Glycyrrhizin Protects against Acetaminophen-Induced Acute Liver Injury via Alleviating Tumor Necrosis Factor α–Mediated Apoptosis

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ABSTRACT

Acetaminophen (APAP) overdose is the leading cause of drug-induced acute liver failure in Western countries. Glycyrrhizin (GL), a potent hepatoprotective constituent extracted from the traditional Chinese medicine liquorice, has potential clinical use in treating APAP-induced liver failure. The present study determined the hepatoprotective effects and underlying mechanisms of action of GL and its active metabolite glycyrrhetic acid (GA). Various administration routes and pharmacokinetics–pharmacodynamics analyses were used to differentiate the effects of GL and GA on APAP toxicity in mice. Mice deficient in cytochrome P450 2E1 enzyme (CYP2E1) or receptor interacting protein 3 (RIPK3) and their relative wild-type littermates were subjected to histologic and bio-chemical analyses to determine the potential mechanisms. Hepatocyte death mediated by tumor necrosis factor α (TNFα)/caspase was analyzed by use of human liver-derived LO2 cells. The pharmacokinetics-pharmacodynamics analysis using various administration routes revealed that GL but not GA potently attenuated APAP-induced liver injury. The protective effect of GL was found only with intraperitoneal and intravenous administration and not with gastric administration. CYP2E1-mediated metabolic activation and RIPK3-mediated necroptosis were unrelated to GL’s protective effect. However, GL inhibited hepatocyte apoptosis via interference with TNFα-induced apoptotic hepatocyte death. These results demonstrate that GL rapidly attenuates APAP-induced liver injury by directly inhibiting TNFα-induced hepatocyte apoptosis. The protective effect against APAP-induced liver toxicity by GL in mice suggests the therapeutic potential of GL for the treatment of APAP overdose.

Introduction

Acetaminophen (APAP) overdose is a leading cause of drug-induced acute liver failure in the United States (Blieden et al., 2014). Because of its prevalence and severity, the U.S. Food and Drug Administration decided to limit and monitor the exposure of high-dose APAP (McCarthy, 2014; Mitka, 2014). Cytochromes P450 enzymes, including CYP2E1, CYP3A11, and CYP1A2, are mainly responsible for the metabolic activation of APAP into its toxic intermediate, N-acetyl-p-benzoquinone imine (NAPQI) (Lee et al., 1996; Manyike et al., 2000; Cheung et al., 2005). High NAPQI production depletes glutathione (GSH), produces NAPQI-protein adducts, triggers mitochondrial oxidative stress, and ultimately initiates hepatocellular apoptosis and/or necrosis with acute liver inflammation.

The therapeutic options for treating APAP hepatotoxicity are limited. The only clinical choice is administration of N-acetyl cysteine. However, it has a narrow therapeutic time window after the onset APAP overdosing, and thus new antidotes are warranted (Lancaster et al., 2015).

In recent years, studies have revealed that some traditional Chinese medicines (TCMs) have therapeutic effects, and TCMs are recognized as potential drugs for ameliorating liver (Zhang and Schuppan, 2014) and other diseases (Chan et al., 2015; Xiong, 2015; Zheng et al., 2015). Notably, glycyrrhizin (GL), also known as glycyrrhizic acid, is one of the most effective medicines extracted from the TCM liquorice for treating liver diseases. GL has potent hepatoprotective (Li et al., 2014b), anti-inflammatory (Honda et al., 2012; Fu et al., 2014; Kim et al., 2015), and neuroprotective effects (Ni et al., 2013; Barakat et al., 2014b), anti-inflammatory (Honda et al., 2012; Fu et al., 2014; Kim et al., 2015), and neuroprotective effects (Ni et al., 2013; Barakat et al., 2014b). GL has been formulated as a drug widely used for treating chronic liver diseases in Asian countries (Li et al., 2014b). Structurally,
GL is a glycoside that is rapidly hydrolyzed to glycyrrhetinic acid (GA) by intestinal bacteria (Akao et al., 1994; Takeda et al., 1996). In a previous study, single intraperitoneal injections of GL were used as an inhibitor of high-mobility group box-1 (HMGB1) to confirm its pathophysiologic role in APAP toxicity (Wang et al., 2013). Pretreatment with GL (400 mg/kg) for 7 consecutive days attenuated APAP-induced hepatotoxicity by reversing fatty acid metabolism (Yu et al., 2014). GL’s hydrolyzed metabolite, GA, was demonstrated to directly protect against several types of liver injury induced by exposure to carbon tetrachloride (Jeong et al., 2002; Chen et al., 2013), free fatty acids (Wu et al., 2008), and toxic bile acids (Gumprecht et al., 2005). However, it remains unknown whether GL’s hepatoprotective effect is derived from GL or its putative active metabolite GA, and how either protects against APAP-induced cell death of hepatocytes.

The type and mechanism of APAP-triggered death of hepatocytes are still topics of debate. The protein complex of receptor interacting protein kinase-1 (RIPK1) and RIPK3, also known as necosome, performs necroptosis (Li et al., 2012). The RIPK1 inhibitor necrostatin-1 (NEC-1) and the RIPK3 inhibitor dabrafenib have been reported to protect against APAP-induced hepatotoxicity both in vivo and in vitro, suggesting that necroptosis plays a role in protection (Li et al., 2014a; 2014b). Two studies used gene knockout mice to elucidate the roles of necosome signaling genes, including Ripk1, Ripk3, and Mikl1, in mediating APAP-induced toxicity. However, these two studies reported different results about the role of RIPK3 in APAP-induced toxicity. One study showed that RIPK3 was an early mediator of APAP-induced toxicity (Ramachandran et al., 2013), and the other study showed no difference in sensitivity to APAP between Ripk3-null mice and their wild-type littermates (Dara et al., 2015). Thus, the role of RIPK3-mediated necroptosis in APAP-induced hepatocyte death remains controversial.

