The accumulation of hydrophobic bile acids results in cholestatic liver injury by increasing oxidative stress, mitochondrial dysfunction, and activation of cell signaling pathways. Licorice root and its constituents have been utilized as antihepatotoxic agents. The purpose of this study was to evaluate the potential modulation by a primary component of licorice root, glycyrrhizin (GL), and its metabolite, 18β-glycyrrhetinic acid (GA), in a hepatocyte model of cholestatic liver injury. Preincubation of fresh rat hepatocyte suspensions with GL or GA reduced glycochenodeoxycholic acid (GCDC)-dependent reactive oxygen species generation, with GA more potent than GL. Interestingly, GL and GA had opposing effects toward GCDC-induced cytotoxicity; GA prevented both necrosis and apoptosis, whereas GL enhanced apoptosis. GCDC promoted activation of caspase 10, caspase 3, and PARP; all were inhibited by GA but not GL. Induction of apoptosis by GCDC was also associated with activation of JNK, which was prevented by GA. Activation of caspase 9 and dissipation of mitochondrial membrane potential were prevented by GA but not GL. In liver mitochondrial studies, GL and GA were both potent inhibitors of the mitochondrial permeability transition, reactive oxygen species generation, and cytochrome c release at submicromolar concentrations. Results from this study suggest that GL exhibits pro-apoptotic properties, whereas GA is a potent inhibitor of bile acid-induced apoptosis and necrosis in a manner consistent with its antioxidantative effect.

Cholestatic liver disorders are characterized by impaired bile flow resulting in the retention of bile constituents and hepatocellular damage. Because there are few effective therapies available, the development of cirrhosis and the need for liver transplantation is a frequent outcome in cholestatic children and adults (1). The accumulation of hydrophobic bile acids within the liver is an important factor in the pathogenesis of cholestatic liver disorders (2). Higher concentrations (≥250 μM) of hydrophobic bile acids, such as glycochendoxycholic acid (GCDC), promote hepatocyte death by necrosis, and lower concentrations cause apoptosis (3–5). Mechanistic studies have revealed that several factors, including physicochemical properties (6) and death receptor activation (7), account for the pro-apoptotic effects of bile acids. Activation of cell stress signaling pathways, including caspases and mitogen-activated protein kinases (MAPK), are strongly implicated in both the initiation and execution of events culminating in apoptotic cell death. However, the toxicity of bile acids is not uniform; for example, taurochenodeoxycholic acid not only stimulates apoptotic pathways but also activates cell survival proteins, such as phosphatidylinositol 3-phosphate kinase or MAPK extracellular signal-regulated kinase (ERK) (8). Thus, there is a complex interplay between cell death and survival signals in bile acid-induced cytotoxicity that determines ultimate cell fate.

Extensive evidence also supports the involvement of mitochondrial pathways in bile acid-induced hepatocyte toxicity, including induction of the mitochondrial permeability transition (MPT) (9). Upon MPT induction, there is a loss of mitochondrial swelling, release of soluble proteins such as cytochrome c and apoptosis-inducing factor from the intermembrane space, and activation of caspase 9. Furthermore, oxidative stress generated by mitochondria plays a role in bile acid-induced cellular toxicity, as demonstrated in liver mitochondria (10, 11) and rat hepatocytes (12, 13) as well as in vitro studies with whole animals exposed to bile acids (14). Moreover, a variety of diverse antioxidants reduce both oxidative stress and bile acid-induced hepatocyte toxicity (11–15).

