Pregnane X receptor mediated-transcription regulation of CYP3A by glycyrrhizin: A possible mechanism for its hepatoprotective property against lithocholic acid-induced injury

Yu-Guang Wang [a,1], Jian-Ming Zhou [a,1], Zeng-Chun Ma [a], Hua Li [b], Qian-De Liang [a], Hong-Ling Tan [a], Cheng-Rong Xiao [a], Bo-Li Zhang [c], Yue Gao [a,*]

[a] Department of Pharmacology and Toxicology, Beijing Institute of Radiation Medicine, Beijing 100850, China
[b] Laboratory of Drug Metabolism and Pharmacokinetics, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China
[c] Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China

ARTICLE INFO

ABSTRACT

Licorice (LE) has been commonly used in traditional Chinese medicine (TCM) for over 4000 years to reconcile various drugs and for hepatic disorders. Glycyrrhizin is the main bioactive component isolated from LE herbs. In the present study we examined the effects of glycyrrhizin on pregnane X receptor (PXR)-mediated CYP3A expression and its hepatoprotective activity. Treatment of HepG2 cells with glycyrrhizin resulted in marked increase in both CYP3A4 mRNA and protein levels. The transcriptional activation of the CYP3A4 gene through glycyrrhizin is PXR-dependent, as shown in transient transfection experiments. Glycyrrhizin activates the DNA-binding capacity of the PXR for the CYP3A4 element responding to xenobiotic signals, as measured by the electrophoretic-mobility shift assay (EMSA). These results indicate that the induction of the hepatic CYP3A4 by glycyrrhizin is mediated through the activation of PXR. The next aim of the current study was to determine whether the activation of PXR and induction of CYP3A by glycyrrhizin prevents hepatotoxicity during cholestasis as a mechanism of hepatoprotection. Mice were pretreated with glycyrrhizin prior to induction of intrahepatic cholestasis using lithocholic acid (LCA). Pre-treatment with glycyrrhizin, as well as the PXR activator pregnenolone 16α-carbontrile (PCN), prevents the increase in plasma ALT and AST activity, multifocal necrosis and prevents an increase in a level of serum LCA level in mice, as compared with the results in the mice treated with LCA alone. Activation of the PXR by glycyrrhizin results in induction of CYP3A11 (CYP3A4 for human) expression and inhibition of CYP7A1 through an increase in small heterodimer partner (SHP) expression. Glycyrrhizin regulates the expression of the gene mentioned above to prevent toxic accumulation of bile acids in the liver and it also protects mouse livers from the harmful effects of LCA. In conclusion, PXR-mediated effects on CYP3A and CYP7A may contribute to the hepatoprotective property of glycyrrhizin against LCA-induced liver injury.

Crown Copyright © 2012 Published by Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Licorice is sometimes thought of as the origin of TCM because it is included in almost 70–80% of Chinese herbal prescriptions. It is known for its detoxifying and hepatoprotective properties as well as many other pharmacological activities [1–3]. Glycyrrhizin is one of the main ingredients isolated from aqueous LE extracts. Several animal models have shown its protective properties in the setting of liver injuries [4,5]. Furthermore, glycyrrhizin has shown a protective effect on cocklebur-induced hepatotoxicity in both human and rat hepatocytes [6], which indicated that glycyrrhizin has hepatoprotective properties [7]. LCA is a hydrophobic secondary bile acid and commonly used to prepare a hepatotoxicity model in experiment animals. Notably CYP3A4 (CYP3A11 rodent homolog) participates in LCA detoxification via 6α-hydroxylation of LCA. Our previously studies have shown that LE or glycyrrhizin induce CYP3A at the mRNA and enzyme levels in rats (data not shown). The aim of the present study is to investigate a protective effect of glycyrrhizin in a LCA induced cholestatic liver model involved CYP3A.

CYP3A4 plays a significant role in the metabolism of approximately half the currently used drugs, including bile acid. A number of herbs have been shown to induce CYP3A4, e.g., St. John's work has been found to accelerate the clearance of several clinically used

medications by inducing CYP3A [8]. Tianxian [9], Ginkgo Biloba [10], hops [11], have also been reported to have CYP induction effects.

