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Effects of glycyrrhizin on biliary transport and hepatic levels of glutathione in rats

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ABSTRACT: The purpose of the current study was to determine whether glycyrrhizin (GL) maintains hepatic glutathione (GSH) levels by inhibiting GSH biliary secretion in normal rats. The effects of glycyrrhizin on hepatic glutathione content, bile flow and biliary secretion of glutathione were examined. Because glutathione is a substrate for multidrug resistance associated protein-2 (Mrp2/ABCC2), the inhibitory effects of GL on Mrp2 in isolated perfused rat liver and in Mrp2-expressing Sf9 membrane vesicles were also examined using the Mrp2 substrate methotrexate (MTX) and estradiol-17-β-glucuronide ($E_2$17G). The hepatic content of glutathione in rats following GL perfusion (43.7 µmol/l) in isolated liver perfusion and GL intravenous treatment (25 mg/kg) was significantly higher than that for the control. A marked and dose-dependent decrease in the excretion of glutathione was observed. In addition, the secretion rate of MTX was decreased by 57% in isolated liver perfusion in GL-treated rats. Moreover the ATP-dependent uptake of $E_2$17G by Mrp2 membrane vesicles was decreased by 75.9% in the 20 µM GL group and by 60.5% in the 2 µM GL group. In conclusion, glycyrrhizin increases hepatic glutathione content possibly through inhibition of Mrp2 which then reduces the biliary excretion of glutathione. Copyright © 2012 John Wiley & Sons, Ltd.

Key words: glycyrrhizin; glutathione; multidrug resistance associated transporters; isolated liver perfusion; membrane vesicles

Introduction

Infection with the hepatitis C virus (HCV) is a worldwide problem. Infection by HCV can result in chronic hepatitis, and then the chronic condition may lead to cirrhosis which is the leading indication for liver transplantation [1]. Licorice has been used as a traditional herbal remedy to treat chronic hepatitis and gastric ulcer and to enhance the actions of a prescribed therapy [2]. Glycyrrhizin (GL) has similar pharmacological effects to licorice and has been used for the treatment of chronic hepatitis for many years with considerable therapeutic effect [3]. It has been reported that glycyrrhizin has protective effects on the hepatic cellular membrane [4], but the mechanism by which glycyrrhizin prevents chronic hepatitis is not fully understood.

Recent studies support the view that oxidative stress and a defective host antiviral immune response, in addition to viral factors, play major roles in the progression of chronic hepatitis C [5,6]. Glutathione is the major endogenous soluble antioxidant in mammalian cells. It protects cells

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against oxidative stress either through serving as a substrate for the antioxidant enzymes that convert peroxides into less harmful substances or as a free radical scavenger via non-enzymic mechanisms [7]. Several studies have shown that glycyrrhizin can affect the hepatic glutathione content in animal models of liver diseases. Nagai *et al*. [8] reported that glycyrrhizin can increase the hepatic glutathione content which was impaired by ischemia–reperfusion and provides partial protection against ischemia–reperfusion damage. A recent experiment [9] also showed that GL attenuated the decrease of glutathione concentration in liver induced by carbon tetrachloride (CCl_4) and protected the acute liver injury in mice. However, the mechanisms by which GL increases hepatic glutathione content remain elusive.

It was reported that more than 80% of glycyrrhizin is excreted in bile [10]. Many transporters are involved in biliary secretion and drug excretion. Mrp2 is the efflux transporter located at the canalicular membrane of a hepatocyte, and translocates glutathione, LTC4, bilirubin, methotrexate (MTX), glucuronide (e.g. estradiol-17-β-glucuronide ($E_2$17G)) or sulfate conjugates and other organic anions from a hepatocyte into the bile canaliculus [11–15]. Shimamura *et al*. [16] investigated the biliary excretion of glycyrrhizin in Eisai hyperbilirubinemic rats (EHBR) and Sprague–Dawley rats dosed with Mrp2 inhibitors and their findings suggested that glycyrrhizin was a substrate of Mrp2. Therefore, glycyrrhizin may share the same transporter with glutathione which is the typical substrate for Mrp2. In addition, glycyrrhizin was found to inhibit the biliary excretion of several other Mrp2 substrates such as SN-38 and SN-38-Glu in rats *in vivo* [17]. The evidence suggests that glycyrrhizin may act as both a substrate and an inhibitor for Mrp2.

Given that both glycyrrhizin and glutathione are substrates for Mrp2, and glycyrrhizin also inhibits Mrp2, glycyrrhizin may affect the biliary excretion of glutathione. Hence the reason for the increase in hepatic total glutathione when glycyrrhizin is administered may be due to inhibition of the transport of glutathione from hepatocytes into bile. The aim of this study was to investigate whether glycyrrhizin can alter the biliary transport and hepatic levels of glutathione *in vivo* and *in vitro*.