Licorice root is an herbal preparation that has been used for decades to reduce liver injury in a number of clinical disorders. In 1977, Suzuki et al. (16) reported that the principal triterpene component of licorice root, glycyrrhizin (GL), benefits patients with chronic hepatitis C infection. Derivatives of licorice root have been used in Asia to treat children with biliary atresia (17), a cholestatic liver disease, although no clinical trials have been reported. Increasing evidence supports the hypothesis that GL, or its hydrolyzed metabolite 18β-glycyrrhetinic acid (GA), protects against several models of oxidant-mediated tox-
ity, including exposure to CCl₄ (18), t-butyl hydroperoxide (19), and ischemia-reperfusion injury (20), with GA generally exhibiting greater hepatic protection than GL. Although several hypotheses have been offered to account for the hepatoprotective effects of GL and GA, the effects of these compounds on molecular and biochemical pathways of cell injury have not been well characterized. Therefore, the purpose of this study was to examine the effects of GA and GL on cell pathways of bile acid-induced cytotoxicity in both freshly isolated rat hepatocyte suspensions and purified liver mitochondrial fractions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sodium glycochenodeoxycholate, lactate dehydrogenase kits, ammonium glycyrhiznin, and 18β-glycyrrhetinic acid were obtained from Sigma. Cyclosporin A (CSa) was purchased from Alexis Biochemicals (San Diego, CA). 2',7'-Dichlorofluorescin diacetate (DCF-DA) and bovine serum albumin (fraction V) were from Eastman Kodak Co. and Calbiochem, respectively. The fluorescent probe JC-1 was obtained from Molecular Probes (Eugene, OR). Primary antibodies against caspase 3, cleaved caspase 9, caspase 10, native and cleaved PARP, phosphorylated and unphosphorylated pS6 MAPK, and phospho-histone H3 were from Cell Signaling Technology (Beverly, MA). All other chemicals were reagent grade or better.

**Isolation of Rat Hepatocytes**—Hepatocytes were isolated by a recirculating collagenase technique from male Sprague-Dawley rats (175–225 g) (Sasco, Inc., Omaha, NE) maintained on a 12-h light-dark cycle and fed standard laboratory rat chow, as described previously (15). Initial hepatocyte viability measured by trypan blue exclusion was always >94%. Fresh hepatocytes were resuspended in a Krebs-Ringer HEPES (KRH) buffer containing 0.2% bovine serum albumin (KRH/BSA) to a concentration of ~1 × 10⁶/ml. This study was approved by the Institutional Animal Care and Use Committee, University of Colorado Health Sciences Center.

**Generation of Reactive Oxygen Species in Rat Hepatocytes**—Generation of reactive oxygen species (ROS) was measured spectrofluorometrically using the ROS-detecting probe, 2',7'-dichlorofluorescin (DCFein), as described previously in detail (21). Briefly, hepatocytes were loaded with DCF-DA for 30 min at 37 °C prior to a 30 min preincubation with graded concentrations of GL or GA. DCF-DA is trapped within cells and deesterified, yielding nonfluorescent dichlorofluorescein (DCFein), as described previously in detail (21). Briefly, hepatocytes were exposed to GCDC (0 or 100 μM) for 4 h in a 37 °C shaking water bath at room temperature, and aliquots were removed for analysis of ROS by measuring DCFein fluorescence at 490 nm excitation and 520 nm emission. The results were expressed as fluorescence units/10⁶ cells.

**Determination of Hepatocyte Apoptosis and Necrosis**—Hepatocyte apoptosis was quantified by determining the percentage of hepatocytes with nuclear morphologic changes of apoptosis (fragmentation and margination of chromatin) detected by fluorescence microscopy of DAPI-stained fixed hepatocytes (13). Necrosis was assessed by the release of lactate dehydrogenase activity from cells and expressed as the percentage of total cellular activity released into the medium (13).

**Mitochondrial Depolarization—Flow cytometric analysis** was performed to determine the effect of GL and GA upon GCDC-dependent mitochondrial depolarization in hepatocytes, as described previously (12). Briefly, freshly isolated hepatocytes were pretreated with 25 μM GL or 10 μM GA for 30 min and then incubated with 100 μM GCDC for 4 h. Aliquots of cells were removed hourly, loaded with 7.6 μM JC-1 or 3 μM propidium iodide for 15 min at 22 °C in the dark, and washed with KRH buffer at 4 °C prior to flow cytometry on a BD Biosciences FACSCalibur using FlowJo software. In actively respiring mitochondria, JC-1 aggregates in the mitochondrial matrix, and the fluorescence of JC-1 aggregates is proportional to the mitochondrial Δψ and indicative of a closed MPT pore. Neither GL nor GA alone affected JC-1 fluorescence. For each time point and treatment 10,000 cells were analyzed. The fluorescence of JC-1 aggregates was determined only in live cells identified through gating for propidium iodide fluorescence.