PXR thought to be the main transcriptional regulator of CYP3A4 gene expression [12]. The ligand-activated PXR forms a heterodimer with the retinoid X receptor (RXR), and subsequently binds to the promoter region of its target genes such as CYP3A4 [13]. PXR functions in bile acid homeostasis. Activation of PXR by PCN induces CYP3A, which is important for LCA detoxification. Previous studies including our study (unpublished) have shown that LE, and its natural constituent glycyrrhizin are able to significantly induce hepatic CYP3A-dependent microsomal monooxygenase activities in male and female mice. In addition, mRNA and protein levels of CYP3A were also affected by prolonged intake of high doses of LE or glycyrrhizin [14]. Previous studies showed specific differences in the induction profile among species. For example, PCN, a strong inducer of CYP3A in rat or mouse, does not induce CYP3A4 in human, while rifampicin, a strong inducer of human CYP3A4, is not an inducer in rat. Though it is already known that glycyrrhizin can induce rat CYP3A1/2 or mouse CYP3A23, the effects of LE and glycyrrhizin on human CYP3A4, as well as the involvement of human PXR in the transcriptional activation of CYP3A4 have not been examined. In the present study, human hepatoma cells HepG2, human CYP3A4 luciferase reporter vector and expression vector pCMX-hPXR were used.

Normal metabolism of bile acid is regulated by feedback regulation, i.e. high levels of bile acid inhibit biosynthesis in the liver through the ligand-activated farnesoid X receptor (FXR) [15]. However, potentially toxic bile acid precursors and hydrophobic secondary bile acids are efficiently 6-hydroxylated by the CYP 3A4 (CYP3A11 in mice) enzyme into less toxic bile acids through activated PXR [15–17]. Consequently, a dynamic balance between biosynthesis and metabolism of bile acids is maintained. LCA has been identified as a toxic hydrophobic secondary bile acid and its accumulation has been shown to induce irreversible liver damage in many animal models [17,18].

PXR has been shown to act as an LCA sensor and accelerate LCA detoxification. The aim of the current study was to characterize the induction of human CYP3A4 by glycyrrhizin and the involvement of human PXR activation in this mechanism. We also attempted to determine whether the activation of PXR and induction of CYP3A by glycyrrhizin prevents LCA-induced hepatotoxicity in vivo during cholestasis as a mechanism of hepatoprotection.

2. Materials and methods

2.1. Drugs and reagents

Glycyrrhizin (>95% purity), rifampicin (RIF) and pregnenolone 16α-carbonitrile (PCN) were purchased from Sigma Chemical Co. (St. Louis, MO). Lithocholic acid (LCA) was purchased from Fluka Chemical Co (Milwaukee, WI). LE was obtained from Tongrentang Pharmacutical Co. (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Los Angeles, USA). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (NEB, Beverly, MA). AMV reverse transcriptase and other regents which were used for RT-PCR were obtained from TaKaRa (Dalian, China). Lipofectamine 2000 and TRIzol reagent were from Invitrogen (Carlsbad, CA). The pGL3-basic vector constructs and Dual-Luciferase reporter assay system were purchased from Promega (Madison, WI). Polyclonal sheep anti-human CYP3A4 antibody was purchased from Chemicon (Temecula, CA). Rabbit anti-sheep IgG (H + L) was obtained from SouthernBiotech (SBA, Birmingham, Alabama). EMSA kit of LightShift® Chemiluminescent was purchased from Pierce (Rockford, IL). All other reagents used were of the highest commercially available quality.

2.2. Aqueous extracts of licorice (LE)

The aqueous product of licorice was extracted twice by immersing 100 g raw radix LE in 1000 ml water for 30 min, followed by boiling of the mixture for another 30 min. After filtering the sample with two-tiered gauze, the extracts were combined and concentrated to a final volume of 50 ml using a rotary evaporator. The concentrated extract contained 2 g/ml LE. The chemical composition reproducibility of LE extract contained glycyrrhizin was monitored by UPLC–TOF/MS (data not shown).