APAP induces a severe inflammatory response in the liver (Lawson et al., 2000) and significant GSH depletion (Matsumaru et al., 2003). Tumor necrosis factor-α (TNFα) potentiates apoptotic hepatocyte death under conditions of low cellular GSH (Collett et al., 1998; Matsumaru et al., 2003). These studies suggest possible synergy with proinflammatory cytokines in mediating hepatic damage. Some studies have suggested that GL prevents the release of proinflammatory cytokines from immune cells (Fu et al., 2014; Kim et al., 2015), making GL a potential immunoregulator. However, the crosstalk among TNFα, APAP, and the effects of GL on this process, especially in hepatocytes, is still poorly understood.

In this study, the hepatoprotective effects of GL and GA against APAP-induced hepatotoxicity were examined to uncover the potential mechanisms involved in the protective effects. The protective effect of GL on APAP-induced liver injury is not derived from the metabolic inhibition of APAP activation and is unlikely to be related to RIPK3/necroptosis. The intraperitoneal or intravenous injection of GL, but not the intraperitoneal injection of GA, had a potent hepatoprotective effect via interference with TNFα-induced hepatocyte apoptosis.

Materials and Methods

Chemicals and Reagents. GL (97%) and GA (99.5%) were purchased from TCI (Shanghai, People’s Republic of China). 18β-Glycyrrhetinic acid (18β-GA; 97%), APAP, NEC-1, ammonium diethylthiocarbamate, ketoconazole, formic acid, dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT), and chlorpropamide were purchased from Sigma-Aldrich (St. Louis, MO). Acetaminophen glutathione disulfide (NAPQ-GSH) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). N-acetyl cysteine–acetylaminothiophenol (NAC–APAP) standard was kindly provided by Professor Bernhard Lauterburg, University of Bern, Switzerland. Recombinant human TNFα was purchased from Peprotech (London, United Kingdom). N-benzylxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethyl ketone (ZVAD-fmk; zVAD) and the GSH/glutathione disulfide (GSGG) assay kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, People’s Republic of China). High-performance liquid chromatography–grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Billerica, MA). Enzyme-linked immunosorbent assay kits for mouse TNFα were purchased from Excel (Shanghai, People’s Republic of China). The annexin V fluorescein isothiocyanate apoptosis detection kit was purchased from BD Biosciences (San Diego, CA), and the fluorescence-activated cell sorting (FACS) analysis was performed on a BD Accuri C6 flow cytometer (BD Biosciences). The In Situ Cell Death Detection Kit, AP was purchased from Roche (Indianapolis, IN).

Experimental Animals and Treatments

Male C57BL/6 wild-type mice (6 to 8 weeks old) were obtained from the Shanghai SLAC Laboratory Animal Center (Shanghai, People’s Republic of China) and allowed free access to food and water until experimental use. The animal room was maintained at 23 ± 1°C with a 12-hour light/dark cycle and 55% ± 5% humidity. The animal studies were approved by the animal ethics committee of China Pharmaceutical University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. C57BL/6 null mice and wild-type mice on a C57BL/6N background were provided by Vishva Dixit (Genentech, South San Francisco, CA), and Cyp2e1–null mice on a 129/CV genetic background were maintained in the U.S. National Cancer Institute, with handling in accordance with animal study protocols approved by the National Cancer Institute Animal Care and Use Committee. Before intraperitoneal dosing of APAP, the mice were fasted overnight (14 hours) with free access to water.

Mice were administered 600 mg/kg of APAP for inducing hepatotoxicity in Cyp2e1–null mice, 300 mg/kg of APAP for inducing hepatotoxicity in wild-type C57BL/6 mice, and 200 mg/kg of APAP for inducing hepatotoxicity in wild-type 129/CV mice. For single GL/GA treatments, GL by intragastric administration was performed at 1 hour before APAP dosing, GL or GA treatment was administered by intraperitoneal injection at 30 minutes before APAP dosing, and GL treatment by intravenous injection was coadministered with APAP dosing. A dose of 50 mg/kg or 100 mg/kg of GL (GL50 or GL100, respectively) and 50 mg/kg of GA (GA50) or 30 mg/kg of 18β-GA was injected to mice; the mice were killed at 0, 0.5, 2, 4, 6, 8, and 24 hours, and serum and livers were collected. For multiple GL injections, mice were pretreated with 50 mg/kg of GL for 7 consecutive days, subjected to APAP overdosing 30 minutes after final GL injection, and sacrificed at 24 hours after APAP challenge. A portion of liver was fixed in 10% formalin solution, and the remaining liver was flash frozen in liquid nitrogen and stored at −80°C for further use. APAP was freshly dissolved in warm saline at 55°C and cooled to 37°C before use. GL was freshly dissolved in saline with the pH adjusted to 7.0–7.2. GA or 18β-GA was freshly dissolved in saline containing 5% Tween 80.

