Glycyrrhizin Alleviates Nonalcoholic Steatohepatitis via Modulating Bile Acids and Meta-Inflammation

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ABSTRACT

Nonalcoholic steatohepatitis (NASH) is the progressive stage of nonalcoholic fatty liver disease that may ultimately lead to cirrhosis and liver cancer, and there are few therapeutic options for its treatment. Glycyrrhizin (GL), extracted from the traditional Chinese medicine liquorice, has potent hepatoprotective effects in both preclinical animal models and in humans. However, little is currently known about its effects and mechanisms in treating NASH. To explore the effects of GL on NASH, GL or its active metabolite glycyrrhetinic acid (GA) was administered to mice treated with a methionine- and choline-deficient (MCD) diet-induced NASH model, and histologic and biochemical analyses were used to measure the degree of lipid disruption, liver inflammation, and fibrosis. GL significantly improved MCD diet-induced hepatic steatosis, inflammation, and fibrosis and inhibited activation of the NLR family pyrin domain-containing 3 (NLRP3) inflammasome. GL significantly attenuated serum bile acid accumulation in MCD diet-fed mice partially by restoring inflammation-mediated hepatic farnesoid X receptor inhibition. In Raw 264.7 macrophage cells, both GL and GA inhibited deoxycholic acid-induced NLRP3 inflammasome-associated inflammation. Notably, both intraperitoneal injection of GL’s active metabolite GA and oral administration of GL prevented NASH in mice, indicating that GL may attenuate NASH via its active metabolite GA. These results reveal that GL, via restoration of bile acid homeostasis and inhibition of inflammatory injury, can be a therapeutic option for treatment of NASH.

Introduction

Nonalcoholic fatty liver disease (NAFLD) affects 25% of the global adult population and has become a leading cause of chronic liver disease in Western countries and increasingly affects the Asian population (Younossi et al., 2016). Early-stage NAFLD is asymptomatic; however, when NAFLD progresses to nonalcoholic steatohepatitis (NASH), patients have a high risk of adverse events including fibrosis, cirrhosis, and NASH-driven hepatocellular carcinoma (Wong et al., 2014, 2016; Zoller and Tilg, 2016). Traditional Chinese medicine has been investigated for treating NASH (Jadeja et al., 2014; Zhang and Schuppan, 2014). Glycyrrhizin (GL) is a potent hepatoprotective constituent extracted from the traditional Chinese medicine liquorice, as revealed by various animal experimental liver injury models (Li et al., 2014; Yan et al., 2016), and thus is clinically used for treating chronic liver diseases in some Asian countries (Li et al., 2014). Clinical prescription preference for GL is found mainly for treating chronic liver diseases, especially for viral hepatitis in China and Japan (Li et al., 2014), whereas preclinical studies of GL in treating liver diseases have mostly focused on viral hepatitis and drug/toxin-induced hepatotoxicity (Li et al., 2014; Yan et al., 2016; Zhou et al., 2016). However, the effect of GL in preventing NASH is largely unknown. Thus far, only glycyrrhetinic acid (GA), the metabolite of GL in vivo, has been shown to prevent high-fat diet-induced NAFLD in rats in vivo and free fatty acid–induced toxicity in HepG2 cells in vitro (Wu et al., 2008). Whether and how GL and GA affect NASH (the progressive stage of NAFLD) is still unknown.

Bile acid homeostasis, which is mainly modulated by the farnesoid X receptor (FXR), is disrupted in experimental NASH mouse models and in clinical human NASH patients (Arab et al., 2017). Dysregulated bile acid disruption correlates with nonalcoholic steatosis scores in obese NAFLD (Bechmann et al., 2013), causes cholestatic liver injury (Schoemaker et al., 2003), and promotes liver carcinogenesis in a NASH-driven hepatocellular carcinoma model in mice (Xie et al., 2016). Furthermore, a bile acid sequestant was found to prevent NAFLD and NASH (Arab et al., 2017), suggesting that suppressing the accumulation of bile acids during NASH could benefit NASH treatment. On the other hand, the NLR family pyrin domain-containing 3 (NLRP3) inflammasome and inhibition of inflammatory injury, can be a therapeutic option for treatment of NASH.
Materials and Methods

GL was demonstrated to inhibit lathionic acid–induced cholestatic liver injury (Wang et al., 2012), bile acid–induced cytotoxicity in rat hepatocytes (Gumprecht et al., 2005), and α-naphthyl isothiocyanate–induced liver injury and bile acid disruption (Wang et al., 2017), indicating a potential role for GL in treating cholestatic liver injury. In addition, GL is also an inhibitor of NLRP3 inflammasome activation in macrophages in vitro and in NLRP3 inflammasome–associated adipose tissue inflammation in mice (Honda et al., 2014). Thus, GL might target the bile acid–NLRP3 inflammasome to elicit its hepatoprotective function for decreasing NASH.

