, angiotensin II (All, 10 nM; Fig. 4D). These data indicate that exogenous S1P has anti-apoptotic effects in primary rat hepatocytes and acts via a S1PR2-dependent signaling pathway.

4. Discussion

In the present study, we show that pro-apoptotic GCDCA increases the level of S1P in primary rat hepatocytes and that inhibition of SphK1 reduces GCDCA-induced apoptosis. We further demonstrate that the protective effect of Ski II occurs independently of various specific kinase pathways and of adaptive gene regulation. However, it is dependent on the mobilization of intracellular \([Ca^{2+}]\) and Ski II inhibits GCDCA-induced \([Ca^{2+}]\) oscillations. We suggest that S1PR2-dependent signaling contributes to the pro-apoptotic effect of GCDCA. By contrast, exogenous S1P promotes cell survival against GCDCA-induced apoptosis via S1PR2-dependent signaling in rat hepatocytes (see Graphical abstract).

SphKs and S1P have been implicated to play a role in apoptosis and survival of liver cells [1,8,18]. SphK2 activation leads to the mitochondrial dysfunction and hepatocyte apoptosis after hepatic ischemia–reperfusion injury [18]. S1P may activate both apoptotic and survival pathways at the same time and the balance between the two pathways determine the result. For instance, S1P is pro-apoptotic in human hepatic myofibroblasts acting via a receptor-independent pathway, while concomitant signaling via S1P receptors is anti-apoptotic [8]. The effect of SphK1 activation and S1P on hepatocyte apoptosis has not yet been elucidated. Hepatocyte apoptosis in response to elevated levels of toxic stimuli, such as bile salts, cytokines and reactive oxygen species, contributes to the pathogenesis of chronic liver diseases. Understanding the mechanisms of hepatocyte injury is therefore of great relevance for the development of therapeutic strategies to prevent hepatocyte apoptosis. Therefore, we studied the role played by SphK1 and S1P in models of bile salt (GCDCA)-induced and cytokine (TNFα)-induced hepatocyte apoptosis in vitro.

In our study, we have used Ski II to inhibit SphK1 signaling. Ski II is a selective inhibitor of SphK1 that does not act on the ATP-binding site of the enzyme and is highly specific for SphK1 up to 60 μM [27]. Using this strategy, our data indicate that GCDCA-induced apoptosis is in part dependent on SphK1 signaling in rat hepatocytes. In addition, we confirmed that GCDCA induces a transient peak in intracellular S1P level, which can act as a signaling lipid in apoptotic pathways. In contrast to a previous report [10], we demonstrate that TNFα-induced apoptosis in rat hepatocytes is not SphK1-dependent. Osawa et al. [10] analyzed the role of SphKs in human hepatoma cell lines and used non-selective (broad range) inhibitors (DMS and DHS) of SphKs. In our study, we analyzed primary rat hepatocytes and focused on the role of SphK1 by applying a highly selective inhibitor (Ski II). These experimental differences may explain the fact that we did not observe an effect of inhibition of SphK1 on TNFα-induced apoptosis.

Endogenous S1P was shown to function as a powerful mediator of \([Ca^{2+}]\) release through a non-InsP3 receptor-mediated mechanism in the ER membrane [33,34]. Furthermore, it has been demonstrated that UTP stimulates \([Ca^{2+}]\) mobilization in a SphK-dependent manner, which is not mimicked by exogenous S1P [35]. Altogether, these reports suggest that endogenous S1P generated by SphK activation induces intracellular \([Ca^{2+}]\) mobilization. GCDCA-induced apoptosis in rat hepatocytes has been shown to coincide with elevated intracellular \([Ca^{2+}]\) levels [17]. Accordingly, we show that GCDCA-induced apoptosis is dependent on intracellular calcium mobilization (since the GCDCA-induced apoptosis was reduced in hepatocytes loaded with the \([Ca^{2+}]\) chelator BAPTA). In our experiments, the GCDCA-induced increase in \([Ca^{2+}]\) is coinciding with \([Ca^{2+}]\) oscillations. Remarkably, not all hepatocytes showed \([Ca^{2+}]\) oscillations in response to bile salts and the \([Ca^{2+}]\) oscillations were not coordinated in hepatocytes. It was previously shown that the lack of coordination of \([Ca^{2+}]\) oscillations in hepatocytes can result either from the absence of InsP3 production by bile salts or from the inhibitory effect of bile salts on the permeability of gap junction channels [36,37]. Although we did not specifically test these mechanisms, they may provide a clue to explain the lack of homogeneity and coordination of \([Ca^{2+}]\) oscillations induced by GCDCA in our experiments. Interestingly, we show...
that GCDCA induces [Ca\(^{2+}\)] oscillations in a SphK1-dependent manner, which is not mimicked by exogenous S1P at the concentration that is anti-apoptotic in hepatocytes. It has previously been shown that exogenous S1P mobilizes intracellular [Ca\(^{2+}\)] in rat hepatocytes [14,38], but this did not present as [Ca\(^{2+}\)] oscillations. Therefore, we suggest that endogenous S1P generated by GCDCA-mediated pathways can act as a second messenger to induce [Ca\(^{2+}\)] oscillations in rat hepatocytes. Inhibition of SphK1 activation inhibits GCDCA-induced apoptosis as well as GCDCA-induced [Ca\(^{2+}\)] oscillations.

