Licorice Compounds Glycyrrhizin and 18β-Glycyrrhetinic Acid Are Potent Modulators of Bile Acid-induced Cytotoxicity in Rat Hepatocytes*

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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 antioxidant 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 glychenodeoxycholic 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 (ERK1/2) (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 polarization, onset 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 vivo 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-

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‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-ter-minal kinase; SAPK, stress-activated protein kinase; DAPI, 4,6-diamidino-2-phenylindole; MPT, mitochondrial permeability transition; CsA, cyclosporin A; DCFA-DA, 2′,7′-dichlorofluorescein diacetate; DCF-Fein, dichlorofluoroscein; GCDC, glycochenodeoxycholic acid; GL, glycyrrhizin; GA, glycyrrhetinic acid; KRB, Krebs-Ringer HEPES (buffer); ROS, reactive oxygen species; MOPS, 4-morpholinopropanesulfonic acid; TRAIL, tumor necrosis factor apoptosis-inducing ligand; PARP, poly(ADP-ribose) polymerase.
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icy, 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 glycerocholate, lactate dehydrogenase kits, ammonium glycyrhrizin, and 1β-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 p53 MAPK, and phosphorylated and unphosphorylated PKC were from Cell Signaling Technology (Beverley, 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.

**Determination of Reactive Oxygen Species in Rat Hepatocytes**—Generation of reactive oxygen species (ROS) was measured spectrofluorometrically using the ROS-detecting probe, 2′,7′-dichlorofluorescin (DCF-DA), 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 dichloflourescein, which is oxidized to the fluorescent DCF by several ROS. 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 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 Me2SO 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 DCF fluorescence measurements at 490 nm excitation and 520 nm emission. The results were expressed as DCFein fluorescence/mg of mitochondrial protein.

**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 DCF fluorescence was measured. GCDC increased DCF 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 achieved 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 ± 11.9% 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 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. 5a 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. 5a 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. 5a 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 (procaspase 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
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 clinical effects are not fully understood, recent studies have suggested that licorice possesses anti-inflammatory, anti-oxidative, and anti-apoptotic properties. Licorice contains two main components: glabridin (GL) and glycyrrhetinic acid (GA). These compounds have been shown to protect against cancer, diabetes, and liver disease, among other conditions.

Effects of GL and GA on Liver Mitochondria—ROS Generation and the Mitochondrial Permeability Transition (MPT)

Previous studies have demonstrated that GL and GA can inhibit the mitochondrial permeability transition (MPT), a process that disrupts mitochondrial function and leads to cell death. The MPT is induced by various stimuli, including bile acids, which are toxic to liver cells.

GL and GA exert their protective effects through different mechanisms. GL is a direct inhibitor of the MPT, whereas GA may be mediated by inhibition of the SAPK/JNK pathway. These findings are supported by the observation that GA exhibits a greater antioxidative and protective effect against mitochondrial damage than GL.

Caspase 9 activation was found to be the key event in the MPT pathway. GL and GA inhibited caspase 9 activation in a dose-dependent manner, suggesting that the anti-apoptotic effects of GA are primarily through a caspase 9-independent mechanism.

In conclusion, GL and GA offer protective effects against liver damage induced by bile acids, suggesting their potential therapeutic applications in liver disease management.

**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 clinical effects are not fully understood, recent studies have suggested that licorice possesses anti-inflammatory, anti-oxidative, and anti-apoptotic properties. Licorice contains two main components: glabridin (GL) and glycyrrhetinic acid (GA). These compounds have been shown to protect against cancer, diabetes, and liver disease, among other conditions.