**SDS-PAGE and Immunoblotting**—Total cell lysates were obtained from hepatocytes for immunoblot analysis as follows. Hepatocytes (3 × 10⁶) were pelleted by centrifugation at 2500 rpm for 5 min, resuspended in KRH buffer (no bovine serum albumin), and recentrifuged to obtain a washed cell pellet. Cells were lysed in 1 ml of a buffer containing 62.5 mM Tris-HCl, pH 6.8, 6 μl urea, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.00125% bromphenol blue followed by a 15-s sonication on ice, as described by Shah et al. (22). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes, and nonspecific proteins were blocked for 1-h incubation in fresh 5% nonfat dry milk. The blots were probed against the appropriate primary antibody by an overnight incubation at 4 °C followed by a 1-h incubation with rabbit anti-horseradish peroxidase with biotinylated horseradish peroxidase utilized as a size marker. On selected blots, β-actin (Oncogene Research, Boston, MA) was probed to demonstrate equal protein loading.

**Mitochondrial ROS Generation**—Fresh rat liver mitochondria were isolated by differential centrifugation through a Percoll gradient as described previously (11). ROS were quantitated spectrofluorometrically using DCFein (11). Briefly, purified mitochondria were resuspended in a buffer containing 5 mM HEPES, 50 mM KCl, 2 mM K₃PO₄, 125 mM sucrose, pH 7.4, treated with 1% Chelex 100 (wash buffer), and loaded with 8 μM DCFein-DA at 28 °C for 30 min. The loaded mitochondria were washed twice with wash buffer, centrifuged at 10,000 × g for 10 min, and finally resuspended in 20 ml of a buffer containing 10 mM MOPS, 100 mM NaCl, 125 mM sucrose, pH 7.4, treated with 1% Chelex 100. Mitochondria were then preincubated with graded concentrations of GL or GA, or Me₃SO solvent vehicle. Solvent alone had no effect on any measurements and was at a concentration of <0.1%. Mitochondria were then incubated with GCDC, and aliquots were removed at specified time points for DCFein fluorescence measurements at 490 nm excitation and 520 nm emission. The results were expressed as DCFein fluorescence/mg of mitochondrial protein.

**MPT and Cytochrome c Content in Liver Mitochondria**—MPT induction was quantitated in purified mitochondria spectrophotometrically at 540 nm as described previously (11). Briefly, hepatic mitochondria were preincubated at 25 °C for 5 min alone or in the presence of GL, GA, or Me₃SO solvent vehicle. Mitochondria were then incubated with GCDC (0 or 100 μM) for 5 min at 25 °C and then centrifuged to pellet the suspension and the precipitate was resuspended in a buffer containing 5 mM HEPES, 50 mM KCl, 2 mM KH₂PO₄, 125 mM sucrose, and 5 μM rotenone. Following this 5-min incubation, the MPT was induced by addition of 100 μM DCFein. Mitochondrial swelling was quantitated by the reduction in absorbance at 540 nm during the 5-min incubation with GCDC. After the MPT experiment, mitochondrial samples were centrifuged at 13,000 × g for 30 min at 4 °C to isolate the mitochondrial pellet for immunoblot analysis of cytochrome c content using anti-cytochrome c mouse monoclonal antibody and a mouse horseradish peroxidase secondary antibody (BD Biosciences).

**Statistical Analysis**—Statistical analysis between groups was conducted by analysis of variance using the Scheffe test or by t test for comparing means from two groups. A p value of <0.05 was considered significant. Values were expressed as means ± S.E.