In Vitro Studies. Human nontumor hepatic LO2 cells were obtained from the Chinese Academy of Sciences (Shanghai, People’s Republic of China). For testing the protective effect of GL or GA against APAP-induced LO2 cell death, LO2 cells were seeded in 96-well plates for MTT assays or 6-well plates for FACS assays and then grown to 70–90% confluence before use. GL, GA, or 18β-GA was dissolved in DMSO as stock solution and diluted 1000 times. APAP was dissolved in Dulbecco’s modified Eagle’s medium. LO2 cells were pretreated with control 0.1% DMSO, or GL, GA, or 18β-GA for 30 minutes before adding APAP. Only 0.1% DMSO-treated cells were set as the control group. For cell viability assays, MTT was added after 18 to 24 hours of APAP treatment. For FACS assays, samples were collected at 10 hours after APAP treatment and analyzed according to the instruction manual protocol for the annexin V fluorescein isothiocyanate apoptosis detection kit.

Serum Aminotransferase Analysis. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were quantified using a standard clinical automatic analyzer or a commercial ALT or AST assay kit (Catachem, Bridgeport, CT).

Quantitative Real-Time Polymerase Chain Reaction. Total tissue RNA extraction was performed by using the RNAiso Plus reagent (TaKaRa Biotechnology, Dalian, People’s Republic of China) according to the manufacturer’s
protocol. Purified total RNA was reverse-transcribed using the Prime Script RT Reagent Kit (Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. Quantitative real-time polymerase chain reaction was performed by using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Bedford, MA) and SyBr Green reagent kit (Takara Biotechnology). Values were normalized to glyceraldehyde-3-phosphate dehydrogenase. Sequences for the primers are listed in Supplemental Table 1.

H&E Staining and Terminal Deoxynucleotidyl Transferase–Mediated Digoxigenin-Deoxyuridine Nick-End Labeling. Formalin-fixed liver tissues were embedded in paraffin and 5-μm-thick sections were cut for H&E staining and terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining according to the manual protocols. Data represent n = 3 in each group for all the analyses.

Liquid Chromatography/Tandem Mass Spectrometry Analysis of GL and GA. Chromatographic separation was obtained on a Waters Acquity I Class UPLC system comprising a binary solvent manager, a flow-through needle autosampler, and column manager using a Waters Acuity BEH C-18 2.1 × 50 mm column. A liquid chromatography/tandem mass spectrometry method was used with multiple reaction monitoring in positive mode for monitoring 823.7−→453.5 for GL, 471.5−→149.1 for GA, and 277−→110.9 for the internal standard chlorpropamide with a slight modification of the mobile phase, as previously described by Li et al. (2013).

Liquid Chromatography with Quadrupole Time of Flight Mass Spectrometry Analysis of NAPQI-GSH. The oxidized APAP intermediate NAPQI, formed in the mouse microsomal incubation system, was trapped by reduced GSH with a slight modification of the in vitro APAP incubation system as described elsewhere (Fan et al., 2014; Jiang et al., 2015). NAPQI-GSH was determined by the liquid chromatography with quadrupole time of flight mass spectrometry (Q-TOF LC/MS) method. Briefly, mouse liver microsomes (final concentration 1 mg/ml protein) were incubated with 500 μM APAP alone and with GL (10 μM, 50 μM, 100 μM, 200 μM, and 500 μM), or GA (18 μg-GA (2 μM, 5 μM, 10 μM, and 20 μM). The reaction was initiated by adding NAPDH, trapped by 5 mM GSH and quenched by adding cold acetonitrile. NAPQI-GSH was identified through accurate mass measurement, compared with authentic standards, and monitored at m/z 457.1393. The internal standard chlorpropamide was monitored at m/z 277.0414 in positive mode. Chromatographic separation was obtained on a Waters Synapt-HDMS Q-TOF mass spectrometer running in positive electrospray ionization mode. The capillary and cone voltage were 3.0 kV and 30 V, respectively. The source and desolvation temperature were 150°C and 400°C. The desolvation and cone gas (nitrogen) were 850 l/h and 70 μl/min. The mobile phases were: A, water with 0.1% formic acid; and B, acetonitrile with 0.1% formic acid (Q). The flow rate was maintained at 0.4 ml/min. The gradient was as follows: initial conditions 98% A for 0.5 minutes, to 80% A at 4 minutes, to 5% A at 8.0 minutes; held for 1 minute; and returning to initial conditions and holding for 2 minutes for column equilibration, with a total running time of 11 minutes.

Statistical Analysis. Data are presented as the mean ± S.E.M. Differences between the control and experimental groups were determined by a two-tailed Student’s t test in GraphPad Prism 6 (GraphPad Software, San Diego, CA). P < 0.05 was considered statistically significant.