In this study, the methionine- and choline-deficient (MCD) diet–induced NASH mouse model was used to examine the hepatoprotective effect and potential mechanisms of GL. GL was found to potently inhibit MCD diet–induced liver lipid accumulation, inflammation, and fibrosis. GL dampened activation of the NLRP3 inflammasome and restored bile acid homeostasis. Moreover, GL also directly inhibited deoxycholic acid–induced NLRP3 inflammasome activation. GL gavage as well as GA significantly prevented MCD diet–induced liver injury, suggesting that GL by oral intake might be applied in the clinic as a therapeutic option for NASH patients.
one-way analysis of variance followed by the Dunnett multiple-comparisons test in GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA) as stated. 

Results

GL Attenuates NASH-Induced Liver Injury. To determine whether GL attenuates MCD diet-induced liver injury in vivo, C57BL/6 mice were treated with GL after MCD diet feeding for 6 weeks, and the GL treatment continued for the last 2 weeks while the mice were fed the matched MCD diet or the MCS diet during the injection (Fig. 1A). GL treatment did not significantly affect food intake and body weight (Supplemental Fig. 1, A and B) or the liver index (Supplemental Fig. 1C) in both MCS and MCD diet-fed mice. Hematoxylin and eosin staining data showed that 50 mg/kg GL sharply decreased hepatic steatosis in MCD diet-fed mice, whereas 50 mg/kg GL treatment alone did not cause liver toxicity (Fig. 1B). Apoptotic hepatocyte death is known to be involved in NASH (Feldstein et al., 2003); therefore, liver tissues were analyzed by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling staining to measure apoptosis. GL treatment significantly reduced the positive staining compared with control saline treatment in MCD diet-fed mice (Fig. 1C). Consistently, the apoptosis marker gene mRNA, B cell leukemia/lymphoma 2 related protein A1c (Bcl2a1c) Bcl2a1c, was also significantly inhibited by GL treatment in MCD diet-fed mice (Supplemental Fig. 1D). Since TNF-α induces apoptosis (Wang et al., 1996) and ACTD/TNF-α is frequently used to mimic apoptotic cell death (Leist et al., 1994), the effects of GL in alleviating ACTD/TNF-α–induced apoptosis were examined in HepG2 cells. GL directly inhibited an ACTD/TNF-α–induced decrease in cell viability at both 13 and 24 hours after ACTD/TNF-α treatment (Supplemental Fig. 1, E and F) and ameliorated the increase in apoptotic cell death as assessed by the FACS assay (Supplemental Fig. 1G), supporting that GL could directly inhibit TNF-α–mediated apoptosis. In addition, serum ALT levels were increased in the MCD diet-fed group but were markedly decreased in the MCD+GL50-treated group (Fig. 1D). NAFLD scores among the MCS, MCD, and MCD+GL50 groups by a double-blinded analysis also showed that the MCD diet induced a significant increase in NAFLD activity, steatosis, and inflammation in livers of the MCD group, which were all markedly alleviated by GL treatment (Fig. 1, E–G). Taken together, these results suggest that GL rescues apoptotic liver injury induced by MCD diet feeding, at least partially through directly inhibition of TNF-α–induced hepatocyte apoptosis.

GL Improves NASH-Related Liver Fibrosis. Liver fibrosis is a hallmark of NAFLD progression to NASH. Masson trichrome staining of collagen was used to measure liver fibrosis, and GL was found to significantly ameliorate NASH-associated collagen deposition induced by MCD diet feeding (Fig. 2A). α-Smooth muscle actin is another
GL significantly dampens MCD-induced liver fibrogenesis.  

(A and B) Masson trichrome staining (A) and immunohistochemistry staining of α-SMA (B) for paraffin-embedded livers.  

(C–F) Levels of αSma (C), Tgfb1 (D), Timp1 (E), and Timp2 (F) mRNAs in the liver.  