**RESULTS**

**GL and GA Reduce GCDC-stimulated ROS Generation**—To determine the effect of licorice compounds on bile acid-induced ROS generation, freshly isolated rat hepatocyte suspensions were incubated for 4 h with 100 μM GCDC, and DCFein fluorescence was measured. GCDC increased DCFein fluorescence linearly in a time-dependent manner when compared with control hepatocytes (35.8 ± 1.5 versus 9.7 ± 0.9 fluorescence units/10⁶ cells at 4 h) (Fig. 1a). Pretreatment with GL decreased ROS generation modestly at all concentrations (0.5–25 μM) (Fig. 1a). GA treatment reduced ROS generation to a greater extent, with all concentrations reducing ROS generation by >60% at 4 h (Fig. 1b). Concentration-effect relationships of the two compounds after 4 h of incubation with GCDC (Fig. 1c) showed that both GA and GL function at low concentrations to reduce bile acid-induced oxidative stress, that maximal effect was reached at low concentrations, and that GA was superior to GL in this effect.

**GL and GA Differentially Regulate GCDC-induced Cytotoxicity**—We next determined the effects of the licorice compounds on cell death pathways. Hepatocytes exposed to 100 μM GCDC underwent a time-dependent increase in necrosis, as evident by the percent of lactate dehydrogenase leakage (38.2 ± 2.2% versus 15.9 ± 1.3% for control cells at 4 h) (Fig. 2). Pretreatment with GL afforded no protection against cell necrosis (Fig. 2a), whereas all concentrations of GA reduced cell necrosis by >60% at 4 h (Fig. 2b). A concentration-effect comparison of GL and GA (Fig. 2c) demonstrates the superior protection afforded at all concentrations by GA.
The differential effects of GL and GA on hepatocytes were more dramatic when DAPI-stained hepatocyte nuclei were examined for apoptosis (Fig. 3). Hepatocytes treated with 100 μM GCDC underwent significant apoptosis by 4 h (31.5 ± 1.6% versus 1.6 ± 0.7% for control cells) (Fig. 4). Preincubating hepatocytes with 25 μM GL enhanced apoptosis by -170–210% after 2 h incubation (Fig. 4a). In contrast, 25 μM GA significantly inhibited GCDC-induced apoptosis by >70% throughout the course of the experiment (Fig. 4b). A concentration-effect analysis of the effects of GL and GA on GCDC-induced apoptosis at 3 h (Fig. 4c) demonstrated that GL enhanced apoptosis at concentrations of 0.5 μM and above, whereas GA protected against apoptosis at ≥10 μM.

Previous experiments have demonstrated that GCDC causes a reduction of mitochondrial membrane potential (indicating the MPT) that precedes induction of apoptosis in hepatocytes (11, 15). Therefore, JC-1 fluorescence was used to determine the effects of GL and GA on bile acid induction of MPT in live hepatocytes. Hepatocytes exposed to GCDC for 3 h (Fig. 5a, red data line) showed a decrease in mitochondrial membrane potential (shift to left of JC-1 aggregate fluorescence curve) when compared with control cells (blue data line). The time course (Fig. 5, b and c) demonstrated a decrease in JC-1 fluorescence by 1 h. Preincubation with 10 μM GL (Fig. 5, a and b, black data line) failed to prevent the dissipation of membrane potential at any time point. However, pretreating cells with 25 μM GA (Fig. 5, a and c, green data line) significantly prevented the reduction in membrane potential for at least 3 h, commensurate with the protection offered against necrosis and apoptosis. GL and GA alone (Fig. 5, a and c, gold data line) had only a slight effect on membrane potential.

Effects of GL and GA on Changes in Caspase and MAPK Activation—Immunoblots of whole cell lysates indicated that pro-caspase 10 was reduced after 3 h incubation with GCDC (Fig. 6a). This apparent activation of caspase 10 was prevented by 25 μM GA but not by 1 or 10 μM GL. Activation of caspase 10 previously has been shown to process executioner caspases 3 and 7 (23). As shown in Fig. 6b, caspase 3 existed predominantly in the native, uncleaved form (pro-caspase 3) in the absence of GCDC (lane 1). However, after 3 h GCDC reduced levels of procaspase 3. Preincubation of hepatocytes with GA prevented loss of procaspase 3, whereas GL yielded no protection at 1 μM, and at 10 μM GL increased the appearance of the
cleaved caspase 3. Similar results were observed by examining the cleavage of PARP, where GCDC promoted PARP cleavage, which was prevented by GA and potentiated by GL (Fig. 6c).