2.3. HepG2 cell cultures and treatment

Human hepatoma cells HepG2 (ECACC No. 85011430) were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FCS), 100 U/ml streptomycin,100 mg/ml penicillin, 4 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate. Cell cultures were maintained in 75-cm^2 flasks at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. HepG2 cells were maintained in 6-well plates, and when growth reached 80% confluency, the cells were treated with RIF and glycyrrhizin in medium without fetal bovine serum for an additional 2, 6 or 12 h prior to harvest.

2.4. RNA isolation and RT-PCR analysis of HepG2 cells

Total RNA of HepG2 cells was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The mRNA level of CYP3A4 was determined by RT-PCR assay with a RNA PCR kit (Takara) per the manufacturer's instructions. Total RNA (0.5 μg) was subjected to the synthesis of the first strand cDNA at a total volume of 10 μL with oligo dT-adaptor primer and AMV reverse transcriptase. The reaction condition used was as follows: 42 °C for 30 min, 99 °C for 5 min and 5 °C for 5 min. The cDNA (2 μl) was subjected to PCR amplification with the following. The parameters: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and the optimum cycle number that was within the exponential range of response for CYP3A4 (35 cycles) and β-actin (30 cycles) was used. PCR primers for CYP3A4 detection were as follows: Sense, 5′-CAATAAGGCACCACCCACCTAT-3′, antisense, 5′-TTCTTGCTGAATCTTTCAGGGAG-3′; β-actin (house-keeping gene) primers were as follows: sense, 5′-CTACAATGAGCTGCGTGTGG-3′, antisense, 5′-TAGCTCTTCTCCAGGGAGGA-3′. Quantitative data normalized to β-actin were obtained from a densitometer and analyzed with the Image J software program.

2.5. Preparation of total protein and Western-blot analysis

HepG2 cells were harvested at 24 h after glycyrrhizin or RIF treatment. HepG2 cells were rinsed twice with ice-cold PBS and lysed with a scraper in PBS containing SDS (0.1%, w/v), NP-40 (1%, v/v), deoxycholic acid and sodium salt (0.5%, w/v). The cell lysates were centrifuged at 12,000 g for 15 min at 4 °C to remove insoluble precipitates. The protein content in each sample was determined by the method of Bradford. Bovine serum albumin was used as the protein standard. Total protein (50 μg) from culture cells were denatured and separated by 12% SDS–polyacrylamide gel electrophoresis (PAGE) and subsequently transferred electrophoretically to nitrocellulose membranes. After nonspecific binding sites were blocked overnight at 4 °C with TTBS (Tween Tris buffered saline) containing 5% skimmed milk, the membrane were incubated with sheep anti-human CYP3A4 antibody from Chemicon at a dilution of 1:1000, overnight at 4 °C. The samples were subsequently incubated with horseradish peroxidase-conjugated rabbit anti-sheep secondary antibody for 2 h and visualized on film

using the SantaCruz ECL detection system. β-Actin was used as a loading control.

2.6. Preparation of pGL3-P3A4 reporter plasmid

The CYP3A4 luciferase reporter vector pGL3-P3A4 contains three copies of the corresponding response element and was constructed following previously described method with slight modifications [19], which carried the xenobiotic responsive enhancer module (XREM) region ($-7836/-7208$), the proximal promoter ($-362/+53$), and the luciferase reporter gene. Oligonucleotide primers for the proximal and distal promoter regions (PXRE) were:

5′-CGGGGGTACCAGATCTGTAGGTGTGGCTTGTTGG-3′
(KpnI site underlined),
5′-CTAGCTAGCTGTTGCTCTTTGCTGGGCTATGTGC-3′
(nheI site underlined),
5′-CCGCTCGAGTCTAGAGAGATGGTTCATTCC-3′
(XhoI site underlined) and,
5′-CCCAAGCTTTCGTCAACAGGTTAAAGGAG-3′