Materials and Methods

Instruments, reagents and animals

A high-performance liquid chromatography (HPLC) system (Model LC-2010 C) was supplied by Shimadzu and consisted of a four-component gradient pump, an on-line degasification device, an auto-sampler, a UV detector and a column oven. A high-speed centrifuge (Model 1612 –1) was supplied by Surgical Instrument Factory, Shanghai Medical Apparatus Company. A thermostat (Model 501 super) was supplied by Shanghai Equipment Factory Co. Ltd. A peristaltic pump (Model Lead-1) was purchased from Baoding Lange Peristaltic Pump Co. Ltd. A microinfusion pump (Model WZS-50) was supplied by Medical Instrument Factory, Zhejiang Medical University. An automatic biochemistry analyser (AU 2700) was supplied by Olympus. An automatic ELISA analyser was supplied by Alisei.

Diammonium glycyrrhizinate (purity > 97%) was a gift from Chia-Tai Tianqing Pharmaceutical Co. Ltd (Lianyungang, China). Methotrexate (MTX) (purity > 98%), indomethacin (purity > 98%) and cefetamet acid (purity > 97%) were kindly provided by Zhejiang Hisun Pharmaceutical Co. Ltd (Taizhou, China), Zhejiang Hailisheng Pharmaceutical Co. Ltd (Zhoushan, China) and Zhejiang Yongning Pharmaceutical Co. Ltd (Taizhou, China), respectively. Glutathione, bile acids and bilirubin assay kits were purchased from Beyotime Institute of Biotechnology (Haimen, China), Sichuan Maker Science and Technology Co. Ltd (Chengdu, China) and Beijing Labo Biotech. Co. Ltd (Beijing, China), respectively. Estradiol-17β-D-glucuronide was purchased from Sigma-Aldrich. Adenosine triphosphate disodium salt (ATP) and adenosine monophosphate disodium salt (AMP) were obtained from Oriental Yeast (Tokyo, Japan). Rat Mrp2-expressing Sf9 membrane vesicles were obtained from GenoMembrane, Inc. (Kanagawa, Japan). All other chemicals were commercially available and of analytical grade.

Male Sprague–Dawley rats weighing 200–320 g were obtained from Zhejiang Laboratory Animal Center. The rats were housed in a 12 h light/dark cycle with free access to standard chow and water *ad libitum*. All animal experiments were approved by the Animal Ethics Committee of China Pharmaceutical University, and performed under a license granted by Jiangsu Science and

Technology Office (China). Every effort was made to minimize stress to the animals.

Liver perfusion

Effect of glycyrrhizin on hepatic glutathione content. The perfused livers were divided into GL and control groups ($n = 5$ for each group). The surgical procedure was described previously [10]. Briefly, the rat was anesthetized with urethane (1. 2 g/kg, i.p.). A midline incision on the abdominal wall was made. The common bile duct was cannulated with a polyethylene tubing (PE-10) for bile collection. Sodium heparin (0.5 ml, 72 U/ml) was injected intravenously. The pyloric vein was tied and the portal vein was cannulated. The liver was perfused (20 ml/min) *in situ* with oxygenated Krebs-Henseleit bicarbonate (KHB) buffer (NaCl 118 mmol/l, KCl 4.7 mmol/l, NaHCO$_3$ 25 mmol/l, KH$_2$PO$_4$ 1.19 mmol/l, MgSO$_4$·7H$_2$O 1.18 mmol/l, CaCl$_2$ 2.5 mmol/l, glucose 11.1 mmol/l) supplemented with sodium taurocholate (8.4 μmol/l) and gassed with 95% O$_2$ and 5% CO$_2$. The liver was dissected free and transferred ($t = 0$) to a perfusion chamber. The temperature in the perfusion chamber and of the perfusion medium was thermostatically controlled at 37°C. Perfusion was performed in a recirculating mode. After 10 min equilibration with GL-free perfusate, the livers in the GL group were perfused with the perfusate containing GL (43.7 μmol/l) for 90 min, while the livers in the control group were perfused with the blank perfusate for 90 min. At the end of the experiment, the liver was taken and homogenized for determination of glutathione content.

Effect of glycyrrhizin on biliary excretion of the Mrp2 substrate MTX. The perfused livers were divided into MTX and MTX plus GL groups ($n = 5$ for each group). The surgical procedure was performed as described above. Perfusion was performed in a non-recirculating mode. After 10 min equilibration with GL free perfusate, the livers in the MTX + GL group were perfused with the perfusate containing GL (14 μmol/l) from 10 to 90 min, while the livers in the MTX group were continued with the GL-free perfusate from 10 to 90 min. In addition, MTX (50 μmol/l) was included in the perfusate from 40 min to 50 min for both groups.

Glycyrrhizin pharmacokinetic experiment. Four rats weighing 200–250 g were anesthetized with urethane (1.2 g/kg, intraperitoneal injection (i.p.)) and the common bile duct was cannulated with polyethylene tubing. The right jugular vein was also cannulated to collect blood samples. After injection of glycyrrhizin (25 mg/kg) into the right femoral vein, blood samples (300 μl each) were collected in heparinized polyethylene tubes at 5, 15, 45, 60, 90, 120 and 240 min. Plasma was separated by centrifugation at $1100 \times g$ for 5 min and then frozen at −20°C pending analysis.

