LICORICE COMPOUNDS, GLYCYRRHIZIN AND 18β-GLYCYRRHETINIC ACID, ARE POTENT MODULATORS OF BILE ACID-INDUCED CYTOTOXICITY IN RAT HEPATOCYTES

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Short Title: Licorice and Bile acid-Induced Toxicity


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ABSTRACT

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 anti-hepatotoxic agents against hepatitis C. 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 (ROS) 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, ROS 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 antioxidative effect.

Keywords: Glycyrrhizin, 18β-glycyrrhetinic acid, bile acid, apoptosis, oxidant stress, mitochondria.
INTRODUCTION

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 glycochenodeoxycholic acid (GCDC), promote hepatocyte death by necrosis, and lower concentrations cause apoptosis (3-5). Mechanistic studies reveal 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 1/2) (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), 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. reported that the principal triterpene component of licorice root, glycyrrhizin (GL), benefited patients with chronic hepatitis C infection (16). 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 that GL, or its hydrolyzed metabolite 18β-glycyrrhetinic acid (GA), protects against several models of oxidant-mediated toxicity, including exposure to CCl₄ (18), t-butyl hydroperoxide (19), and ischemia-reperfusion injury (20), with GA generally exhibiting greater hepatic protection than GL. While several hypotheses are 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.
MATERIALS AND METHODS

Materials

Sodium glycochenodeoxycholate, LDH kits, ammonium glycyrhrizin and 18-beta glycyrhretinic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Cyclosporin A (CsA) was purchased from Alexis Biochemicals (San Diego, CA). 2’,7’-dichlorofluorescein diacetate (DCF-DA) and BSA (fraction V) were from Eastman Kodak (Rochester, NY) and Calbiochem (La Jolla, CA), 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 p38 MAPK, and phosphorylated and unphosphorylated SAPK/JNK were purchased 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 175-225 gm male Sprague-Dawley rats (Sasco, Inc., Omaha, NE) maintained on a 12 hour light-dark cycle and fed standard laboratory rat chow, as previously described (15). Initial hepatocyte viability measured by trypan blue exclusion was always >94%. Fresh hepatocytes were resuspended in a Krebs Ringers HEPES (KRH) buffer containing 0.2% BSA (KRH/BSA) to a concentration \( \approx 1 \times 10^6 \text{/ml} \). This study was approved by the Institutional Animal Care and Use Committee of the University of Colorado Health Sciences Center.

Measurement of Reactive Oxygen Species in Rat Hepatocytes

Generation of reactive oxygen species (ROS) was measured spectrofluorometrically using the ROS detecting probe, 2’,7’-dichlorofluorescein (DCF-DA), as previously described in detail (21). Briefly, hepatocytes were loaded with DCF-DA for 30 minutes at 37°C prior to a 30
minute preincubation with graded concentrations of GL or GA. DCF-DA is trapped within cells and deesterified yielding nonfluorescent dichlorofluoroscin, which is oxidized to the fluorescent DCFein by several ROS. Hepatocytes were exposed to GCDC (0 or 100 µM) for 4 hours in a 37°C shaking water bath under room air and aliquots were removed for analysis of ROS by measuring DCFein fluorescence at 490 nm excitation and 520 nm emission. Results were expressed as fluorescence units/10⁶ cells.

**Determination of Hepatocyte Apoptosis and Necrosis**

Hepatocyte apoptosis was quantitated 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 determined by the release of lactate dehydrogenase (LDH) activity from cells and expressed as the percentage of total cellular activity that was released into the media (13).

**Mitochondrial Depolarization**

Flow cytofluorometric analysis was performed to determine the effect of GL and GA upon GCDC-dependent mitochondrial depolarization in hepatocytes, as previously described (12). Briefly, freshly isolated hepatocytes were pretreated with 25 µM GL or 10 µM GA for 30 minutes and then incubated with 100 µM GCDC for 4 hours. Hourly aliquots of cells were removed, loaded with 7.6 µM JC-1 or 3 µM propidium iodide (PI) for 15 minutes at 22°C in the dark, and washed with KRH buffer at 4°C prior to flow cytometry on a Becton Dickinson FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) using Flojo software. In actively respiring mitochondria within cells, JC-1 aggregates form and the intensity of their fluorescence at 590 nm is proportional to the mitochondria Δψ and indicative of a closed MPT pore. Neither GL or 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 PI fluorescence.