(HindIII site underlined), the distal promoter amplified products were then cloned into the HindIII/XhoI sites of the pGL3-basic vector, and the reconstructed plasmid was named pGL3-enhancer. Finally, the proximal promoter amplified products were subcloned into the KpnI/nheI sites of pGL3-enhancer to create the pGL3-P3A4. The pRL-TK vector (Promega, Madison, WI), which encodes the renilla luciferase gene regulated by the herpes simplex virus thymidine kinase (HSV-TK) promoter, was used as a control vector to normalize the results of transient transfection assays. The expression vector pCMX-hPXR was kindly provided by Dr. Xinqiang Zhu (Zhejiang University, Hangzhou, ZJ). All constructs were confirmed by sequence analysis.

2.7. Transient transfections and reporter gene assays

Approximately 200,000 HepG2 cells were seeded into each well of 24-well plates and cultured in 1 ml of DMEM supplemented with 10% FBS, at 37 °C under a humidified atmosphere consisting of 95% air and 5% CO_2. The following day, the cells in each well were transiently transfected with 500 ng pGL3-P3A4, 250 ng pCMX-hPXR (provided by Dr. Xinqiang Zhu, Zhejiang University, Hangzhou, ZJ) and 50 ng pRL-TK using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's procedures. After a 6 h incubation in the transfection mixture, the transfected cells were incubated with fresh medium (serum free) containing either RIF (10 μM) or various concentrations of glycyrrhizin for an additional 12, 24 or 36 h. At the end of the incubation time, the cells were washed once with phosphate-buffered saline (PBS) and harvested. The reporter enzyme activities were measured with the dual-luciferase reporter assay system (Promega) and 20/20n luminometer (Turner Biosystems, Sunnyvale, CA). Firefly luciferase activity of the reporter plasmids was then normalized to renilla luciferase activity of the vector pRL-TK, and the radio of the normalized signal of Glycyrrhizin or RIF-treated cells to that of vehicle-treated cells was calculated to determine the degree of activation. At least three independent transfections were performed for each time point.

2.8. Electrophoretic mobility shift assays

HepG2 cells were treated for 8 h with DMSO, glycyrrhizin and RIF in growth media. Nuclear proteins were isolated using NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce, Rcokford, IL). Oligonucleotides used for EMSA were commercially synthesized (Invitrogen). For EMSA assay 5 μg nuclear extracts was mixed with 5× binding buffer containing poly (dI-dC), DTT, Glycerol, EDTA, NaCl, $MgCl_2$ and Tris in optimized concentrations. After a 10 min incubation at 4 °C, a 2 μl sample containing the PXR-

binding site of the CYP3A4 as well as proximal and distal promoter Oligonucleotides labeled with 5′-biotin (5′-GAATGAACTTGCT-GACCCTCTATATGAACTCAAAGGAGGTCAGTG-3′ and its complementary) which was commercially synthesized by Invitrogen was added. To determine the specificity of oligonucleotide, binding, a 400× fold excess of unlabeled pregnane X response element (PXRE) was added to extracts of glycyrrhizin and RIF treated cells for competition analysis. After incubating for an additional 10 min at 4 °C, the samples were run on a 6% polyacrylamide gel with 0.5× TE buffer (45 mM Tris, pH 7.5, 45 mM boricacid, 2 mM EDTA). The gel was then dried and analyzed using the chemiluminescent nucleic acid detection module Kit (Pierce, Rockford, IL).

2.9. Animals and treatments

Seven-week-old male BALB/c mice weighing between 20 and 25 g were obtained from the animal center of Academy of Military Medical Sciences (Beijing, China). The mice were kept in a pathogen-free animal facility under a standard 12 h light/dark cycle and allowed free access to food and water. All surgical procedures and animal care were approved by the Ethics Committee of the institution. To induce intrahepatic injury in mice, the secondary bile acid LCA was administered. This has become a useful model for liver damage and has previously been described [20]. All inducers were dissolved in pyrogen-free saline except LCA and PCN, which were dissolved in camellia oil. All the animals were pre-treated with chemical inducers (LE aqueous extracts, 10 g/kg/d; glycyrrhizin, 10–50 mg/kg; PCN, 42 mg/kg) or pyrogen-free saline for 7 days via i.p. injection, and on the fourth day, LCA (125 mg/kg) treatment was initiated via i.p. injections twice daily for another 4 days.