(G–J) Levels of Cola1 (G), Cola2 (H), Mmp2 (I), and Mmp9 (J) mRNAs in the liver. Data are presented as means ± S.D. Statistical differences between experimental groups were determined by the two-tailed t test (n = 6–8 in each group). *P < 0.05; **P < 0.01; ***P < 0.001 vs. the MCD group; ##P < 0.01; ###P < 0.001 vs. the MCS group. Colla, collagen; Mmp, matrix metalloproteinase; Sma, smooth muscle actin; Tgfb1, transforming growth factor β-1; Timp, tissue inhibitor of metalloproteinases. Original magnification, ×20. Scale bar, 50 μm.
sensitive marker of liver fibrosis. MCD diet-induced upregulation of α-smooth muscle actin expression was significantly decreased by GL treatment at the protein level as determined by both immunohis- tochemical staining (Fig. 2B) and mRNA analysis (Fig. 2C). GL also significantly dampened the expression of fibrogenetic gene mRNAs including transforming growth factor \( b \)-1, tissue inhibitor of metalloproteinases 1 and 2, collagen 1 and 2, and matrix metalloproteinases 2 and 9 (Fig. 2, D–J). These data demonstrate that GL significantly improves NASH-related liver fibrogenesis.

**GL Restores NASH-Related Hepatic Lipid Accumulation.** In NASH, lipotoxicity caused by accumulating lipids in the liver is a major trigger of liver toxicity (Fuchs and Sanyal, 2012). Therefore, the effect of GL in MCD diet-induced lipid disruption was determined. Compared with the control vehicle-treated MCD group, intraperitoneal injection of 50 mg/kg GL significantly attenuated MCD diet-induced lipid accumulation in the liver, as revealed by Oil Red O staining (Fig. 3A). Compared with the MCS group, liver TG and TC levels were both significantly increased in the MCD group, and both were significantly decreased by GL treatment (Fig. 3, B and C). In contrast, serum TG and TC levels were significantly decreased after MCD diet feeding, both of which were also normalized by GL treatment (Fig. 3, D and E). Similarly, GL treatment also normalized MCD diet-induced disruption of serum high-density lipoprotein cholesterol and low-density lipoprotein cholesterol levels (Fig. 3, F and G). These data strongly suggest that GL treatment could improve MCD diet-induced lipid disruption in both the liver and serum.

Systematic lipid homeostasis is maintained by balanced mutual regulation of de novo lipid synthesis and lipid degradation by oxidation and subsequent ketogenesis (Tessari et al., 2009). To examine how GL restores MCD diet-induced dyslipidemia, lipid regulatory pathways were analyzed. Compared with MCS diet-fed mice, MCD diet feeding significantly increased expression of the liver X receptor and sterol regulatory element-binding transcription factor Srebp1 mRNA, and the downstream lipid synthesis gene mRNA, *Fas*. Although GL treatment decreased the induction of liver X receptor, Srebp1a, and Srebp1c mRNA, it showed no significant effect on the mRNA level of *Fas* and significantly restored MCD diet-decreased stearoyl-CoA desaturase 1 mRNA, both of which are encoded by two key lipogenic genes.
GL treatment was inferred to influence the de novo synthesis of bile acids in the liver. Therefore, FXR-SHP-CYP7A1 signaling, the predominant pathway for bile acid synthesis, was analyzed. Hepatic FXR signaling was inhibited and Cyp7a1 mRNA was upregulated in MCD diet-fed mice compared with MCD diet-fed mice. As expected, GL treatment significantly activated FXR, evidenced by upregulation of Shp mRNA and the downstream suppression of Cyp7a1 mRNA, whereas Fxr mRNA itself was also significantly increased by GL treatment compared with the vehicle-treated group in the mouse NASH model (Fig. 4, C–E). In contrast, bile salt export pump mRNA was not significantly influenced by GL treatment (Fig. 4F), suggesting a role of GL in inhibiting de novo bile acid synthesis.