**SDS-PAGE and Immunoblotting**

Total cell lysates were obtained from hepatocytes for immunoblot analysis as follows. Hepatocytes (3 x 10^6) were pelleted by centrifugation at 2500 rpm x 5min, resuspended in KRH buffer (no BSA), and recentrifuged to obtain a washed cell pellet. Cells were lysed in 1ml of a buffer containing 62.5 mM Tris-HCl pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.00125% bromophenol blue, followed by a 15 sec. sonication on ice, as described by Shah et al. (22). Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes and non-specific proteins were blocked by an hour 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 an hour incubation with rabbit anti-HRP and biotinylated HRP 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 previously described (11). ROS were quantitated fluorimetrically using DCF-ein (11). Briefly, purified mitochondria were resuspended in a buffer containing 5 mM HEPES/50 mM KCl/2 mM KH₂PO₄/125 mM sucrose pH 7.4, treated with 1% Chelex 100 (wash buffer) and loaded with 8 μM DCF-DA at 28°C for 30 min. The loaded mitochondria were washed twice with wash buffer, centrifuged at 10,000g 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 DMSO solvent vehicle. Solvent alone had no effect on any measurements and was at a concentration =0.1%.
Mitochondria were then incubated with GCDC and aliquots removed at specified time points for DCFein fluorescence measurements at 490 nm excitation and 520 nm emission. Results were expressed as DCFein fluorescence/mg mitochondrial protein.

**MPT and Cytochrome c Content in Liver Mitochondria**

MPT induction was quantitated in purified mitochondria spectrophotometrically at 540 nm as previously described (11). Briefly, hepatic mitochondria were pre-incubated at 25°C for 5 minutes alone or in the presence of GL, GA, or the MPT blocker, CsA, prior to the addition of 100 µM CaCl₂, 5 mM sodium succinate and 5 µM rotenone. Following this 5 minute incubation the MPT was induced by addition of 100 µM GCDC. Mitochondrial swelling was quantitated by the reduction in absorbance at 540 nm during the 5 minute incubation with GCDC. After the MPT experiment, mitochondrial samples were centrifuged at 13,000g x 30 minutes at 4°C to isolate the mitochondrial pellet for immunoblot analysis of cytochrome c using anti-mouse cytochrome c antibody and anti-mouse HRP secondary antibody (BD Pharmingen).

**Statistical Analysis**

Statistical analysis between groups was conducted by ANOVA using the Schefe 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 ± SEM.
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 hours with 100 µM GCDC and DCF fluorescence measured. GCDC increased DCF fluorescence linearly in a time-dependent manner when compared with control hepatocytes (35.8 ± 1.5 vs 9.7 ± 0.9 at 4 hours) (Figure 1a). Pretreatment with GL decreased ROS generation modestly at all concentrations (0.5-25 µM) (Figure 1a). GA treatment reduced ROS generation to a greater extent, with all concentrations reducing ROS generation by >60% at 4 hours (Figure 1b). Concentration-effect relationships of the two compounds after 4 hours incubation with GCDC (Figure 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 % LDH leakage (38.2 ± 2.2 vs 15.9 ± 1.3 for control cells at 4 hours) (Figure 2). Pretreatment with GL afforded no protection against cell necrosis (Figure 2a), whereas all concentrations of GA reduced cell necrosis by >60% at 4 hours (Figure 2b). A concentration-effect comparison of GL and GA (Figure 2c) demonstrates the superior protection afforded at all concentrations of GA.