Results

Intraperitoneal Administration of GL Reduces APAP-Induced Hepatotoxicity. To determine whether GL attenuates APAP-mediated damage in vivo, a nonlethal dose of APAP (300 mg/kg) was administered to wild-type mice and serum liver enzyme levels and histology were measured to determine hepatocellular toxicity after 24 hours. Serum ALT and AST were sharply increased in the vehicle/APAP-treated group, and they were markedly decreased in the GL/APAP-treated group (Fig. 1, A and B). Necrotic areas were markedly decreased in liver from GL-treated mice (Fig. 1C). Because APAP-induced liver toxicity is associated with increased inflammation (Lawson et al., 2000; Liu et al., 2004), the APAP-induced inflammatory response was analyzed. Secreted TNFα in serum was assessed in the APAP-administered mice treated with or without GL. Serum TNFα levels were significantly reduced by

Fig. 1. GL pretreatment reduces serum transaminases, improves liver histology, and normalizes inflammation. (A, B) Serum ALT and AST levels. (C) H&E staining of liver sections. Original magnification, 20×; black scale bar, 50 μm. (D) Serum TNFα levels. (E-G) Relative mRNA level of TNFα, Il-1β, and Il-6 (n = 6–8 in each group). Control, saline-treated control mice; APAP, saline/APAP-treated mice; GL50 + APAP, GL 50 mg/kg/APAP-treated mice; GL100 + APAP, GL 100 mg/kg/APAP-treated mice. Data are expressed as mean ± S.E.M. *P < 0.05, **P < 0.01, and ***P < 0.001 versus control mice. *P < 0.05, **P < 0.01, and ***P < 0.001 versus APAP-overdosed mice.
GL-treatment (Fig. 1D). Moreover, expression of mRNAs encoding the proinflammatory cytokines TNFα, interleukin 6 (IL-6), and IL-1β were normalized in the liver by pretreatment with GL (Fig. 1, E–G). Taken together, these results suggest that GL attenuated both liver toxicity and inflammation induced by APAP administration.

**Intravenous, but Not Oral Administration of GL Inhibits APAP-Induced Hepatotoxicity.** In the clinic, GL is administrated orally or intravenously, so we determined the effect of GL by intragastric administration or intravenous injection in APAP-caused liver injury. A single gavage administration of GL50 and GL100 showed no effect in

![Fig. 2. Intragastric administration of GL fails to prevent APAP-induced hepatotoxicity. (A) H&E-stained liver sections 24 hours after APAP challenge. Original magnification, 20×; black scale bar, 50 μm. (B, C) Serum ALT and AST levels. (D, E) GL and GA levels in serum and liver. Control, saline-treated control mice; APAP, saline/APAP-treated mice; GL50 (po) + APAP, GL50 mg/kg (oral)/APAP-treated mice; GL100 (po) + APAP, GL100 mg/kg (oral)/APAP-treated mice; GL50 (ip) + APAP, GL50 mg/kg (i.p.)/APAP-treated mice. Data are expressed as mean ± S.E.M., n = 4–5 in each group. ###P, 0.001 versus control mice; **P, 0.01 versus APAP-overdosed mice.](image-url)

![Fig. 3. Intravenous injection of GL inhibits APAP-induced hepatotoxicity. (A) H&E-stained liver sections 4 hours after APAP challenge. Original magnification, 20×; black scale bar, 50 μm. (B, C) Serum ALT and AST levels. (D, E) GL and GA levels in serum and liver. Data are expressed as mean ± S.E.M., n = 5–8 in each group for all analyses. Control, saline-treated control mice; APAP, saline/APAP-treated mice; GL50 (iv) + APAP, GL 50 mg/kg (i.v.)/APAP-treated mice; GL100 (iv) + APAP, GL 100 mg/kg (i.v.)/APAP-treated mice; GL100 (iv), only GL 100 mg/kg (i.v.)-treated mice. ###P, 0.001 versus control mice; ***P, 0.001 versus APAP-overdosed mice.](image-url)
attenuating APAP-induced liver injury at 24 hours after APAP dosing (Fig. 2, A–C). GL was not detected, and GA was significantly detected in both serum and liver at 24 hours after APAP challenge (Fig. 2, D and E). GL50 by intraperitoneal injection was used as a positive control. Indeed, it is known that GL is hydrolyzed to GA by intestinal bacteria (Akao et al., 1994; Takeda et al., 1996). In contrast, intravenous injection of GL50 and GL100 decreased APAP-induced hepatocytic death (Fig. 3A) and markedly attenuated the increased serum ALT and AST levels (Fig. 3, B and C). By intravenous injection of GL50, the serum concentrations of GA were less than 1 μM in GL-alone injected mice and GL/APAP-treated mice, respectively, whereas approximately a 700-fold increase of GL was detected in the serum of GL/APAP-treated mice (Supplemental Fig. 1A and Fig. 3D). In the liver, the concentration of GA was 2 ng/mg, and a 50-fold increase of GL to approximately 100 ng/mg was detected (Supplemental Fig. 1B and Fig. 3E). Similarly, after intravenous administration of GL100, the serum and liver GA levels were markedly lower than GL levels (Fig. 3, D and E). These data indicate that GL attenuates liver injury only after intraperitoneal and intravenous administration and not after oral administration.

GL, Not GA, Attenuates APAP-Induced Liver Damage. The observation that intravenous/intraperitoneal injection but not oral administration of GL showed a protective effect suggest that GL itself, and not the metabolite GA, directly contributed to GL’s protective effect. To determine the compound responsible for GL’s hepatoprotective effect, we investigated the pharmacokinetics and pharmacodynamics of injected GL after APAP dosing.