To explain how GL could activate FXR in MCD diet-fed livers, the possibility that GL could act as a FXR ligand was explored. In mouse primary hepatocytes and human HepG2 cells in vitro, no significant FXR activation was induced by either GL nor GA treatment (Supplemental Fig. 2, A and B). In mice in vivo, both single GL injection and multiple GL injection failed to activate FXR (Supplemental Fig. 2C). Consistently, both GL and GA failed to activate FXR, whereas GW4064 (3-(2,6-Dichlorophenyl)-4-(3′-carboxy-2-chlorostilben-4-yl) oxymethyl-5-isopropylisoxazole) induced FXR reporter luciferase by 20-fold as a positive control in HepG2 cells (Supplemental Fig. 2D, E). In contrast, bile salt export pump mRNA was not significantly influenced by GL treatment (Fig. 4F), suggesting a role of GL in inhibiting de novo bile acid synthesis. However, it is likely that GL activates liver FXR/SHP/CYP7A1 signaling and rescues MCD-induced bile acid disruption. (A) Serum level of free bile acids α-MCA, β-MCA, CA, DCA, UDCA, and HDCA. (B) Serum level of conjugated bile acids T-β-MCA, T-CA, T-DCA, T-CDCA, T-UDCA, T-HDCA, and G-CA. (C-F) Hepatic mRNA levels of Fxr, Shp, Cyp7a1, and Bsep. Data are presented as means ± S.D. Statistical differences between experimental groups were determined by the two-tailed t test (n = 6–8 in each group). *p < 0.05; **p < 0.01; ***p < 0.001 vs. the MCD group; #p < 0.05; ##p < 0.01; ###p < 0.001 vs. the MCS group, Bsep, bile salt export pump; CDCA, chenodeoxycholic acid; G-CA, glyco- cholic acid; HDCA, hyodeoxycholic acid; UDCA, ursodeoxycholic acid.

**Fig. 4.** GL activates liver FXR/SHP/CYP7A1 signaling and rescues MCD-induced bile acid disruption. (A) Serum level of free bile acids α-MCA, β-MCA, CA, DCA, UDCA, and HDCA. (B) Serum level of conjugated bile acids T-β-MCA, T-CA, T-DCA, T-CDCA, T-UDCA, T-HDCA, and G-CA. (C-F) Hepatic mRNA levels of Fxr (C), Shp (D), Cyp7a1 (E), and Bsep (F). Data are presented as means ± S.D. Statistical differences between experimental groups were determined by the two-tailed t test (n = 6–8 in each group). *p < 0.05; **p < 0.01; ***p < 0.001 vs. the MCD group; #p < 0.05; ##p < 0.01; ###p < 0.001 vs. the MCS group, Bsep, bile salt export pump; CDCA, chenodeoxycholic acid; G-CA, glyco- cholic acid; HDCA, hyodeoxycholic acid; UDCA, ursodeoxycholic acid.
damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns to receptors such as Toll-like receptor (TLR) to increase the transcription of NLRP3 and pro–IL-1β, followed by an activation step (signal 2) induced by the recruitment of apoptosis-associated speck-like protein containing a caspase recruitment domain to activate the NLRP3 inflammasome to convert pro-CASP1 into active CASP1, which in turn processes pro–IL-1β into mature IL-1β (Guo et al., 2015). Since GL is a known inhibitor of the NLRP3 inflammasome (Honda et al., 2014), we tested whether GL inhibited activation of the NLRP3 inflammasome in the NASH model. Compared with MCS diet-fed mice, MCD diet feeding induced a significant 2- to 4-fold increase in upstream Tlr4, Tlr9, and Myd88 mRNAs, all of which were normalized by GL treatment (Fig. 5, A–C). Similarly, the MCD diet induced Casp1, Nlrp3, and apoptosis-associated speck-like protein containing a caspase recruitment domain mRNAs by 5.5-, 4.8-, and 7.7-fold, respectively, which were significantly reduced by GL treatment (Fig. 5, D–F). These data suggest that GL could dampen the signal 1 activation of NLRP3 inflammasome in MCD diet-fed mice. Western blot analysis was performed to test the protein levels of pro-CASP1, active CASP1, pro–IL-1β, and active IL-1β. The MCD diet induced upregulation of signals 1 and 2 compared with MCS diet treatment. GL treatment decreased the protein expression of pro-CASP1 and pro–IL-1β (Fig. 5G), confirming GL’s effect in inhibiting signal 1 of NLRP3 inflammasome activation. Furthermore, GL treatment was also found to inhibit the MCD diet-induced upregulation of active CASP1 (p20) and cleaved IL-1β (Fig. 5G). In addition, GL significantly inhibited the upregulation of downstream proinflammatory cytokine mRNAs Tnfa, Il6, and Il1b (Fig. 5, H–J) and serum release of IL-1β protein (Fig. 5K), demonstrating a significant improvement in NASH-related meta-inflammation by GL. These data demonstrate that GL treatment could dampen MCD diet-induced TLR/NLRP3 inflammasome activation and the related meta-inflammation.