Caspase 9 is activated following cytochrome c release from mitochondria. Immunoblot analysis of cleaved caspase 9 revealed an increase of caspase 9 after incubation with GCDC (Fig. 6d), which was only mildly reduced by GA and was potentiated by 10 μM GL (Fig. 6d). In selected experiments, β-actin was probed to demonstrate equal loading of all lanes (Fig. 6e). Taken together, these data support the hypothesis that the anti-apoptotic effects of GA is primarily through a caspase 9-independent mechanism.

Activation of MAPK has been recently reported to be involved in cell signaling cascades involved in bile acid cytotoxicity (24, 25). We examined activation of the two members of the MAPK family implicated in bile acid toxicity, p38 MAPK and JNK; the latter is also a member of the SAPK family (Fig. 7). Band densities of phosphorylated MAPK were expressed relative to total MAPK levels and then adjusted to control samples, which were normalized to a value of 1.0. The effects of GL and GA on phosphorylation of p38 MAPK in GCDC-treated cells are shown in Fig. 7a. The ratio of phosphorylated to total p38 MAPK remains relatively unchanged by treatment with GCDC (Fig. 7a, lane 2), GA (lane 3), or GL (lanes 4 and 5), suggesting that apoptosis was not p38 MAPK-dependent. In Fig. 7b, the ratio of phosphorylated to total JNK density was increased by exposure to GCDC alone (1.9-fold, lane 2). Pretreatment with GA (Fig. 7b, lane 3) prevented JNK phosphorylation, whereas GL (lanes 4 and 5) had no significant effect. These data support a role for JNK activation during GCDC-induced apoptosis, suggesting that the anti-apoptotic effect of GA may be mediated by inhibition of the SAPK/JNK pathway. 

Effects of GL and GA on Liver Mitochondria—ROS generation and induction of the MPT in liver mitochondria by bile acids have been implicated in hepatocellular death by necrotic and apoptotic mechanisms. Therefore, we next examined the direct effects of GL and GA on mitochondrial function. Incubation of purified liver mitochondria with 100 μM GCDC resulted in a linear increase in ROS generation (Fig. 8). Preincubation with GL inhibited ROS generation in a dose-dependent manner at low concentrations (0.1–1 μM), reaching a plateau at 1.0 μM GL, which reduced ROS generation by >60% at 10 min (Fig. 8a). GA reduced ROS generation >60% at all concentrations (0.1–10 μM) (Fig. 8b). These treatment effects were comparable with the cytotoxicity responses observed in Figs. 1 and 2, with GA exhibiting a greater antioxidative and protective effect than GL.

Next, the effects of GL and GA on GCDC-induced MPT in purified mitochondria were compared. As shown previously (11, 13), GCDC induced the MPT when incubated for 5 min with succinate-energized mitochondria (Fig. 9). In the current study, a dose-dependent decrease in the magnitude of the MPT was observed when mitochondria were preincubated with 0.1–1.0 μM GL (Fig. 9a), with a reversal of this effect as the concentration of GL was increased to 5 or 10 μM. GA inhibition of the MPT was almost identical to that observed with GL, including the reversal of protection observed above 1 μM (Fig. 9b). Neither GL nor GA (up to 25 μM) incubated alone with mitochondria induced the MPT (data not shown). Because of the magnitude of MPT inhibition by low (including submicromolar) concentrations of GL and GA, we compared these compounds against CsA, a direct blocker of the MPT (Fig. 9c). On an equimolar basis, both GL and GA offered protection against the GCDC-induced MPT almost equal to that provided by CsA.

Subsequent to induction of the MPT by GCDC, mitochondria released substantial amounts of cytochrome c (Fig. 9d). Preincubation with graded concentrations of GL and GA showed similar protection against loss of cytochrome c, paralleling the reduction of MPT magnitude (Fig. 9, a and b).