Biliary excretion experiment

Effect of a single dose of glycyrrhizin on bile flow, biliary secretion of glutathione, bile acids and bilirubin. The rats were divided into control and GL groups ($n = 5$ for each group). The rats were anesthetized with urethane (1.2 g/kg, i.p.) and the common bile duct was cannulated with polyethylene tubing (PE-10). Bile samples were collected at 0, 0.5, 1, 2, 3, 4 and 5 h after a bolus injection of GL (25 mg/kg) or saline (for the controls). Bile volume was determined by weight assuming a density of 1 g/ml. Bile samples were frozen at −20°C pending determination of glutathione, bile acids and bilirubin.

Effect of different doses of glycyrrhizin on the biliary secretion of glutathione. The rats were divided into control and GL groups ($n = 5$ for each group). The rats were anesthetized and the common bile duct was cannulated as described above. The right jugular vein and the right femoral vein were cannulated for blood sampling and drug administration, respectively. The rats received three bolus injections of glycyrrhizin in a loading dose of 6.75, 6.75 and 20.25 mg/kg at 0 min (1 h after bile duct cannulation), 60 min and 120 min, respectively. Each bolus injection was followed by the continuous infusion of GL at 12, 24 and 60 mg/h/kg into the right femoral vein, respectively. The loading and maintenance doses of GL used were based on pharmacokinetic parameters obtained from the GL pharmacokinetic study described above. A steady state plasma concentration of GL was attained immediately after the start of the infusion. Bile samples were collected at 30 min intervals over a period of 240 min. Blood samples

were taken at 20, 40, 80, 100, 140 and 160 min and used to determine the plateau plasma concentration during each infusion period. The rectal temperature of the animals was kept at 37°C using a heat lamp throughout the experiment. At the end of the experiment, the liver was taken and homogenized for the determination of glutathione content.

Transport studies with membrane vesicles expressing rat Mrp2

The inhibitory effect of GL on rat Mrp2 transport was measured as uptake of Mrp2 substrate $E_2 17 G$ into rat Mrp2-expressing Sf9 membrane vesicles according to the manufacturer's protocol with a minor modification. The ATP-dependent transport activity was calculated by subtracting the $E_2 17G$ uptake in the presence of AMP from the $E_2 17G$ uptake in the presence of ATP. In brief, membrane vesicles (50 μg of protein) were suspended in the transport buffer (50 mM MOPS-Tris (pH 7.0), 70 mM KCl, 7.5 mM $MgCl_2$ and 2 mM glutathione). For the determination of inhibitory effects of GL on $E_2 17G$ uptake by Mrp2, membrane vesicles were preincubated with $E_2 17G$ in the presence (2 μM and 20 μM) or absence of GL at 37°C for 5 min and then rapidly mixed with the reaction mixture containing 4 mM ATP or AMP with an ATP-regenerating system (10 mM creatine phosphate and 100 μg/μl creatine phosphokinase), and 75 μl of the reaction mixture was incubated for 5 min. The transport reaction was terminated by the addition of 200 μl of ice-cold buffer containing 40 mM MOPS-Tris (pH 7.0) and 70 mM KCl. The stopped reaction mixture was then filtered through a 0.45 μm HAWP filter (Millipore Corporation, Billerica, MA) and washed five times with 200 μl of stop solution per well.

Drug analysis

The high-performance liquid chromatography (HPLC) system (Shimadzu, Model LC-2010C) consisted of a four-component gradient pump, an on-line degasification device, an auto-sampler, a UV detector and a column oven.

Analysis of glycyrrhizin in plasma

The pretreatment and analysis of plasma samples for HPLC analysis of glycyrrhizin was modified from our previous study [10]. Ninety μl plasma was taken into an Eppendorf tube, and added to 10 μl indomethacin (internal standard, 100 μg/ml) and 300 μl methanol. The tube was vortex mixed and then centrifuged at $9600 \times g$ for 10 min. The supernatant was transferred to another clean Eppendorf tube and centrifuged at $9600 \times g$ for 10 min. An aliquot (20 μl) of supernatant was injected directly onto the HPLC column (Lichrospher C8 column, 250×4.6 mm, 5 μm particle size). The absorbance of the eluate was measured at 254 nm. The column temperature was maintained at 30°C. The mobile phase for the GL assay consisted of methanol and 0.05 M ammonium acetate (pH 6.0) at a volume ratio of 61 to 39, respectively, and was pumped into the column at a flow rate of 1 ml/min. The retention times of glycyrrhizin and indomethacin were 7.4 min and 14.0 min, respectively.