Mice were pretreated with a single intraperitoneal injection of GL100 30 minutes before APAP (300 mg/kg) challenge, and were killed at 2, 4, 8, and 24 hours after APAP (Fig. 4A). GL and GA distribution in liver and serum were then determined. During the time course of GL/APAP challenge, GL markedly attenuated APAP-caused liver injury. Intriguingly, the potent protective effect was observed even at the earliest time point of 2 hours, suggesting that GL could affect the initiation of APAP-induced toxicity and rapidly inhibit liver injury (Fig. 4, B and C). Moreover, GA levels peaked at 8 hours in liver and 4 hours in serum, while GL levels were time-dependently decreased in both serum and liver in GL/APAP-treated mice (Fig. 4, D and E) and in GL/saline-treated control mice (Supplemental Fig. 1, C and D).

Given that GA attenuates liver injury, ALT and AST would be expected to be decreased in a time-dependent manner. However, ALT and AST were markedly increased from 8 hours after APAP administration in the GL/APAP-challenged group (Fig. 4, B and C). The potent protective effect of GL and increase in liver damage commencing at 8 hours were also confirmed by histology (Supplemental Fig. 1E). These data suggested that GL, and not GA, directly attenuated APAP hepatotoxicity.

To examine the direct effect of GA in combating APAP-induced liver injury, GAS50 (approximately equimolar to GL100) was injected to wild-type mice by a single intraperitoneal injection before APAP (300 mg/kg) challenge. All mice were killed at 24 hours after APAP dosing.
administration and subjected to histologic and serum analysis. In serum, GA was only detected in GA-treated mice and not detected in the GL-treated mice (Fig. 5A). In the liver, approximately a 10-fold increase of GA was detected in GA-treated mice compared with GL-treated mice (Fig. 5B). Thus, GA was efficiently absorbed and detected in both serum and liver. Histology revealed that the necrotic areas that developed after APAP in the GA-treated group were not decreased as compared with the nontreated group, while the areas were significantly decreased in the GL-treated group (Supplemental Fig. 2A).

In agreement with this result, ALT and AST levels were also decreased in the GL/APAP-treated group. In contrast, these markers in the GA-treated group were moderately but not significantly decreased (Fig. 5, C and D). Furthermore, the effect of 18β-GA showed no significant effect in preventing APAP-induced liver injury (Supplemental Fig. 2, B–D) whereas 18β-GA was efficiently absorbed in both serum and liver (Supplemental Fig. 2, E and F). These data demonstrate that the effects of GA derived from hydrolyzed GL in the GL-treated group in vivo could be excluded.

The direct effect of GL and the hydrolyzed form GA were next analyzed in vitro by use of human hepatic-derived LO2 cells. Cell survival rates were determined after APAP treatment together with GL, GA, or 18β-GA. GL was found to significantly inhibit APAP-induced cell death in vitro (Supplemental Fig. 3A) whereas neither GA nor 18β-GA showed protective potency against cytotoxicity induced by APAP (10 mM) (Supplemental Fig. 3, B and C). This protective effect was more remarkable at a higher dose of APAP (20 mM). GL significantly rescued the survival rate of APAP-treated cells from approximately 10% to 40%, even at the low concentration of 5 μM (Fig. 5E) whereas both GA and 18β-GA showed no significant protective effect at various concentrations (Fig. 5, F and G). Both concentrations of GA and 18β-GA used included the maximum nontoxic concentration in the hepatic cells (Supplemental Fig. 3, D–F). These data demonstrate that GL and not GA directly prevents APAP-caused liver injury by inhibiting hepatocyte damage.

Hepatoprotective Effect of GL Is Unrelated to the Metabolic Activation of APAP. To clarify the possible mechanisms, we first asked whether the hepatoprotective effect of GL against APAP toxicity was from the direct interference of APAP metabolic activation. We found that treatment with a single or multiple injections of GL alone to mice had no significant effect on the expression of Cyp2e1, Cyp3a11, or Cyp1a2, although both treatments significantly attenuated APAP-induced down-regulation of such enzymes (Fig. 6, A–C). Similar results were obtained from the in vitro mouse primary hepatocytes study; no significant change in the Cyp2e1, Cyp3a11, or Cyp1a2 mRNA levels was observed with GL, GA, or 18β-GA (Supplemental Fig. 4, A–F).

APAP hepatotoxicity is mainly induced by NAPQI, which subsequently binds with hepatic GSH and forms NAPQI-GSH. To test whether GL and/or GA directly inhibited APAP bioactivation, an APAP/mouse liver microsome incubation system was used. A peak at the same retention time (1.9 minutes) with NAPQI-GSH authentic standard was found in the in vitro APAP incubation system whereas no peak was found when we removed NAPDH from the APAP incubation system (Supplemental Fig. 5A). In this system, we found that GL, GA, and 18β-GA did not significantly influence APAP metabolic activation in vitro whereas the positive CYP3A11 inhibitor (ketoconazole) and CYP2E1 inhibitor (ammonium diethyldithiocarbamate) significantly inhibited the formation of NAPQI-GSH (Fig. 6, D–F).

In mice, the hepatotoxic NAPQI produced from APAP metabolic activation is very unstable and has been known to subsequently form with hepatic GSH and forms NAPQI-GSH. To test whether GL and/or GA directly inhibited APAP bioactivation, an APAP/mouse liver microsome incubation system was used. A peak at the same retention time (1.9 minutes) with NAPQI-GSH authentic standard was found in the in vitro APAP incubation system whereas no peak was found when we removed NAPDH from the APAP incubation system (Supplemental Fig. 5A). In this system, we found that GL, GA, and 18β-GA did not significantly influence APAP metabolic activation in vitro whereas the positive CYP3A11 inhibitor (ketoconazole) and CYP2E1 inhibitor (ammonium diethyldithiocarbamate) significantly inhibited the formation of NAPQI-GSH (Fig. 6, D–F).