2.10. Serum bile acid extraction and quantitation of LCA in serum by UPLC–TOF/MS

Twelve hours after the final treatment, mice were anesthetized with CO_2, and blood samples were collected by cardiac puncture. Serum was collected and stored at -80 °C until used for LCA analysis. 1 volume sample was mixed with 4 volumes chilled acetone, shaken vigorously and left on ice for 5 min before centrifugation at 10,000g for 3 min. The UPLC separation was performed using a Waters Acquity UPLC system (Waters, Milford, MA, USA), equipped with a binary solvent delivery system, an autosampler. The column used was a Waters Acquity BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters, Milford, MA, USA). The mobile phases were water with 3 mM ammonium formate (A) and acetonitrile with 3 mM ammonium formate (B). The gradient was the following: linear gradient from 5% B to 30% B (0–1 min), 30% (1–5 min), 30% B to 70% B (5–10 min), 30% B to 100% B (10–15 min), 100% B to 5% B(15–16 min). The flow rate was 0.45 ml/min. The injection volume was 15 μl. MS detection was performed on a Waters Synapt Mass Spectrometor (Waters, Milford, MA, USA). LCA was detected as the molecular ions $[M-H]^- = 375.2889$ in a negative selected ion monitor mode of TOF/MS. LCA concentrations in serum are calculated by comparison to standard solutions of LCA.

2.11. AST and ALT assay

The determination of serum ALT and AST levels using a biochemical auto analyzer [10].

2.12. Histology of liver

The left lobe of the liver was removed in all treated mice and immediately fixed in a 4% formaldehyde–phosphate-buffered saline solution, embedded in paraffin, sectioned into 5 μm slices, and stained with hematoxylin and eosin, according to a standard

staining protocol. Samples were examined under a light microscope. Representative liver sections from the same lobe of 3 mice per group were examined and scored by a pathologist.

2.13. RNA isolation and RT-PCR analysis of mice

To investigate the effects of LCA, LE or glycyrrhizin on CYP3A11, CYP7A1 and SHP mRNA expression in mice liver, the animals were either treated with chemical inducers (LE aqueous extracts, 10 g/kg/d; glycyrrhizin, 10–50 mg/kg; PCN, 42 mg/kg) or pyrogen-free saline for 7 days via i.p. injections, or LCA (125 mg/kg) treatment was initiated via i.p. injections twice daily for another 4 days. Total RNA was isolated from mouse liver tissue using the TRIzol regent (invitrogen) according to the manufacturer's instructions. RT-PCR assay was performed as previously described. The primers were synthesized (Invitrogen) according to sequence designs previously described [21,22].

2.14. Statistical analyses

Data are expressed as mean ± SD and the two-tailed student's t test was used for comparisons between groups. $P < 0.05$ was regarded as a significant difference between groups.

3. Results

3.1. Concentration and time-dependent inductions of CYP3A4 mRNA expression by glycyrrhizin

To investigate the effect of glycyrrhizin treatment on CYP3A4 mRNA transcription, RT-PCR was used to determine CYP3A4 mRNA levels in HepG2 cells treated with 50, 100, 250 and 500 μM glycyrrhizin or 10 μM RIF as a positive control for 12 h. As shown in Fig. 1A, CYP3A4 mRNA levels significantly increased in RIF-treated HepG2 cells when compared to the solvent control, indicating that the HepG2 cells can serve as a sensitive and selective model for assessing the regulation of CYP3A4 transcription by drugs and other xenobiotics. The increased CYP3A4 mRNA levels were also observed in glycyrrhizin treated cells for all the exposure levels. However, the alteration of CYP3A4 mRNA levels by glycyrrhizin was concentration-dependent over the range from 50 to 500 μM. Subsequently, the time course of CYP3A mRNA expression caused by glycyrrhizin was investigated using 250 μM glycyrrhizin. The maximum level of CYP3A4 transcription was reached after 6 h of incubation, though an elevated level was also observed after a 12 h incubation (Fig. 1B). The results confirmed that glycyrrhizin can induce CYP3A4 mRNA expression.