Analysis of MTX in bile

The bile samples were diluted 1 in 50 with distilled water. Ninety μl diluted bile was transferred into an Eppendorf tube and 10 μl cefetamet acid (internal standard, 150 μg/ml) added. The tube was vortex-mixed for 2 min and then centrifuged at $9600 \times g$ for 10 min. An aliquot (20 μl) of supernatant was injected directly into an HPLC column (Lichrospher C18 column, 250 mm $\times$ 4.6 mm, 5 μm particle size). The absorbance of the eluate was measured at 310 nm. The column temperature was maintained at 40°C. The optimal composition of the mobile phase for the GL assay was found to be methanol: ammonium acetate (0.05 M, pH 6.0) at a ratio of 18:82 (v/v) and the flow rate was set at 1 ml/min. The retention time of GL and cefetamet acid was 9.8 and 8.2 min, respectively.

Analysis of $E_2 17G$

The filters were soaked in 200 μl 80% methanol containing internal standard (osalmide) for 15 min in Eppendorf tubes. After ultrasonication for 5 min, the tubes were centrifuged at 12000 rpm for 5 min. The concentrations of $E_2 17G$ and osalmide in the medium were quantified using an HP1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) and a AB API 4000 triple–quadrupole mass spectrometer (Applied Biosystems, USA) equipped with an electrospray ionization interface used to generate negative ions $[M-H]^-$. The

compounds were separated on a reversed-phase C18 column (50 mm × 2.1 mm, 5 μm particles, Sepax Technologies, Delaware, USA) connected to an Dikma EasyGard IIC18 guard column (10 mm 2.1 mm, 5 μm particles, Dikma Technologies, USA) at 20°C. The two mobile phases consisted of (A) 0.1% (v/v) formic acid aqueous solution and (B) methanol containing 0.1% (v/v) formic acid. The compounds were eluted by a gradient shown as follows: 0–1.5 min 98% B; 1.51–3 min 2% B; 3 min–5 min 98% B. Approximately, 0.2 ml/min of the effluent was introduced into the interface of the mass spectrometer by a valved three-way split. The turbo ion spray interface was operated in negative ion mode at 5500 V and 500°C. The mass transitions for $E_2 17G$ and osalmide were m/z 447.5 → 74.9 and 228.1 → 92.8, respectively.

Analysis of glutathione, bile acids and bilirubin in bile

Glutathione was measured using a commercial glutathione assay kit. Briefly, after reduction of GSSG into GSH in the bile and liver samples, the total glutathione concentration was assayed by the formation of a chromophoric thione. The absorbance measured at 412 nm is directly proportional to the glutathione concentration [18]. Bile acids and bilirubin were measured using a commercial bile acids assay kit and a bilirubin assay kit. Briefly, bile acids were oxidized by 3α-hydroxysteroid dehydroxygenase to form a chromophoric Thio-NADH. The absorbance measured at 405 nm was directly proportional to the bile acid concentration [19]. Bilirubin was determined using a modified Jendrassik-Gróf method [20]. In the presence of an accelerator (caffeine), total bilirubin couples with sulfanilic acid to form a red dye (azobilirubin), the color intensity of which is proportional to the concentration of bilirubin.

Statistical analysis

The results were expressed as mean ± standard deviation for the indicated numbers of experiments, and the data were compared using an unpaired two-tailed t-test, or a single factor ANOVA with Tukey's multiple comparison test (respectively). A probability (p) of less than 0.05 was considered

statistically significant. Tukey's multiple means that the number of rats in every group was equal, and the study is an exploratory trail.

Results

Effects of glycyrrhizin on hepatic glutathione content in isolated liver perfusion

To characterize the effect of glycyrrhizin on hepatic glutathione content, GL was perfused continuously throughout the experiment. At the end of the experiment, the hepatic glutathione content (5.05 ± 0.69 μmol/g liver, $n = 5$) was significantly higher ($p < 0.05$) than that for the control (4.05 ± 0.40 μmol/g liver, $n = 5$).

Effect of single injection of glycyrrhizin on glutathione biliary excretion

When glycyrrhizin was administered intravenously in rats, the glutathione secretion rate decreased immediately and fell to a minimum value at 0.5 h, partially recovering between 0.5 to 1 h, and returned to the control group levels at 2 h (Figure 1). At 0.5 h, the glutathione secretion rate (0.28 ± 0.29 nmol/min/100 g body weight (b.w.) was significantly decreased compared with the control group (4.26 ± 1.41 nmol/min/100 g b.w.). However, no significant change was observed in the bile flow. However, the biliary secretion rate of bilirubin appeared slightly decreased (0.34 ± 0.02 μmol/min/100 g b.w.) when compared with the control group (0.42 ± 0.12 μmol/min/
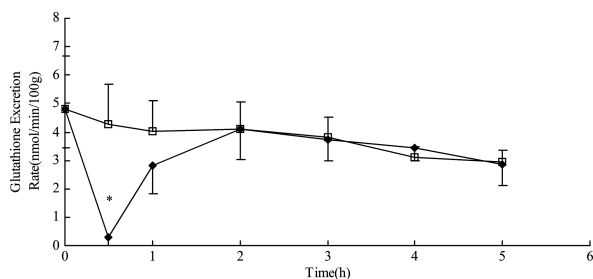

Figure 1. Effects of a single i.v. dose of glycyrrhizin (25 mg/kg) on biliary secretion of glutathione in anesthetized rats. The data are expressed as mean ± SD ($n = 5$) for the GL (filled diamonds) and the control (open squares) group. *$p < 0.05$ compared with the corresponding value in the control group

100 g b.w.) at 0.5 h, but the difference was not significant ($p = 0.1$). The bile acids secretion rate in the GL group was similar to that in the control group throughout the experiment (Table 1).