The differential effects of GL and GA on hepatocytes were more dramatic when examining DAPI-stained hepatocyte nuclei for apoptosis (Figure 3). Hepatocytes treated with 100 µM GCDC underwent significant apoptosis by 4 hours (31.5% ± 11.9 vs 1.6 ± 0.7% for
control cells) (Figure 4). Preincubating hepatocytes with 25 µM GL enhanced apoptosis by approximately 170-210% after 2 hours incubation (Figure 4a). In contrast, 25µM GA significantly inhibited GCDC-induced apoptosis by >70% throughout the course of the experiment (Figure 4b). A concentration-effect analysis of the effects of GL and GA on GCDC-induced apoptosis at 3 hours (Figure 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 hours (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) (Figure 5a). The time course (Figure 5b-c) demonstrated a decrease in JC-1 fluorescence by 1 hour. Preincubation with 10 µM GL (black data line in panel a and b) failed to prevent the dissipation of membrane potential at any time point. However, pretreating cells with 25 µM GA (green data line in panel a and c) significantly prevented the reduction in membrane potential for at least 3 hours, commensurate with the protection offered against necrosis and apoptosis. GL and GA alone (gold data line in panels a and c) had only a slight effect on membrane potential.

**Effects of GL and GA on Changes in Caspase and MAP Kinase Activation**

Immunoblots of whole cell lysates indicated that procaspase 10 was reduced after 3 hours of incubation with GCDC (Figure 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 has been previously shown to process executioner caspases 3 and 7 (23). As shown in Figure 6b, caspase 3 existed
predominantly in the native, uncleaved form (procaspase 3) in the absence of GCDC (lane 1). However, after 3 hours GCDC reduced levels of pro-caspase 3. Preincubation of hepatocytes with GA prevented loss of pro-caspase 3, whereas GL yielded no protection at 1 μM, and at 10 μM increased appearance of the cleaved caspase 3. Similar results were observed by examining cleavage of PARP, where GCDC promoted PARP cleavage, which was prevented by GA and potentiated by GL (Figure 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 (Figure 6d), which was only mildly reduced by GA, and potentiated by 10 µM GL (Figure 6d). In selected experiments, β-actin was probed to demonstrate equal loading of all lanes (Figure 6e). Taken together, these data support 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 also a member of the stress-activated protein kinase (SAPK) family (Figure 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 Figure 7a. The ratio of phosphorylated/total p38 MAPK remains relatively unchanged by treatment with GCDC (lane 2), GA (lane 3), or GL (lanes 4 and 5), suggesting that apoptosis was not p38 MAPK-dependent. In Figure 7b, the ratio of phosphorylated/total JNK density was increased by exposure to GCDC alone (1.9-fold, lane 2). Pretreatment with GA (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, and suggests 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 effects of GL and GA directly on mitochondrial function. Incubation of purified liver mitochondria with 100 µM GCDC result in increased generation of ROS within 1 minute that continued to rise linearly (Figure 8). Preincubation with GL inhibited ROS generation in a dose-dependent manner at low concentrations (0.1-1 µM), plateauing at 1.0 µM GL which reduces ROS generation by >60% at 10 minutes (Figure 8a). GA more significantly reduced ROS generation with all concentrations (0.1-10 µM) yielding >60% inhibition to a similar extent at 10 minutes of GCDC incubation (Figure 8b). These treatment effects were comparable to the cytotoxicity responses observed in Figures 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 induces the MPT when incubated for 5 minutes with succinate-energized mitochondria (Figure 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 (Figure 9a), with 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 (Figure 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 (Figure 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 (Figure 9d). Preincubation with graded concentrations of GL and GA showed similar protection against loss of cytochrome c, paralleling the reduction of MPT magnitude (Figure 9a 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 clinical 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 by acting 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 α-tocopherol, β-carotene, or the coenzyme Q analog, idebenone (13,15,30). The relative degree of antioxidant activity of GL and GA (Figure 1) correlated well with the capacity of each compound to suppress GCDC-induced cellular necrosis (Figure 2). However, the potentiation of apoptosis by GL (Figure 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 µM.