In mice, the hepatotoxic NAPQI produced from APAP metabolic activation is very unstable and has been known to subsequently form NAPQI-GSH and NAC-APAP (Chen et al., 2008). While we were determining the extent of APAP metabolic activation in vivo, we found that the serum level of NAPQI-GSH and NAC-APAP at 3 hours after APAP injection were not significantly changed by either a single GL injection or multiple GL injections (Fig. 6, G). Meanwhile, we analyzed the GSH/GSSG ratio in APAP-administered and GL-treated liver. In time course experiments, 300 mg/kg of APAP administration resulted in a rapid depletion of GSH in the liver at 2 and 4 hours after administration; GL pretreatment did not alter the liver GSH/GSSG ratios at either time point (Supplemental Fig. 5B).

CYP2E1 has a significant role in the metabolic activation of APAP, as Cyp2e1-null mice show resistance to APAP toxicity (Lee et al., 1996). Analysis of the relationships between the protective effect of GL and CYP2E1-mediated APAP activation revealed that 200 mg/kg of APAP administered to wild-type129/CV mice or a
high dose (600 mg/kg) of APAP administered to Cyp2e1-null mice induced liver injury, as revealed by the increased serum ALT and AST as well as severe hepatocytes death. In contrast, the injury markers were robustly inhibited by intraperitoneal injection of GL50 both 6 hours and 24 hours after APAP challenge in both wild-type (Fig. 7, A and B) and Cyp2e1-null mice (Fig. 7, C and D). Histology showed a similar attenuation of APAP-induced toxicity by GL in wild-type (Supplemental Fig. 6A) and Cyp2e1-null mice (Supplemental Fig. 6B). These results revealed that GL’s protective effect did not depend on the metabolic activation of APAP and CYP2E1 expression. Together, these results strongly support that the hepatoprotective effect of GL is unrelated to the metabolic activation of APAP.

RIPK3 Is Not Involved in APAP Toxicity or the Hepatoprotective Effect of GL. Recent studies revealed a type of necrosis involved in programmed cell death known as necroptosis (Li et al., 2012). RIPK1 and RIPK3 are both central players in TNFα-induced necroptosis (Zhang et al., 2009; Li et al., 2012), with the involvement of its downstream cellular signaling mixed lineage kinase domain-like protein (MLKL) (Sun et al., 2012; Zhao et al., 2012). In
particular, RIPK3 was confirmed to have an essential role in ameliorating APAP-induced liver injury (Ramachandran et al., 2013). To confirm whether APAP exposure influenced RIPK3 expression, we measured $\text{Ripk}3$ mRNA levels. APAP induced a 2- to 3-fold increase of $\text{Ripk}3$ mRNA as early as 2 hours (Supplemental Fig. 7A). Upstream signaling of RIPK3, $\text{Tnf}a$, and downstream $\text{Mlkl}$ mRNA were also elevated (Supplemental Fig. 7, B and C). GL treatment significantly attenuated both of the $\text{Ripk}3$ and $\text{Tnf}a$ mRNA levels whereas no significant decrease of $\text{Mlkl}$ mRNA by GL treatment was noted (Supplemental Fig. 7, A–E). These results suggest that RIPK3-mediated necroptosis is possibly involved in APAP toxicity and GL’s protective effect.

We further investigated with the use of $\text{Ripk}3$-null mice. The histologic observations (Fig. 8, A and B) and levels of serum ALT and AST (Fig. 8, C and D) showed no differences when we compared $\text{Ripk}3$-null mice and their wild-type littermates at 6 hours after APAP challenge. Furthermore, GL robustly and significantly attenuated APAP toxicity in both genotypes. These results suggested no involvement of RIPK3 in APAP-induced liver toxicity and that GL’s protective effect against APAP toxicity was not through RIPK3/necroptosis. Conversely, these data suggest that RIPK3 up-regulation is possibly only a consequence, that RIPK3-mediated necroptosis is not a mediator of APAP-induced hepatocyte death, and that GL’s protective effect does not depend on RIPK3.
observed in GL/APAP-treated mice during the time course of the APAP challenge (Fig. 9). Analysis of mRNA encoding BCL2a1c, which is an apoptosis marker, also showed a 2- to 3-fold increase in APAP-challenged livers whereas the GL-treated groups showed a significant decrease in expression (Supplemental Fig. 7F).

The factors that initiate apoptotic hepatocyte death after APAP challenge remain largely unknown. TNFα, one of the apoptosis-inducible factors (Colell et al., 1998; Matsumaru et al., 2003), may mediate APAP-induced hepatocyte death. In vivo, APAP-inducible factors (Colell et al., 1998; Matsumaru et al., 2003), APAP-treated mice; GL + APAP, GL 100 mg/kg/APAP-treated mice. Control, saline-treated mice; GL, only GL 100 mg/kg-treated mice; APAP, saline/APAP-treated mice.


Fig. 9. GL blocks TUNEL-positive staining at the early stage after APAP challenge. TUNEL-staining analysis of paraffin-embedded livers. Original magnification, 20×.