Pharmacokinetics of glycyrrhizin in rats and dose-dependent inhibitory effect of glycyrrhizin on glutathione biliary excretion

After a single intravenous injection of glycyrrhizin (25 mg/kg), the glycyrrhizin concentration decreased from 353 ± 21.1 μg/ml to 10.8 ± 4.76 μg/ml, with a mean half-life of about 24 ± 4 min ($n = 4$) (Figure 2). The dose regimen used for bolus and infusion of GL was based on the pharmacokinetic parameters obtained from the above single dose GL pharmacokinetic study (Figure 2). After the rats received three bolus injections of GL in a loading dose of 6.75, 6.75 and 20.25 mg/kg at 0, 60 and 120 min, followed by a continuous infusion of GL at 12, 24 and 60 mg/h/kg, respectively, three steady state concentrations (50, 100 and 300 μg/ml) of GL in plasma were attained at 0–60, 60–120 and 120–180 min, respectively. As shown in Figure 3, prior to administration of GL (−60–0 min), the values of glutathione biliary excretion rates were similar between the GL and the control groups. After GL administration at different doses, the glutathione biliary excretion rates were reduced by 57%, 86% and 98% when the steady state concentrations of GL were 50, 100 and 300 μg/ml, respectively, showing a dose dependent response. The hepatic content of glutathione (5.91 ± 0.38 μmol/g liver, $n = 5$) in rats receiving a single dose of GL (25 mg/kg) was significantly higher ($p < 0.01$) than for the control (4.37 ± 0.42 μmol/g liver, $n = 5$).
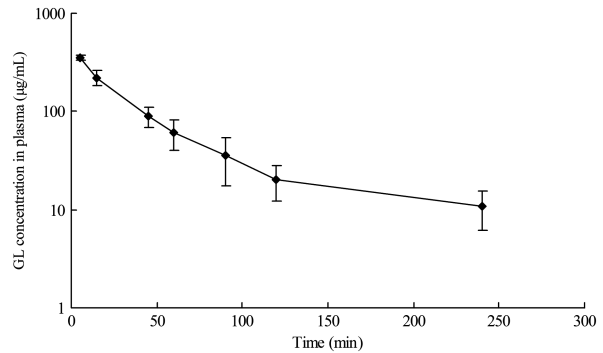

Figure 2. Plasma glycyrrhizin concentration–time profile in anesthetized rats with biliary cannulation after a single intravenous injection of GL (25 mg/kg). The data are presented as mean $\pm$ SD ($n = 4$)
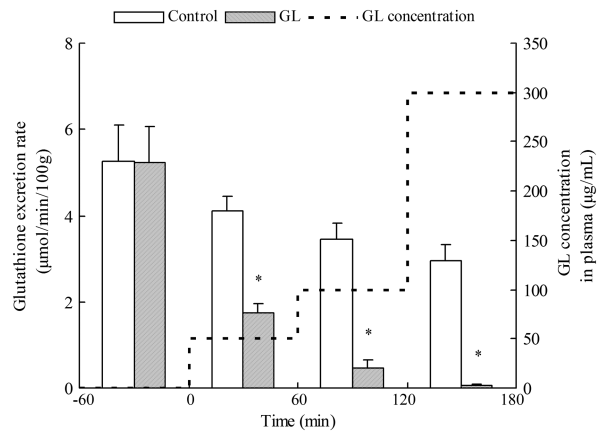

Figure 3. Inhibitory effect of glycyrrhizin on glutathione biliary excretion. Three steady state concentrations (50, 100 and 300 μg/mL) of GL in plasma are illustrated by the dashed line. The data for biliary excretion rate of glutathione are presented as mean $\pm$ SD ($n = 5$). *$p < 0.05$, GL-treated group is significantly different from the control group

Table 1. Bile flow and biliary secretion rates of bile acids and bilirubin after bolus injection of glycyrrhizin (GL)