3.2. Effect of glycyrrhizin on CYP3A4 protein expression

Because glycyrrhizin can induce CYP3A4 expression at the mRNA level, Western blot analysis of the glycyrrhizin-treated cells was performed to determine whether the sustained induction of CYP3A4 was accompanied by corresponding modulation of protein expression. As shown in Fig. 2, compared to the solvent control, the CYP3A4 protein expression levels were significantly increased in both RIF- and glycyrrhizin-treated samples. The concentration-dependent elevation was also observed for glycyrrhizin treated cells over a concentration range of 50–500 μM, which further confirmed that glycyrrhizin is a CYP 3A4 inducer.

3.3. Activation of human PXR by glycyrrhizin

PXR is a key transcription factor in regulating CYP3A4 gene expression by binding to the transcriptional region of the CYP3A4
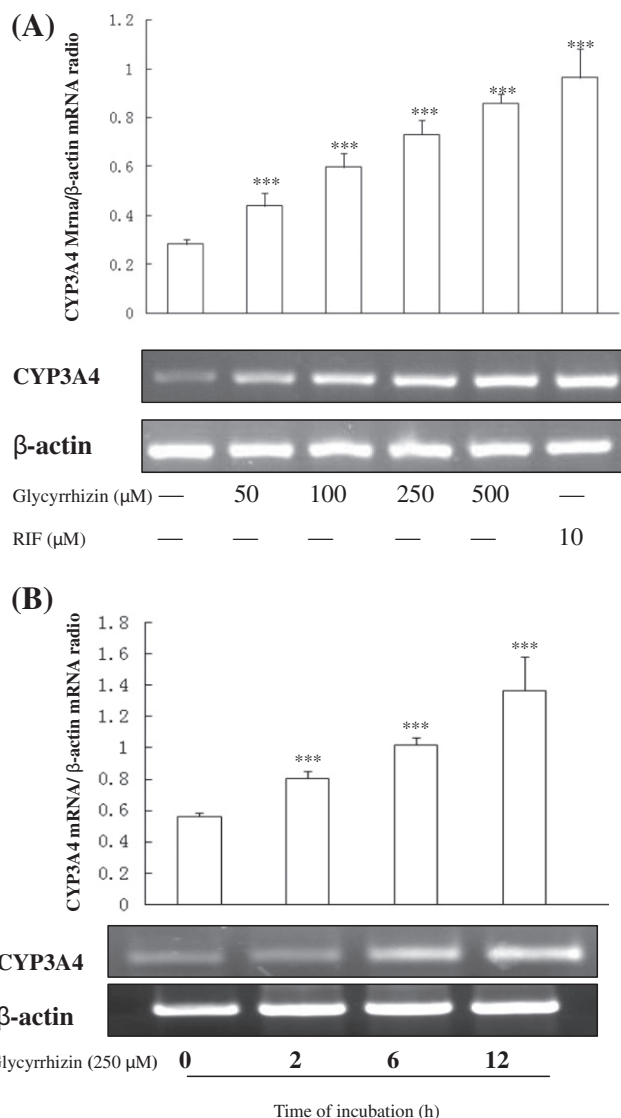