Since previous reports and our results indicate that bile acids accumulate in NASH and may be the main trigger for activation of the NLRP3 inflammasome (Arab et al., 2017; Hao et al., 2017), we explored the possibility of whether GL could directly inhibit bile acid–induced NLRP3 inflammasome activation. DCA was shown to induce NLRP3 inflammasome activation and related meta-inflammation (Arab et al., 2017; Hao et al., 2017). GL treatment significantly reduced DCA-induced NLRP3 inflammasome activation and related meta-inflammation (Fig. 5, A–J).
activation in vitro, which at least partially explains GL mRNAs (Fig. 6). These data demonstrate that GL and its metabolite Il1b significantly decreased DCA-induced upregulation of inflammasome activation. In Raw 264.7 cells, both GL and GA decreased the MCD diet-induced increase in ALT and AST levels (Fig. 7, B and C). Similarly, GL gavage at a dose of 50 mg/kg also significantly ameliorated the MCD diet-induced increase in serum ALT and AST levels (Fig. 7, D and E). Histologic analysis showed that 30 mg/kg GA ameliorated MCD diet-induced hepatic steatosis (Fig. 7, F and G). Similarly, GL gavage at a dose of 50 mg/kg improved liver lipid overloading (Fig. 7, H and I). These results indicate that GA may be required for the hepatoprotective effects of GL for the treatment of NASH.

Discussion

GL and its active metabolite GA have been demonstrated to be hepatoprotective; however, they have not yet been examined for their activities toward NASH. This study shows that GL, via its active metabolite GA, could decrease MCD diet-induced NASH, as revealed by improved hepatic steatosis, inflammation, and fibrosis. Mechanistically, GL may restore NASH-induced dysregulation of bile acids and lipids and the resultant meta-inflammation.

Many factors such as cholesterol crystal (Duewell et al., 2010), ceramides (Chaurasia and Summers, 2015), and secondary bile acids (Hao et al., 2017) are known to accumulate in NASH livers and could act as DAMPs to induce NLRP3 inflammasome activation. Activation of the NLRP3 inflammasome results in the release of IL-1β, together with other proinflammatory cytokines such as TNFα, which triggers an inflammatory cascade that ultimately promotes pathologic development of NASH. Thus, accumulated DAMPs, via activation of the NLRP3 inflammasome and other inflammatory pathways, may represent core pathologic triggers in NASH development. In our study using the MCD diet-induced NASH model, GL was shown to inhibit NLRP3 inflammasome activation accompanied by restoring abnormal serum bile acid accumulation, and GL could normalize the dysregulated lipids and bile acids and thereby reduce meta-inflammation. Since several bile acids are known to activate NLRP3/inflammasome signaling, GL may inhibit the NLRP3 inflammasome via the regulation of FXR signaling, GA for 30 minutes and then treated with 200 μM DCA for an additional 4 hours to further perform mRNA analysis. *P < 0.05; **P < 0.01; ***P < 0.001 vs. the DCA group; #P < 0.05; ##P < 0.01; ###P < 0.001 vs. DMSO group. DMSO, dimethylsulfoxide.

Fig. 6. Both GL and GA significantly inhibit DCA-induced Tnfa, Nlrp3, and Il1b mRNA. (A–C) Effect of GL in DCA-induced mRNA of Tnfa (A), Nlrp3 (B), and Il1b (C) mRNAs in Raw 264.7 cells. (D–F) Effect of GA in DCA-induced Tnfa (D), Nlrp3 (E), and Il1b (F) mRNAs in Raw 264.7 cells. Data are presented as means ± S.D. Statistical differences were determined by one-way analysis of variance followed by the Dunnett multiple-comparisons test among multiple-group comparisons (n = 3 per group). Raw 264.7 cells were pretreated with 0.1% DMSO, GL, or GA for 30 minutes and then treated with 200 μM DCA for an additional 4 hours to further perform mRNA analysis. *P < 0.05; **P < 0.01; ***P < 0.001 vs. the DCA group; #P < 0.05; ##P < 0.01; ###P < 0.001 vs. DMSO group. DMSO, dimethylsulfoxide.

inflammasome activation (Hao et al., 2017) and was thus used as a model to test GL’s effect in inhibiting bile acid–induced NLRP3 inflammasome activation. In Raw 264.7 cells, both GL and GA significantly decreased DCA-induced upregulation of Tnfa, Nlrp3, and Il1b mRNAs (Fig. 6). These data demonstrate that GL and its metabolite GA could directly suppress DCA-induced NLRP3 inflammasome activation in vitro, which at least partially explains GL’s role in dampening MCD diet-induced NLRP3 inflammasome activation in vivo.