Time (h)	0	0. 5	1	2	3	4	5
Bile flow (μl/min/100 g b.w.)							
Control	5.02 ± 1.22	3.90 ± 0.97	4.30 ± 0.89	4.45 ± 0.54	4.17 ± 0.41	4.03 ± 0.33	3.72 ± 0.34
GL	4.72 ± 0.98	3.96 ± 0.79	3.95 ± 0.61	4.18 ± 0.64	4.34 ± 0.68	3.81 ± 0.30	3.67 ± 0.42
Bile acids secretion rate (μmol/min/100 g b.w.)							
Control	57.1 ± 21.9	34.9 ± 9.41	31.8 ± 10.6	31.0 ± 10.5	29.0 ± 8.78	23.0 ± 5.80	19.4 ± 5.14
GL	64.6 ± 14.3	35.7 ± 9.41	32.9 ± 9.51	29.9 ± 6.34	25.8 ± 4.94	24.7 ± 3.95	18.1 ± 5.48
Bilirubin secretion rate (μmol/min/100 g b.w.)							
Control	0.58 ± 0.19	0.42 ± 0.12	0.44 ± 0.13	0.50 ± 0.13	0.58 ± 0.23	0.57 ± 0.26	0.62 ± 0.32
GL	0.62 ± 0.07	0.34 ± 0.02	0.48 ± 0.08	0.49 ± 0.05	0.50 ± 0.12	0.58 ± 0.13	0.62 ± 0.18

The data are presented as mean $\pm$ SD ($n = 5$).

Effects of glycyrrhizin on MTX biliary excretion in isolated perfused rat liver

After administration of MTX (50 μmol/l) for 10 min, the maximum biliary secretion rate of MTX was 3.07 ± 1.68 nmol/min/g liver at 60 min. Pretreatment of livers with GL (14 μmol/l) reduced the maximum biliary secretion rate of MTX by 65% to 1.09 ± 0.36 nmol/min/g liver and the attained time was delayed at 70 min (Figure 4). In the control group, the cumulative amount of MTX via biliary excretion from 40 to 90 min was 279.0 ± 103.5 nmol/g liver. In contrast, in the presence of GL (14 μmol/l) in the perfusate, the amount of MTX excreted was reduced by 57% to 118.9 ± 22.9 nmol/g liver in the GL group (Figure 4). Thus, GL markedly reduced the biliary secretion of an Mrp2 substrate.

The inhibitory effect of glycyrrhizin on primary active transport of $E_2$17G by rat Mrp2

The inhibitory effect of GL on the biliary excretion of Mrp2 substrates GSH and MTX *in vivo* and in the isolated perfused rat liver implied that there is a contribution of Mrp2 to the inhibition of GSH efflux through canalicular membranes. Therefore, the study examined the effect of GL on the transport of $E_2$17G, a model substrate of Mrp2, using membrane vesicles prepared from

Sf9 cells transfected with the rat Mrp2 gene. In the absence of GL (control group), the uptake of $E_2$17G by membrane vesicles in the presence of ATP was almost five times that in the presence of AMP (Figure 5A), suggesting that the transport of $E_2$17G through the membrane vesicles was primarily ATP-dependent by Mrp2. Accordingly, the Mrp2 mediated uptake was assessed by the subtraction of the uptake in the presence of AMP from the uptake in the presence of ATP. Compared with the control group, the uptake of $E_2$17G by rat Mrp2-expressing vesicles was significantly decreased by 75.9% in the 20 μM GL group ($p < 0.05$), and by 60.5% in the 2 μM GL group, although the difference was not significant ($p = 0.07$) (Figure 5B). The percentage of the ATP-dependent transport of $E_2$17G in 2 μM and 20 μM GL group were only 11.4% and 6.99%, respectively, compared with 29% of the control group (Figure 5C). These results suggested that GL does have an inhibitory effect on rat Mrp2 mediated transport which may be the possible mechanism of the decreased GSH biliary excretion and increased GSH hepatic content observed *in vivo* and in the isolated perfused rat liver model.

Discussion

Glutathione plays several physiological roles in cellular antioxidation and detoxification and it is also crucial for maintaining a normal immune response [7]. A decrease or depletion of glutathione levels has been shown to change the intracellular biochemical environment and cellular structure, resulting in severe oxidative stress damage and even cell death [21]. An increase of glutathione levels during hepatic disease could improve cell viability. For example, the administration of glycyrrhizin to experimental animals with liver disease increased the hepatic glutathione content which was impaired under disease conditions and protected the liver against damage [8,9]. However, whether GL can alter the hepatic glutathione content in normal rats has not been reported yet. The effect of GL on hepatic glutathione content in the isolated perfused liver in normal rats was characterized. The results presented above indicate that GL can not only increase hepatic glutathione
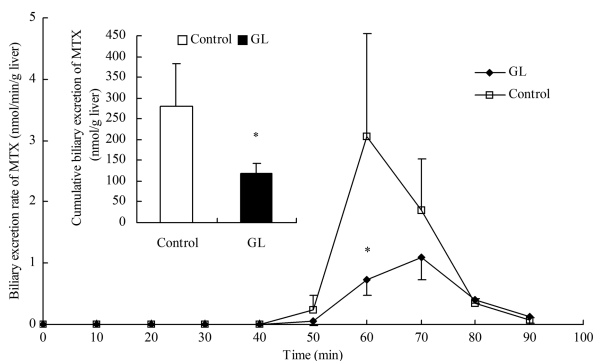