Fig. 1. Effects of glycyrrhizin treatment on the expression of CYP3A4 mRNA in HepG2 cells. (A) Concentration-dependent effects of glycyrrhizin treatment on the CYP3A4 mRNA level in HepG2 cells. (B) Time course of CYP3A4 mRNA induction by glycyrrhizin in HepG2 cells. RIF treatment was used as the positive control. The housekeeping gene, β-actin was used as the loading control. Results represent the mean of three independent experiments and the bands shown are from a representative experiment. Asterisks indicate a statistically significant difference compared with untreated cells. (***$P < 0.001$).
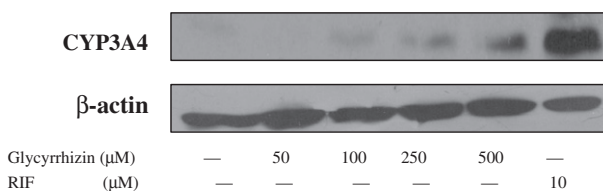

Fig. 2. Concentration-dependent effects of glycyrrhizin treatment on CYP3A4 protein level in HepG2 cells. RIF treatment was used as the positive control and the housekeeping gene, β-actin was used as the loading control.

promoter. To investigate possible mechanisms underlying the induction of CYP3A4 by glycyrrhizin and the ability of glycyrrhizin to transactivate PXR, a plasmid containing the proximal and distal CYP3A4 promoter regions and an expression vector of human PXR were transiently transfected into HepG2 cells. Subsequently, the

cells were treated with 10 μM RIF (positive control) or various concentration of glycyrrhizin. The luciferase reporter gene expression was then determined at different treatment times. Similar to the results obtained from mRNA transcription analysis, the induction of PXR transactivation in glycyrrhizin-treated cells was also dose-dependent in the dose range of 50–500 μM ($P < 0.05$) (Fig. 3A). PXR transactivation in cells exposed to 10 μM RIF, a known PXR activator and CYP3A4 inducer, was far greater (>10-fold) than in glycyrrhizin treated cells (2- to 5-fold). In the time-dependent PXR activation assay, the 1.6–3.6-fold increases in luciferase activity were observed at 12, 24 and 36 h for the glycyrrhizin treated group, when compared to the solvent control group ($P < 0.05$) (Fig. 3B). The highest activity of the luciferase reporter gene group was measured at the 36 h time point, suggesting the sustained transcriptional activation of the CYP3A4 promoter. The strong correlation between the luciferase reporter gene activity and the CYP3A4 mRNA and protein levels indicate that, similar to most CYP3A4 inducers, the induction effect of glycyrrhizin on CYP3A4 was also mediated by transactivation of PXR.

3.4. Nuclear glycyrrhizin-PXR/PXRE complexes in vitro

Glycyrrhizin led to a concentration- and time-dependent increase in CYP3A4 mRNA expression in HepG2 cells, which may
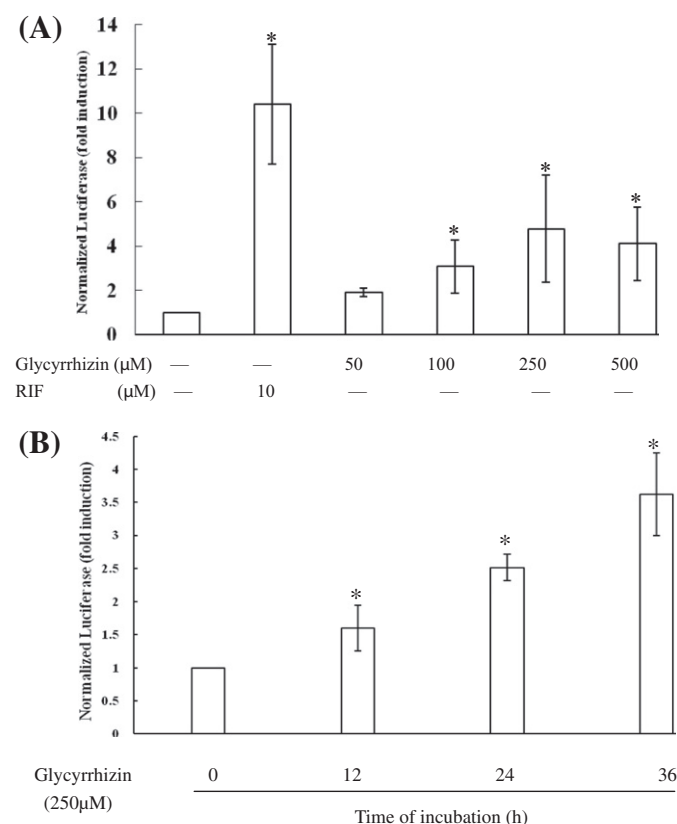
Fig. 3. Effects of glycyrrhizin on PXR activation in transfected HepG2 cells. (A) HepG2 cells were transfected with expression vectors encoding human PXR (pCMX-hPXR) and the reporter plasmid (pGL3-P3A4) gene. Treatment of transfected cells with vehicle (0.1% DMSO), RIF (10 μM) or increasing concentrations of glycyrrhizin ((50, 100, 250 and 500 μM) for 36 h prior to harvest. (B) HepG2 cells were transfected with expression vectors encoding human PXR (pCMX-hPXR) and reporter plasmid (pGL3-P3A4) gene.Ttransfected cells lysates at 12 to 36 h after exposure to glycyrrhizin (250 μM). All transfections were normalized according to Renilla-luciferase, which was used as the internal control for transfection. Data represent the mean ± S.D. of three individual transfections. Asterisks indicate a statistically significant difference compared with untreated cells. (*$P < 0.05$).