**DISCUSSION**

Licorice root has long been utilized as an herbal remedy against a variety of ailments in Asian cultures (26). Although the precise biological mechanisms responsible for these clin-

---

**Fig. 3. Fluorescence microscopy of hepatocyte nuclei labeled with DAPI.**

Hepatocytes were incubated with no additions (A), with 100 μM GCDC alone (B), or in the presence of 25 μM GA (C) or 10 μM GL (D) for 3 h. After the incubation, cells were fixed, cytofuged onto a slide, and stained with DAPI for fluorescence microscopy. Only those cells that had fragmented nuclei or marginalized chromatin (depicted by arrows) were considered apoptotic. Scale bar (in the lower right corner) = ~10 μm.
ical benefits are unknown, evidence from experimental studies document that GL and its major metabolite by intestinal metabolism, GA, are protective in whole animals and cultured hepatocytes (18, 27). Several hypotheses have been put forward to account for the hepatic protection offered by these compounds including stimulation of cytochrome P-450 and glutathione S-transferase activities (27) or their activity as an antioxidant through glutathione preservation (18). Although these compounds are commonly used in herbal preparations purported to be of benefit in cholestatic liver disease (17), their biological effects in cholestatic liver injury have not been characterized. Therefore, the current study was performed to determine the effects of GL and GA on pathways involved in bile acid-induced cytotoxicity.

The results of this study reveal GL and GA to be potent modulators of bile acid-induced cytotoxicity with GL enhancing GCDC-induced apoptosis and GA significantly inhibiting both necrotic and apoptotic cell death. Micromolar concentrations of GL enhanced GCDC-induced activation of several pro-apoptotic pathways, including caspase 10 and JNK signaling. In contrast, GA inhibited these signaling pathways and afforded significant protection against cytotoxicity. This protective role of GA was consistent with its antioxidant effect, although other potential effects of GA (28, 29) were not explored. Interestingly, the potentiation of GCDC-induced apoptosis by GL was independent of its modest reduction of ROS generation. Importantly, the protective effect of GA in hepatocytes was

**FIG. 4.** Effects of GL and GA on GCDC-induced hepatocyte apoptosis. Hepatocytes were exposed to 100 μM GCDC for 4 h in the absence or presence of 25 μM GL (a) or GA (b). Aliquots were removed hourly for quantitation of apoptosis as described under “Experimental Procedures.” The percent of apoptotic cells after 3 h of exposure to GCDC was plotted versus concentrations of GL and GA (c). Results are from at least six separate experiments and expressed as mean ± S.E.

**FIG. 5.** Effects of GA and GL on GCDC-induced mitochondrial depolarization in rat hepatocytes. Isolated rat hepatocytes were treated with 100 μM GCDC alone (red line), or combined with 10 μM GL (black line) or 25 μM GA (green line). Aliquots were removed hourly and loaded with JC-1 and propidium iodide as described under “Experimental Procedures.” In a, a representative plot of JC-1 aggregate fluorescence is shown after 3 h, indicating mitochondrial depolarization in GCDC-treated cells compared with control hepatocytes (blue line), which was prevented by GA but not GL. The time course of JC-1 aggregate formation is depicted in b and c, demonstrating protection by GA but not GL. Neither GL nor GA alone (gold lines) affected mitochondrial depolarization. Results are from three separate experiments and expressed as mean ± S.E.

**FIG. 6.** Immunoblot analysis of caspases and PARP from rat hepatocytes treated with GCDC. Whole cell lysates obtained from hepatocytes after 3 h of incubation were separated by SDS-PAGE and immunoblotted as described under “Experimental Procedures.” For each blot, the lane assignments were as follows: lane 1, control; lane 2, 100 μM GCDC; lane 3, 100 μM GCDC + 25 μM GA; lane 4, 100 μM GCDC + 1 μM GL; and lane 5, 100 μM GCDC + 10 μM GL. Blots were probed with antibodies raised against caspase 10 (a), caspase 3 (b), native and cleaved PARP (c), cleaved caspase 9 (d), and β-actin (e). These are representative results from 2–4 separate hepatocyte preparations.
accompanied by inhibition of the MPT in live cells, ROS generation, cytochrome c release from mitochondria, and caspase 9 activation.