In prior studies of hepatocyte apoptosis, Yoshikawa et al. (31) reported that GL inhibited TNFα-, 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. 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, 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 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 that coincubation of GCDC with TRAIL (tumor necrosis factor-apoptosis inducing ligand), a death receptor cell signaling agent, promoted caspase 10 cleavage. There are differences between our study and that of Higuchi et al. that could account for the differing effects of bile acids on caspase 10. Higuchi et al used HuH-7 cells transfected with a sodium-dependent transporting polypeptide in culture for 12 hours 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, are associated with bile acid-induced apoptosis (24,25,38), whereas activation of ERK may suppress apoptosis (39,40). 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 this report, JNK activation, commensurate with oxidative stress, were key signals 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, the dependence of caspase 10 in our model on ROS generation and JNK activation is consistent with the observation of Chaudhary et al. (43), and suggests 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 vs 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 previously characterized (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 appearance of both GL and GA in plasma of animals (50), although conversion of GL to GA is believed to occur primarily in the small intestinal mucosa (51). IV administration of GL in normal men and 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 10µM in humans ingesting licorice (54), similar to 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.
REFERENCES


FIGURE LEGENDS

Figure 1. Effects of GL and GA on ROS generation in isolated rat hepatocyte suspensions exposed to GCDC. Hepatocytes (10^6/ml) were loaded with 10 μM DCF-DA for 30 minutes prior to preincubating cells with various concentrations of GL (a) or GA (b) followed by exposure to 100 μM GCDC. Aliquots were removed at 2 and 4 hours for DCF fluorescence and expressed as DCFein fluorescence/10^6 cells, as described in Materials and Methods. The 4 hour DCFein values for GCDC-exposed hepatocytes are plotted vs. concentrations of GL and GA (c). Results are from at least 6 separate experiments and expressed as mean ± SEM.

Figure 2. Effects of GL and GA on cellular necrosis in isolated rat hepatocyte suspensions exposed to GCDC. Hepatocytes were preincubated with various concentrations of either GL (a) or GA (b) for 30 minutes prior to the addition of 100 μM GCDC. Aliquots were removed at 2 and 4 hours and necrosis assessed as % LDH released, as described in Materials and Methods. The 4 hour % LDH released values for GCDC-exposed hepatocytes were plotted vs. concentrations of GL and GA (c). Results are from at least 6 separate experiments and expressed as mean ± SEM.

Figure 3. Fluorescence microscopy of hepatocyte nuclei labeled with DAPI. Hepatocytes were incubated with no additions (A), or with 100 μM GCDC alone (B) or in the presence of 25 μM GA (C) or 10 μM GL (D) for 3 hours. After the incubation, cells were fixed, cytotoxed onto a slide and stained with DAPI for fluorescence microscopy. Only those cells which had fragmented nuclei or margantiated chromatin (depicted with arrows) were considered apoptotic. The bar in the lower right corner is approximately 10 μm.
**Figure 4.** Effects of GL and GA on GCDC-induced hepatocyte apoptosis. Hepatocytes were exposed to 100 µM GCDC for 4 hours in the absence or presence of 25 µM GL (a) or GA (b). Hourly aliquots were removed for quantitation of apoptosis as described in Materials and Methods. The % of apoptotic cells after 3 hours exposure to GCDC were plotted vs. concentrations of GL and GA (c). Results are from at least 6 separate experiments and expressed as mean ± SEM.

**Figure 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 PI as described in Materials and Methods. In panel (a) a representative plot of JC-1 aggregate fluorescence is shown after 3 hours 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 panels (b and c), demonstrating protection by GA but not GL. Neither GL nor GA alone (gold lines) affected mitochondrial depolarization. Results are from 3 separate experiments and expressed as mean ± SEM.