Figure 4. Effect of GL on biliary excretion of the typical Mrp2 substrate, MTX in the isolated perfused rat liver. The livers were perfused with GL (14 μmol/l) or GL free buffer from 10 to 90 min after 10 min equilibration. Between 40–50 min, MTX (50 μmol/L) was included in the perfusate. The biliary excretion of MTX was determined from 40 to 90 min in the presence or absence of GL. The data are expressed as mean $\pm$ SD ($n = 5$). $*p < 0.05$, GL-treated group is significantly different from the control group
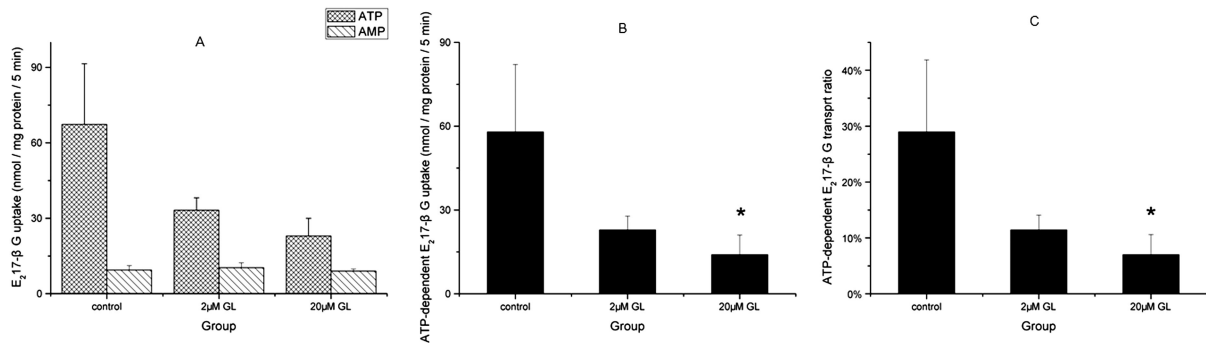
Figure 5. Inhibitory effect of glycyrrhizin on E_217G uptake by rat Mrp2-expressing Sf9 membrane vesicles. (A) Total uptake of E_217G through membrane vesicles in the presence of ATP or AMP in three different groups. (B) The absolute amount of ATP-dependent E_217G uptake by membrane vesicles in the absence (control) and presence of different concentrations of GL (2 μM; 20 μM). (C) The ratio of ATP-dependent E_217G uptake by membrane vesicles to the exposure dose in the beginning of three different groups. The data are expressed as mean $\pm$ SD ($n = 3$) for the GL and the control group. *$p < 0.05$ compared with the corresponding value in the control group

content in rats under disease conditons, but also under normal condition *in vitro* and *in vivo*.

Previous studies reported that glycyrrhizin inhibited azathioprine-induced glutathione depletion in human hepatocytes, and the significant enhancement of intracellular glutathione in both human and rat hepatocytes by GL treatment could be due to GL inhibiting the free radical-generating enzymes and the mitochondrial permeability transition, thus reducing the accumulation of reactive oxygen species and increasing the glutathione level [22]. However, for glutathione in the liver, there are several reasons that may also lead to an enhancement of the hepatic glutathione level. Glutathione is synthesized intracellularly and the reaction is catalysed by γ-glutamylcysteine synthetase (γ-GCS) and GSH synthase [21]. Then Mrp1, Mrp4 and Mrp5 appear to mediate GSH export from hepatocytes into blood, whereas Mrp2 contributes to the transport process from hepatocyte into the bile [23–26]. Finally, GSH is metabolized by the actions of γ-glutamyl transpeptidase and dipeptidase located on the biliary ductular epithelium [27]. These findings indicated that several factors might be involved in the alteration of hepatic GSH content after GL administration, including γ-GCS, GSH synthase, MRP, γ-GT and dipeptidase etc.

Our previous study revealed that the biliary excretion rate of GL was very high and 85% of the dose was excreted through biliary secretion within one hour of intraportal venous injection of GL (7.5 mg/kg) *in vivo* [10]. Due to the

extremely large amounts of GL excreted into bile, this study mainly focused on its effect on glutathione efflux. However, other factors in raising hepatic GSH content cannot be excluded, such as enhancement of the activity of γ-GCS, GSH synthase, γ-GT or dipeptidase, and inhibition of Mrp1, Mrp4 or Mrp5. In the isolated liver perfusion experiment, the hepatic GSH concentration was determined at the end of the study and it was found that the hepatic GSH concentration was increased significantly in the GL-treated group. The single dose GL study suggests that GL inhibited the glutathione secretion rate into bile, supporting the hypothesis that GL increases the hepatic glutathione level by affecting the glutathione efflux into bile.