Previous studies have associated increased oxidative stress with the severity of bile acid-induced cytotoxicity in hepatocyte suspensions (15, 25) and in whole animals receiving parenterally administered bile acids (14). Correspondingly, oxidative stress and cytotoxicity were attenuated by antioxidants including /H9251-tocopherol, /H9252-carotene, or the coenzyme Q analog, idebenone (13, 15, 30). The relative degree of antioxidant activity of GL and GA (Fig. 1) correlated well with the capacity of each compound to suppress GCDC-induced cellular necrosis (Fig. 2). However, the potentiation of apoptosis by GL (Fig. 3) implicates activation of other apoptotic signaling pathways or inhibition of cell survival cascades. In contrast, GA continued to demonstrate marked anti-apoptotic effects even at concentrations as low as 0.5 M. In prior studies of hepatocyte apoptosis,
Yoshikawa et al. (31) reported that GL inhibited tumor necrosis factor-α but not Fas-dependent apoptosis in HepG2 cells at concentrations that significantly enhanced apoptosis in our study. However, ROS generation, mitochondrial function, and caspase activation were not addressed in that study (31). In the current study, the reduction of oxidant stress and the cytoprotective effect by GA were consistent with another study that reported an antioxidative role of GA in the amelioration of carbon tetrachloride-induced liver injury (18).

One well characterized caspase-dependent pathway responsible for bile acid-induced hepatocyte apoptosis requires death receptor activation of caspase 8 as a response to formation and aggregation of a death-induced signaling complex (DISC) (32). Upon activation of caspase 8, signaling through the mitochondrial pathways results in downstream caspase 3 cleavage, activating the nuclear enzyme PARP, which is responsible for nuclear degradation. A closely related homolog to caspase 8, caspase 10, is another target of Fas ligand and TRAIL-induced activation (33, 34) and has been reported to promote apoptosis in certain cell types (35, 36). Caspase 10 exists as four known isoforms and is expressed in many tissues including liver and skeletal muscle (23). Despite its being implicated as an inducer of apoptosis, the role of caspase 10 in bile acid-induced cytotoxicity has not been examined. In our studies, procaspase 10 levels in rat hepatocyte suspensions underwent increased proteolysis in the presence of GCDC, which was prevented by GA. In a recent study, Higuchi et al. (37) found no activation of caspase 10 by GCDC alone but found that co-incubation of GCDC with TRAIL, a death receptor cell signaling agent, promoted caspase 10 cleavage. There are differences between our study and that of Higuchi et al. (37) that could account for the differing effects of bile acids on caspase 10; those authors used HuH-7 cells transfected with a sodium-dependent transporting polypeptide in culture for 12 h, and our study utilized freshly isolated rat hepatocytes. The role of caspase 10 in bile acid-induced cytotoxicity requires further study.

Previous studies indicate that activation of p38 and JNK, via the SAPK pathway, is associated with bile acid-induced apoptosis (24, 25, 38), whereas activation of ERK may suppress apoptosis (39, 40, 44). Both p38 and JNK, activated by stress and inflammatory stimuli, regulate AP-1 transcription factor and its component, c-Jun, by phosphorylation reactions (41). In our study, JNK activation, commensurate with oxidative stress, was a key signal in GCDC cytotoxicity. It has been proposed that oxidative stress itself is responsible for JNK activation (33, 34) and has been reported to promote apoptosis (33, 34) and has been reported to promote apoptosis. Upon activation of caspase 8, signaling through the mitochondrial pathways results in downstream caspase 3 cleavage, activating the nuclear enzyme PARP, which is responsible for nuclear degradation. A closely related homolog to caspase 8, caspase 10, is another target of Fas ligand and TRAIL-induced activation (33, 34) and has been reported to promote apoptosis in certain cell types (35, 36).

In conclusion, low concentrations of GL resulted in enhanced bile acid-induced apoptosis of isolated hepatocytes through activation of caspases and the SAPK pathway member, JNK. Conversely, GA inhibited these pathways, prevented bile acid-induced mitochondrial depolarization, reduced oxidative stress, and protected against apoptosis and necrosis. Further evaluation of these compounds are warranted in regard to a potential role in treating cholestatic liver disease and other liver diseases associated with increased oxidative stress.