Control  GL

2 h: APAP  2 h: GL+APAP

4 h: APAP  4 h: GL+APAP

8 h: APAP  8 h: GL+APAP

24 h: APAP  24 h: GL+APAP

Considering that the time course of TNFα release coincides well with that of the APAP toxicity, the involvement of TNFα in potentiating APAP-induced hepatocyte apoptosis needs to be considered. To validate this assumption, we tested the potentially synergistic role of TNFα with APAP toxicity in vitro. TNFα aggravated APAP-induced cytotoxicity in a dose-dependent manner in LO2 cells, although TNFα alone showed no significant effect on the cell viability (Fig. 10C). Interestingly, the pan-caspases inhibitor zVAD, but not the small necrosis inhibitor NEC-1, significantly inhibited TNFα/APAP-induced cytotoxicity (Fig. 10, C and D). The protective effect of zVAD was also observed in APAP-induced cytotoxicity (Fig. 10E). These results indicate that caspases-dependent apoptosis is a critical factor in both APAP and TNFα/APAP-induced cytotoxicity.

Furthermore, addition of GL, GA, or 18β-GA to the culture medium revealed that GL (Fig. 10D and Supplemental Fig. 3G) but not GA or 18β-GA (Supplemental Fig. 3, H and I) attenuated TNFα/APAP-induced cell death. FACS analysis further indicated that, although TNFα (20 ng/ml) alone did not induce apoptosis, TNFα/APAP markedly induced apoptotic cell death. In addition, apoptosis was potently inhibited by GL addition (Fig. 10F). These data demonstrate that GL, but not GA, attenuated APAP-induced hepatocyte damage via inhibiting the release of TNFα and its downstream caspase-dependent apoptotic signaling.

Discussion

In this study, GL, but not its active metabolite GA, was found to prevent APAP-induced liver injury via inhibiting TNFα/caspase-mediated apoptotic hepatocyte death. The hepatoprotective effect of GL was unlikely to be from the direct interference of APAP metabolic activation or through RIPK3-mediated necroptosis. Intraperitoneal or intravenous injection of GL, but not oral administration, had a potent protective effect against APAP-induced liver toxicity. Moreover, with GL as a chemical probe, this study revealed that the release of TNFα upon APAP challenge might be an important factor in APAP-induced hepatotoxicity via potentiating APAP-induced apoptotic cell death of hepatocytes.

One of the major challenges in ascertaining the hepatoprotective effects of GL is how to differentiate the role of GL and its bioactive metabolite GA. It is widely claimed that the hepatoprotective effect of GL is due to its metabolite GA; GL is rapidly metabolized to GA by the gut bacteria, so GL was previously assumed to be a prodrug. However, in the present study, evidence is provided that GL itself and not its bioactive metabolite GA contributes to the hepatoprotective effect against APAP toxicity. The transformation of GL to GA mainly happens in the intestine, so examining the differential effects of GL via different routes of administration would be helpful for differentiating the roles of GL and GA.

First, we found, unexpectedly, that intravenous or intraperitoneal injection of GL, but not intragastric administration, exerted a significantly protective effect. Second, a pharmacokinetics–pharmacodynamics correlation analysis demonstrated that GL, rather than GA, contributes to the hepatoprotective effect of GL, proving the potential application of “reverse pharmacokinetics” in the mechanistic studies of natural compounds (Hao et al., 2014). Finally, in vitro studies further revealed that GL, rather than GA, protected against APAP-induced hepatocyte damage. All these data strongly suggest that GL and not its metabolite GA possesses hepatoprotective activity against APAP-induced liver injury.

Given that GA shows a potent protective effect in liver injury caused by other toxicants, including carbon tetrachloride, bile acids, and free
fatty acids both in vivo and/or in vitro (Jeong et al., 2002; Gumpricht et al., 2005; Wu et al., 2008; Chen et al., 2013), GL and GA are considered to exert hepatoprotective effects by different mechanisms. Because formulations of GL for both intravenous and oral administration are available in the clinic (Li et al., 2014b), our data suggest that in the therapy of APAP-induced liver injury, intravenous, not oral, formulations of GL should be used.

CYP2E1-mediated activation of APAP is mainly responsible for initiating the cascade of APAP hepatotoxicity through formation of NAPQI (Lee et al., 1996). However, a single dose of GL alone did not change the CYP2E1 expression in our study and did not inhibit CYP2E1 activity in a previous study (Paolini et al., 1999). Conversely, GL significantly increased APAP-induced CYP2E1 down-regulation. The results in Cyp2e1-null mice further provide direct evidence that GL’s protective effect is not dependent on CYP2E1 activity. Moreover, GL and GA have little effect on the metabolism of APAP and the enzymes involved in APAP metabolic activation. All these data strongly support that GL prevents APAP-induced liver injury independent of CYP2E1 and not through direct inhibition of APAP metabolic activation.

The role of RIPK3/necroptosis in APAP-induced toxicity is highly controversial (Ramachandran et al., 2013; Zhang et al., 2014). Another study suggested that RIPK3 was an early mediator in APAP toxicity and that RIPK3 protein, though absent at baseline, was induced in the liver at the early phase of APAP toxicity (An et al., 2013; Ramachandran et al., 2013; Zhang et al., 2014). Ripk3-null mice were reported to be protected at 6 hours after APAP overdosing, but the protective effect was lost at 24 hours after APAP challenge (Ramachandran et al., 2013). In our study, APAP also induced RIPK3 expression, at least at the mRNA level, which partially supports a previous study (Ramachandran et al., 2013). Unexpectedly, after further testing in Ripk3-null mice, no significant difference of sensitivity to APAP was found between Ripk3-null mice and their wild-type littermates, but GL markedly attenuated APAP-induced liver injury in both genotypes, suggesting no involvement of RIPK3 in APAP toxicity.