Bile is formed when secretory components such as bile acids and glutathione are excreted at concentrations that are sufficiently high to generate osmotic water flow into the bile canaliculi [28]. Bile acid is responsible for the bile acid dependent flow (BADF), while glutathione is responsible for the bile acid independent flow (BAIF) [29,30]. Therefore, as GL decreased glutathione secretion this could theoretically lead to reducing BAIF. However, the data showed that administration of GL did not influence the bile flow. Similarly, Ichikawa *et al*. [31] reported that there is no significant difference in the bile flow rate between the GL-treated and the non-treated rats. However, other authors reported that GL administration facilitated bile excretion when compared with the

control rats [32]. These different results may be due to different dosages and routes of GL administration to rats, different rat strains, lack of sufficient number of animals within the study and different surgery procedures for bile duct cannulation. Nevertheless, there is no literature reporting that GL decreases bile flow. The mechanism by which GL did not decrease bile flow, although glutathione excretion decreased, may be that GL itself has a choleretic effect [32], overriding the transient negative impact of the decreased glutathione secretion on bile flow, or other unknown mechanisms might exist. This study also indicates that GL does not change the normal physiological environment of the liver as determined by the lack of effect of GL on bile flow, bilirubin and bile acids excretion, in addition to the positive effect of GL on the hepatic glutathione level.

The study also characterized the effect of different doses of GL on the inhibition of glutathione efflux. Due to the half-life of GL being very short [10], three different doses of GL in a single rat were designed through different loading doses and intravenous infusion at fixed sequential times to avoid individual differences. The concentrations (50 and 100 µg/ml) used in the study refer to the published GL pharmacokinetic study in humans [33], and it was found that the hepatic GSH content increased significantly with the increase of GL. In order to see the trend clearly, the concentration was extended and a higher concentration added (300 µg/ml), and it was shown the higher GL concentration, the lower the biliary GSH excretion rate. The result suggests that the inhibitory effect of GL on glutathione efflux was dose-dependent. This, along with the finding that the inhibition was reversible in the single dose of GL study, indicates that an inhibition of transporter may be involved. Drug–drug interactions between GL and several Mrp2 substrates have been reported in published articles. For example, GL was also found to inhibit other Mrp2 substrates such as SN-38 and SN-38-Glu in rats *in vivo*, and GL can reverse the cisplatin resistance in hepatocellular carcinoma cells through inhibition of Mrp2 [17,34]. To determine whether GL might affect biliary transport of Mrp2 substrate, an Mrp2 substrate, MTX, was chosen and the effect of GL on MTX biliary excretion investigated. The concentration of MTX in the perfusate when the

biliary excretion rate of MTX was altered was monitored and it was found that GL significantly reduced the biliary excretion rate of MTX with an insignificant effect on the concentration of MTX in perfusate, implying that GL, at least in part, inhibited the biliary excretion clearance of Mrp2 substrate MTX. In addition, the results show that when the concentration of GL was increased to 300 µg/ml, the biliary excretion of GSH was decreased to only 2% of the control group. Therefore, GL possibly inhibited the main GSH transport pathway from hepatocytes into bile. Paulusma *et al*. found that biliary GSH excretion was hardly detectable in Mrp2 deficient rats, thus GSH is transported from hepatocytes into bile mainly through Mrp2 [35]. Therefore, the decrease of excretion for glutathione may be attributed to the competitive inhibition of Mrp2 by GL.

To further validate the inhibitory effect of GL on the transport of Mrp2 substrate, a high selectivity *in vitro* study was conducted using rat Mrp2-expressing Sf9 membrane vesicles. Probably because Mrp2 mediates low-affinity (high K_m) transport of GSH which led to extremely low transport rates at the relatively low concentration used, in the present study no significant differences of Mrp2-mediated uptake amount were observed between the ATP and AMP groups when GSH was the substrate (results are not shown). This result was also consistent with previous studies in canalicular membrane vesicles (CMVs) from rat livers [35]. Considering this, $E_2$17G, a more typical substrate than MTX in the *in vitro* study, was chosen as a model Mrp2 substrate according to the present (Figure 5B) as well as previous studies [14,15] to investigate the effects of GL on the transport function of Mrp2. The results suggested that GL could inhibit Mrp2 mediated substrate transport. At a concentration of 20 µM, GL significantly reduced ATP-dependent $E_2$17G uptake compared with the control group. While 2 µM GL was less effective, suggesting that the inhibitory effect of GL on Mrp2 transport was dose dependent. This *in vitro* study directly validated the inhibitory effect of GL on the transport of Mrp2 substrate, which may be the most possible mechanism of the increased GSH concentration in liver while decreasing GSH biliary excretion by GL *in vivo* and in the isolated rat perfused liver.

Conclusion

In conclusion, our findings reveal that increased hepatic glutathione level may be due to the inhibition of biliary excretion of glutathione partly through the inhibition of Mrp2 by GL, and in the meantime GL has no effect on bile flow, biliary excretion of bile acids and bilirubin.

Conflict of Interest

The authors state there were no conflicts of interest.

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