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In conclusion, GL and GA offer protective effects against liver damage induced by bile acids, suggesting their potential therapeutic applications in liver disease management.
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 antioxidative 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...
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 /H9262 M. In prior studies of hepatocyte apoptosis,

**FIG. 7.** Immunoblot analysis of MAPK in rat hepatocytes treated with GCDC. Whole cell lysates obtained from control 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 /H9262 M GCDC; lane 3, 100 /H9262 M GCDC + 25 /H9262 M GA; lane 4, 100 /H9262 M GCDC + 1 /H9262 M GL; and lane 5, 100 /H9262 M GCDC + 10 /H9262 M GL. Blots were probed with antibodies raised against total and phosphorylated p38 MAPK (a) and total and phosphorylated SAPK/JNK (b). Levels of activation by GCDC in the absence or presence of GL or GA are graphically depicted in bar graphs and are expressed as the ratio of the relative density of phosphorylated/total protein, with control cells standardized to 1.0. The results are from three separate hepatocyte preparations, * denotes statistical significance (p < 0.05) of control hepatocytes versus GCDC-treated cells.

**FIG. 8.** Effects of GL and GA on ROS generation in purified liver mitochondria treated with GCDC. Percoll gradient-purified liver mitochondria were loaded with DCF-DA, preincubated with various concentrations of GL (a) or GA (b), and then incubated with 100 /H9262 M GCDC. Aliquots were removed at designated time points, and DCF fluorescence was expressed as DCF fluorescence/mg of protein. Results are from 3–4 separate experiments and expressed as mean ± S.E.

**FIG. 9.** Effects of GL and GA on GCDC-induced MPT and cytochrome c release from purified mitochondria. Rat liver mitochondria were preincubated for 10 min with 0–10 /H9262 M GL (a) or GA (b) prior to induction of MPT by 100 /H9262 M GCDC. Mitochondrial swelling was monitored at 540 nm as described under “Experimental Procedures.” Results are from 3–4 separate experiments and expressed as mean ± S.E. In panel c, the inhibitory potency of GA and GL are compared with CsA in a representative experiment. After the 5-min incubation of mitochondrial with GCDC, mitochondria were isolated and immunoblotted for cytochrome c content (d) as described under “Experimental Procedures.”
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 (25, 42). This sequence of events is supported by the differential effects of GA and GL on ROS generation and JNK activation. In addition, in our model the dependence of caspase 10 on ROS generation and JNK activation is consistent with the observation of Chaudhary et al. (43), suggesting that increased ROS generation may be the upstream event that triggers JNK and caspase activation in bile acid-induced hepatocyte toxicity.

In addition to death receptor-initiated cytotoxicity, bile acids also promote cell death by direct effects on mitochondrial structure and homeostasis (12, 15, 45, 46). Consistent with previous findings (15, 25), GCDC increased generation of ROS, caused mitochondrial depolarization, and activated caspase 9 in rat hepatocytes. GA, providing a more robust antioxidative effect than GL, prevented this apoptotic pathway, whereas GL failed to afford protection. However, in contrast to their dichotomous effects on GCDC toxicity in hepatocytes, submicromolar concentrations of GL and GA similarly blocked the MPT and release of cytochrome c in purified liver mitochondria exposed to GCDC. Thus the differential effects of GA versus GL on cytotoxicity do not appear to be related to direct effects on mitochondria. However, it must be emphasized that although cellular uptake of GL and GA has been characterized previously (47, 48), mitochondrial uptake, transport, and metabolism have not been examined.

Several factors must be considered prior to examining the possible beneficial or modulatory roles of these compounds in hepatobiliary disorders, including achievable tissue concentrations, mode of administration, metabolism and potential toxicity. Orally ingested GL undergoes hydrolysis by β-glucuronidase in the intestine resulting in GL monoglucuronide and, ultimately, GA, which is absorbed into the bloodstream (49). Intravenous administration of GL-containing compounds results in the appearance of both GL and GA in the plasma of animals (50), although conversion of GL to GA is believed to occur primarily in the mucosa of the small intestine (51). Intravenous administration of GL in healthy men and in hepatitis C patients achieved maximal plasma concentrations of 29 µg/ml (approx 36 µM) (52) and 120 µM (53), respectively, whereas orally administered GL yielded no detectable plasma GL and only very low (<200 ng/ml) concentrations of GA (52). In another study, plasma GA levels reached 10 µM in humans ingesting licorice (54), similar to the protective concentrations of GA in our study.

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.

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