Our data on RIPK3/APAP toxicity are in agreement with a recent study that suggested no involvement of RIPK3 in mediating APAP toxicity (Dara et al., 2015). Our present work also revealed that APAP significantly induced Mlkl mRNA, a downstream target of RIPK3, and that GL did not decrease APAP-induced Mlkl mRNA up-regulation. Therefore, although RIPK3 up-regulation was observed upon APAP toxicity, it may not be an initiating factor in APAP toxicity.
The RIPK1 inhibitor NEC-1 was shown to inhibit APAP toxicity both in vivo and in primary hepatocytes in vitro (An et al., 2013; Ramachandran et al., 2013; Takemoto et al., 2014). In LO2 cells, both GL and the pan-caspase inhibitor zVAD, but not NEC-1, prevented APAP-induced cell death, suggesting that GL may act through a caspase-dependent apoptosis pathway, rather than via RIPK1/RIPK3-dependent necroptosis, in modulating APAP-induced hepatotoxicity.

Additional studies focused on elucidating how GL protected against apoptotic cell death of hepatocytes. TUNEL-positive staining and apoptotic hepatocyte death (which can be potentiated by TNFα) in vitro suggest the possible involvement of hepatic apoptosis in APAP toxicity. The previous reports on the involvement of apoptotic cell death in the APAP model have been controversial (Bouloures et al., 2002; Matsumaru et al., 2003). It is widely believed that necrosis represents the exclusive cell death in APAP-overdosed livers with no detection of caspase activation (Jaeschke et al., 2006; Jaeschke et al., 2011). However, we found positive TUNEL staining at 2, 4, and 8 hours after APAP treatment, but the positive staining was sharply decreased at 24 hours. Because the necrotic areas were significantly increased across the time course, the transfer from apoptosis to late necrosis possibly explains the current observation (Possamai et al., 2013). Therefore, caspase-dependent apoptosis may be dominant in the early phase of APAP-induced hepatocyte death, which can be aggravated by APAP-induced release of TNFα, but may be rapidly transferred to typical necrosis or necroptosis through an unknown mechanism. Thus, GL may block caspase-dependent apoptotic cell death at the very early stage but not the latter necrotic phase of APAP toxicity.

TNF-deficient or TNF receptor type 1 (TNFR1)-deficient mice are not protected against APAP toxicity (Nagai et al., 2002; Chiu et al., 2003; James et al., 2005), indicating that TNFα/TNFγR1 signaling is probably not an important factor in mediating APAP toxicity. However, TNFα, as a pleiotropic cytokine, can antagonize liver injury by facilitating liver regeneration, especially at low concentrations, and overrelease of TNFα potentially aggravates hepatotoxicity. Thus, the results of TNF or TNFγR1 knockout mice possibly represent an integrated effect of the two roles. The role of TNF overrelease in aggravating APAP toxicity is still highly possible.

In our study, a direct role of TNFα in potentiating APAP-caused hepatocyte apoptosis in vitro was demonstrated in LO2 cells. These results agree with previous studies in primary hepatocytes (Coelle et al., 1998; Matsumaru et al., 2003; Gandhi et al., 2010). GL shows a direct effect against TNFα/APAP-induced apoptotic cell death in LO2 cells, supporting that GL has a direct antiapoptotic effect. Of interest, APAP treatment induced an increase in the release of TNFα in serum, and GL repressed APAP-triggered release of TNFα. Therefore, the prevailing evidence suggests that APAP-induced TNFα overrelease at the very early phase of toxicity can potentially enhance APAP-induced hepatocyte death. Because GL is also an immunoregulator (Honda et al., 2012; Fu et al., 2014; Kim et al., 2015; Wu et al., 2015), it seems that GL could block both APAP-triggered TNFα release and its downstream apoptotic cascade.

In conclusion, the present study provides evidence that GL rather than its metabolite GA contributes to GL’s protective effect in APAP-induced toxicity. GL’s protective effect is independent of CYP2E1 and not through interference with APAP metabolic activation or RIPK3-mediated necroptosis. APAP-induced caspases-dependent apoptosis can be potentiated by TNFα and inhibited by GL. GL can suppress the APAP-induced inflammatory response and decrease TNFα release. Collectively, GL, not GA, potently prevents APAP toxicity through TNFα/caspase-mediated apoptotic cell death. Translationally, our study suggests that GL via intravenous injection but not oral intake is a potential option for clinical application. However, it is important to note that when GL was intravenously injected at 2 hours after APAP overdosing, when the liver was severely damaged, no significant hepatoprotective effect was observed in our study, although both multiple injections of GL and a single injection of GL showed a potent protective effect as a positive control (Supplemental Fig. 8, A and B). This suggests that GL alone may have limited clinical efficacy for combating APAP-induced liver injury. Farnesoid X receptor agonists could combat APAP toxicity via the promotion of liver regeneration, which is a latter phase of APAP toxicity (Xie et al., 2016). It would be of interest to determine whether the timely administration of GL in the early phase and farnesoid X receptor agonists in the latter phase would confer a synergy in combating APAP toxicity.

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Authorship Contributions

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References