REFERENCES

1. Li, M. K., and Crawford, J. M. (2004) Semin. Liver Dis. 24, 21–42
2. Greim, H., Czygan, P., Schaffner, F., and Popper, H. (1972) Biochem. Med. 8, 280–286
3. Gumpricht, E., Devereaux, M. W., Dahl, R. H., and Sokol, R. J. (2000) Toxicol. Appl. Pharmacol. 164, 102–111
4. Patel, T., Bronk, S. F., and Gores, G. J. (1994) J. Clin. Investig. 94, 2183–2192
5. Chico, P., Romagnoli, E., Aicardi, G., Suozzi, A., Porti, G. C., and Roda, A. (1997) Histochem. J. 29, 875–883
6. Powell, A. A., LaRue, J. M., Batta, A. K., and Martinez, J. D. (2001) Biochem. J. 356, 481–486
7. Faubion, W. A., Guicciardi, M. E., Miyoshi, H., Bronk, S. F., Roberts, P. J., Svingen, P. A., Kaufmann, S. H., and Gores, G. J. (1999) J. Investig. 103, 137–145
8. Rust, C., Kurnitz, L. M., Paya, C. V., Moscat, J., Simari, R. D., and Gores, G. J. (2000) J. Biol. Chem. 275, 20210–20216
9. Bernardi, P., Petrelli, V., Di Lisa, F., and Forte, M. (2001) Trends Biochem. Sci. 26, 112–117
10. Rodrigues, C. M., Fan, G., Wang, P. Y., Kren, B. T., and Steer, C. J. (1998) Mol. Med. 4, 165–178
11. Sokol, R. J., Straka, M. S., Dahl, R., Devereaux, M. W., Yerushalmi, B., Gumpricht, E., Elkins, N., and Everson, G. (2001) Pediatr. Res. 49, 519–526
12. Gumpricht, E., Dahl, R. H., Yerushalmi, B., Devereaux, M. W., and Sokol, R. J. (2002) J. Biol. Chem. 277, 25823–25830
13. Gumpricht, E., Dahl, R., Devereaux, M. W., and Sokol, R. J. (2004) Pediatr. Res. 55, 814–821
14. Sokol, R. J., McKim, J. M., Goff, M. C., Ruyle, S. Z., Devereaux, M. W., Han, D., Packer, L., and Everson, G. (1998) Gastroenterology 114, 164–174
15. Yerasumalhi, B., Dahl, R. H., Yerushalmi, B., Gumpriech, E., and Sokol, R. J. (2001) Hepatology 33, 616–626
16. Suzuki, H., Ohta, T., Takino, T., Fujisawa, K., and Hirayama, C. (1977) Igaku no Ayumi 102, 562–568
17. Sokol, R. J., Mack, C., Narakiewicz, M. R., and Karrer, F. M. (2003) Pediatr. Gastroenterol. Nutr. 37, 4–21
18. Jeong, H. G., You, H. J., Park, S. H., Moon, A. R., Chung, Y. C., Kang, S. K., and Chun, H. K. (2002) Pharmacol. Res. 46, 221–227
19. Kinjo, J., Hirakawa, T., Tsuhihashi, R., Nagao, T., Okawa, M., Nohara, T., and Okabe, H. (2003) Biol. Pharm. Bull. 26, 1357–1360
20. Nagai, T., Egahara, Y., Yamasaka, Y., and Kohno, M. (1991) Arch. Environ. Contam. Toxicol. 20, 432–436
21. Sokol, R. J., Winkler-Roob, B. M., Devereaux, M. W., and McKim, J. M. (1995) Gastroenterology 109, 1249–1256
22. Shah, G. M., Kaufmann, S. H., and Poirier, G. G. (1995) Anal. Biochem. 232, 251–254
23. Fernandes-Almeini, T., Armstrong, R.C., Krebs, J., Srinivasula, S. M., Wang, L., Bullrich, F., Frita, I. C., Trapani, J. A., Tomaesoli, K. J., Litwack, G., and Almeini, E. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7464–7469
24. Qiao, L., Studer, E., Leach, K., McKinstry, R., Gupta, S., Decker, R., Kukreja, R., Valerio, K., Nagarkatti, P., El Deiry, W., Molkentin, J., Schmidt-Ullrich, R.