GA, the Active Metabolite of GL, Improves MCD Diet-Induced Liver Injury. Structurally, GL is a glycoside that is hydrolyzed into its metabolite GA by intestinal bacteria after oral administration (Akao et al., 1994; Takeeda et al., 1996). To test whether GA influences NASH, mice were treated with the MCD diet for 6 weeks and then either intraperitoneally injected with GA (30 mg/kg) or intragastrically administered GL by gavage (50 mg/kg) for the last 3 weeks (Fig. 7A). GA as well as GL gavage did not significantly change the body weight, liver weight, and liver index (Supplemental Fig. 5). GA significantly decreased the MCD diet-induced increase in ALT and AST levels (Fig. 7, B and C). Similarly, GL gavage at a dose of 50 mg/kg also significantly ameliorated the MCD diet-induced increase in serum ALT and AST levels (Fig. 7, D and E). Histologic analysis showed that 30 mg/kg GA ameliorated MCD diet-induced hepatic steatosis (Fig. 7, F and G). Similarly, GL gavage at a dose of 50 mg/kg improved liver lipid overloading (Fig. 7, H and I). These results indicate that GA may be required for the hepatoprotective effects of GL for the treatment of NASH.

In addition to restoring lipid and bile acid homeostasis, GL could also directly inhibit DCA-induced NLRP3 inflammasome activation, suggesting that it may target multiple nodes in treating NASH. In line with our findings, GL was previously shown to inhibit NLRP3 inflammasome activation induced by diverse DAMPs or pathogen-associated molecular patterns in bone marrow–derived macrophages (Honda et al., 2014). MCC950 (N-[(1,2,3,5,6,7-hexahydro-s-indacen-4-yl)amino] carbonyl]-4-(1-hydroxy-1-methylethyl)-2-furansulfonamide sodium salt), an NLRP3 selective inhibitor, was recently demonstrated to improve NAFLD pathology and fibrosis (Mridha et al., 2017), suggesting that the NLRP3 inflammasome might be a potential target for NASH therapy. Moreover, it was found that MCD
diet-induced steatosis sensitized cholestatic liver injury and dysregulated bile acid synthesis and transport (Lionarons et al., 2016). Our results, together with these previous findings, strongly indicate that dysregulated metabolism of lipids and bile acids and the resultant meta-inflammation is a pivotal event in NASH development.

GL administered by the oral route shows extremely low bioavailability (approximately 1%) and most GL by oral intake is hydrolyzed into GA in the gut by intestinal bacteria, which is then absorbed and elicits its pharmacological effects (Nose et al., 1994; Yamamura et al., 1995; Takeda et al., 1996). In line with this point, GL gavage and direct GA intraperitoneal injection could both combat experimental NASH, suggesting that the effect of GL against MCD diet-induced NASH is largely through its primary metabolite GA. In contrast, GL (but not GA) contributes to the hepatoprotective effect of GL in ameliorating acataminophen-induced liver injury (Yan et al., 2016). Thus, it is important to note that although both GL and GA have efficacy in various types of hepatic injury, the exact effects and thereby precise clinical applications of these agents need to be carefully considered.

In summary, the herbal medicine-derived compound GL may be a therapeutic option for treating NASH, particularly that harbors the symptom of cholestasis. Mechanistically, GL and/or GA may target bile acid–mediated meta-inflammation and block the reciprocal FXR-NLRP3 inflammasome pathway. All of the data obtained from our MCD diet-induced NASH model demonstrate that GL and GA may be potentially effective against NASH. However, it is important to note that the MCD diet-induced NASH model, despite the presented liver steatosis and inflammation, does not show obesity or insulin resistance or mimic the aberrant increase of serum lipids, all of which are common pathophysiological characteristics of human NASH (Hebbard and George, 2011).

Thus, studies that use a long-term high-fat and/or high-sugar diet feeding model would help further confirm the translational potential of GL and GA in combating metabolic syndrome–related NASH.