[Ca\(^{2+}\)] oscillations are implicated in the regulation of different cellular processes in hepatocytes including glucose metabolism and cell proliferation [39,40]. [Ca\(^{2+}\)] oscillations are reported to regulate transcriptional activity in cells [41,42] although our data indicate that neither GCDCA-induced apoptosis [16] nor the protective action of Ski II (present study) required adaptive gene regulation. Some cholestatic bile salts have been shown to increase intracellular [Ca\(^{2+}\)] and induce [Ca\(^{2+}\)] oscillations in rat hepatocytes [37,43]. In the present study, we suggest that bile salt-induced [Ca\(^{2+}\)] oscillations in rat hepatocytes promote apoptosis; however, the exact pathway of the [Ca\(^{2+}\)] oscillation-induced events in regulating apoptosis remains unclear. It is reported that TUDCA (a choleretic anti-apoptotic bile salt [16]) can also induce [Ca\(^{2+}\)] oscillations in the H4-IIE cell line [44]; however, this finding could not be confirmed in rat hepatocytes [37]. In addition, it is reported that TUDCA plays an important role in maintaining intracellular calcium homeostasis by reducing thapsigargin-induced release of Ca\(^{2+}\) from ER stores, resulting in the maintenance of mitochondrial membrane potential and cell survival [45]. It is therefore highly likely that cholestatic bile salt-induced [Ca\(^{2+}\)] oscillations in rat hepatocytes constitute a pro-apoptotic signal in these cells.

Activation of MAP-kinase signaling, PI3-kinase signaling and PKC signaling pathways in hepatocytes plays an important role in determining the balance between death and survival in response to stress [16,46–49]. In addition, S1P and S1P receptors are reported to interact with these kinases [8,10,28,50]. We hypothesized that the inhibition of these kinases would re-sensitize Ski II treated hepatocytes to GCDCA-induced apoptosis, but our data demonstrate that inhibition of ERK, PI3-kinase and/or PKC pathways is not involved in the protective effect of Ski II.

In the present study, we observe opposing functions for exogenous S1P versus endogenous S1P. Many reports indicate that S1P acts as a first messenger on cell surface S1P receptors in a paracrine and/or autocrine manner (reviewed in [51]). We suggest that in GCDCA-induced apoptosis, the main autocrine and/or paracrine role of S1P as a first messenger is to activate S1PR\(_{2}\)-dependent survival signaling. It has been shown that in rat hepatocytes exogenous S1P can activate ERK1/2 and Akt via interaction with S1PR\(_{2}\) [28]. Additionally, activation of ERK1/2 and Akt signaling pathways contributes to hepatocyte survival [16]. Activation of these pathways could contribute to the protective effect
of exogenous S1P against GCDCA-induced apoptosis. Interestingly, the GCDCA-induced activation of S1PR1-dependent signaling (likely by se-
creted endogenously generated S1P) is pro-apoptotic for rat hepatocyte-
cells; however, this pathway does not seem to play a major role in
GCDCA-induced apoptosis as the antagonist of S1PR1-reduced GCDCA-
induced apoptosis only by 20%. Therefore, our evidence that GCDCA-
induced apoptosis is a SphK1-dependent event indicates that the generat-
ed endogenous S1P acts as a second messenger to promote cell apoptosis
in rat hepatocytes. Therefore, inhibiting SphK1 in hepatocytes could be an
effective novel anti-apoptotic strategy in the treatment of cholestatic liver
disease.

In summary, the results of this study demonstrate that GCDCA in-
duces apoptosis in rat hepatocytes in a SphK1-dependent manner and
suggests that endogenously generated S1P acts as a second messenger
to induce [Ca++) oscillations and apoptosis in rat hepatocytes.

Supplementary data to this article can be found online at http://
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