**Figure 6.** Immunoblot analysis of caspases and PARP from rat hepatocytes treated with GCDC. Whole cell lysates obtained from hepatocytes after 3 hour incubation were separated by SDS-PAGE and immunoblotted as described in Materials and Methods. For each blot 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 (panel a), caspase 3 (panel b), native and
cleaved PARP (panel c), cleaved caspase 9 (panel d), and β-actin (panel e). Results are representative results from 2-4 separate hepatocyte preparations.

**Figure 7.** Immunoblot analysis of MAPK in rat hepatocytes treated with GCDC. Whole cell lysates obtained from control hepatocytes after 3 hours incubation were separated by SDS-PAGE and immunoblotted as described in Materials and Methods. For each blot 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 total and phosphorylated p38 MAPK (panel a) and total and phosphorylated SAPK/JNK (panel b). Levels of activation by GCDC in the absence or presence of GL or GA are graphically depicted in bar graphs and were expressed as the ratio of the relative density of phosphorylated/total protein, with control cells standardized to 1.0. The results are from 3 separate hepatocyte preparations. *denotes statistical significance (p<0.05) of control hepatocytes vs. GCDC treated cells.

**Figure 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 µM GCDC. Aliquots were removed at designated time points and DCF fluorescence expressed as DCFein fluorescence/mg protein. Results are from 3-4 separate experiments and expressed as mean ± SEM

**Figure 9.** Effects of GL and GA on GCDC-induced MPT and cytochrome c release from purified mitochondria. Rat liver mitochondria were preincubated for 10 minutes with 0-10 µM GL (a) or GA (b) prior to induction of MPT by 100µM GCDC. Mitochondrial swelling was
monitored at 540 nm as described in Materials and Methods. Results are from 34 separate experiments and expressed as mean ± SEM. In panel (c) the inhibitory potency of GA and GL were compared with CsA in a representative experiment. After the 5 minute incubation of mitochondrial with GCDC, mitochondria were isolated and immunoblotted for cytochrome c content (d), as described in Materials and Methods.
Figure 1

(a) Graph showing DCF fluorescence per 10^6 cells over time for different concentrations of GCDC.

(b) Graph showing DCF fluorescence per 10^6 cells over time for different concentrations of GA.

(c) Graph showing DCF fluorescence at 4 hours in the presence of GCDC for different concentrations of GL and GA.
Figure 3
Figure 4
Figure 5
Figure 6

- a) Caspase 10
- b) Caspase 3 (35kDa), Cleaved Caspase 3 (17kDa)
- c) PARP (116kDa), Cleaved PARP (89kDa)
- d) Cleaved Caspase 9 (17kDa)
- e) β-Actin

1  2  3  4  5
Figure 7

**a)**

- **Phospho-p38 MAPK**
- **Total p38 MAPK**

**Relative Density**

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<td>1.5</td>
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**b)**

- **Phospho-SAPK/JNK**
- **Total SAPK/JNK**

**Relative Density**

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<td>*</td>
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</tbody>
</table>

*Significant difference*
Figure 9

a) 

Δ Abs 540nm/5min

μM GCDC: 0, 100, 100, 100, 100, 100, 100, 100
μM GL: 0, 0, 0.1, 0.25, 0.5, 1.0, 5.0, 10.0

b) 

Δ Abs 540nm/5min

μM GCDC: 0, 100, 100, 100, 100, 100, 100, 100
μM GA: 0, 0, 0.1, 0.25, 0.5, 1.0, 5.0, 10.0

c) 

Absorbance 540nm

Minutes

GCDC/1μM CsA
GCDC/1μM GL
GCDC/1μM GA
Control

GCDC

d) cyto c

GCDC

CsA 0.5 1.0 5.0 10.0
GL (μM)

GA (μM)
Licorice compounds, glycyrrhizin and 18β-glycyrrhetinic acid, are potent modulators of bile acid-induced cytotoxicity in rat hepatocytes
Eric Gumpricht, Rolf Dahl, Michael W. Devereaux and Ronald J. Sokol

J. Biol. Chem. published online January 10, 2005

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