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Authorship Contributions

Participated in research design: Yan, H. Wang, G. Wang, Gonzalez, Hao.
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References

Supplemental Data

Title: Glycyrrhizin alleviates non-alcoholic steatohepatitis via modulating bile acids and meta-inflammation

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Supplemental Figure 1. GL decreases MCD-induced apoptotic signaling activation, while shows no significant effect in MCD-induced body weight loss, food intake, and liver index. (A), Body weight ratio, the ratio of body weight at the end of experiment to body weight before MCD diet feeding. (B), Average food intake record for the first week after GL injection, all mice were single-caged and food intake was recorded every day for 7 days. (C), Liver index, ratio of liver weight to body weight. (D), Levels of liver apoptotic signaling, Bcl2a1c mRNAs. (E and F), GL treatment alleviates ACTD/TNFα-induced HepG2 cell death at 13 h (E) and 24 h (F) after TNFα challenge by CKK-8 kit. (G), GL treatment alleviates ACTD/TNFα-induced HepG2 apoptosis by FACS assay at 13 h after TNFα challenge. Data are presented as means ± SD. Statistic
differences determined by one-way ANOVA followed by Dunnett’s multiple comparisons test among multiple-group comparisons. For mouse experiments, n=6-8 in each group. Bcl2a1c, B-cell leukemia/lymphoma 2 related protein a1c. MCS, MCS diet-fed mice treated with saline; MCD, MCD diet-fed mice treated with saline; MCD+GL50, MCD diet-fed mice treated with 50 mg/kg of GL. ###p<0.001 versus MCS group. *p<0.05 and **p<0.01 versus MCD group. For cell culture experiments, HepG2 cells were pretreated with 0.1% DMSO or various concentration of GL for 30 minutes in the presence or absence of 0.3 μM of ACTD, and then 30 ng/mL of recombinant human TNFα were added to induce cell death. CCK-8 kit assay or FACS assay was performed at 13 h or 24 h after TNFα treatment to test the cell viability. n=6-12 for F and G, and n=3 for H. ###p<0.001 versus control group. ***p<0.001 versus 0.1% DMSO group.
Supplemental Figure 2. Effect of GL in FXR activation in vivo and in vitro. (A-C), Effect of GL in the mRNA expression of FXR target genes in primary mouse hepatocytes (A), HepG2 cells (B), and in mice (C). Primary hepatocytes isolated from 8-week old male mice or HepG2 cells were treated with 50 μM of GL, 10 μM of GA or control vehicle 0.1% DMSO (n=3-6 per group) for 24 h in 10% DMEM culture medium; mice were treated with control vehicle (Vehicle group), 50 mg/kg of GL for a single injection (Single GL50 group) or 7 consecutive days (Multiple GL50 group), and then livers were collected for mRNA analysis at the end of experiments, n=5 mice in each group. (D), Effect of 200 μM of GL and 15 μM of GA in LPS-induced FXR inactivation in HepG2 cells. (E), Effect of 50 μM or 200 μM of GL, 5 μM or 15 μM of GA, 4 μM of GW4064 in the FXR luciferase reporter activity; HepG2 cells in 10% DMEM culture medium (n=3 per group). Data are presented as means ± SD. Statistic differences determined by one-way ANOVA followed by Dunnett’s multiple comparisons test among multiple-group comparisons. #p<0.05; ##p<0.01 and ###p<0.001 versus control vehicle-treated group. *p<0.05 versus LPS-treated group.
Supplemental Figure 3. GA as well as GL gavage shows no significant effect in body weight, liver weight, and liver index. (A), Body weight ratio, the ratio of body weight at the end of experiment to body weight at Day 0 of the experiment. (B), Liver weight. (C), Liver index, ratio of liver weight to body weight. (D), Body weight ratio, the ratio of body weight at the end of experiment to body weight at Day 0 of the experiment. (E), Liver weight. (F), Liver index, ratio of liver weight to body weight. Data are presented as means ± SD. Statistic differences determined by one-way ANOVA followed by Dunnett’s multiple comparisons test among multiple-group comparisons. #p<0.05; ##p<0.01 and ###p<0.001 versus MCS group. n=5 in each group. MCS, MCS diet-fed mice treated with saline; MCD, MCD diet-fed mice treated with saline;
MCD+GL50(P.O.), MCD diet-fed mice treated with 50 mg/kg of GL by gavage. MCD+GA30, MCD diet-fed mice treated with 30 mg/kg of GA.