have been accomplished by nuclear translocation of the PXR and formation of the transcription-activating complex resulting in binding to the PXRE sequences. To test this hypothesis, we performed an EMSA assay using nuclear extracts of glycyrrhizin- or RIF-treated HepG2 cells. After cells were treated with the indicated concentrations of glycyrrhizin for 8 h, nuclear extraction was performed and the resulting extracts were subjected to the EMSA assay. When compared to the solvent control (Fig. 4 lane 2), 10 μM RIF (positive control) caused an obvious increase in the DNA-binding ability of PXR to oligonucleotides containing the PXRE (Fig. 4 lanes 6). A significantly enhanced binding of PXR to the PXRE consensus sequence was also observed for the glycyrrhizin-treated cells with a concentration-dependent increase over the range of 100–500 μM. The maximal increase was seen at 500 μM (Fig. 4 lane 5). The specificity of this band as a PXR-mediated gel shift was confirmed by pretreatment of the nuclear extracts from cells treated with 250 μM glycyrrhizin (Fig. 4 lane 7). The binding shift was abolished in the presence of excess concentrations of unlabelled PXRE. Together with results from RT-PCR, Western-blot and reporter assay, induction of human CYP3A4 at the mRNA and protein levels by glycyrrhizin is mediated through activation of PXR and initiated at the step of PXR-PXRE binding.

3.5. Glycyrrhizin protects the liver against LCA induced liver injury

PXR has been shown to act as an LCA sensor and accelerate LCA detoxification. Thus, we postulated that glycyrrhizin-activated PXR and induced CYP3A may protect the liver against LCA-induced liver toxicity. To define the protective function of glycyrrhizin against LCA induced liver toxicity, the LCA-treated mice were simultaneously treated with glycyrrhizin or PCN, a potent PXR ligand. The livers of the treated mice were sampled and evaluated by histopathology and quantitative measures of the liver markers ALT and AST. Mice in the control group had normal ALT and AST levels (Fig. 5A and B) as well as normal histopathology with minimal hepatocellular vacuolization (Fig. 6A).

Highly elevated ALT, AST and LCA levels (Fig. 5A and B) and severe necrosis (Fig. 6B) were detected in mice after a 7-day treatment with LCA, indicating LCA-induced hepatotoxicity and cholestasis. However, the hepatotoxicity of LCA could be prevented significantly by co-administration of glycyrrhizin (Fig. 6D–F), LE (Fig. 6C) or PCN (Fig. 6G). As shown in Fig. 5, serum AST and ALT levels rapidly increased after a 7-day treatment with LCA, but the elevated serum levels of AST and ALT were markedly prevented by glycyrrhizin (10–50 mg/kg) and LE aqueous extracts (10 g/kg/ d). Glycyrrhizin at a dose of 50 mg/kg exhibited 98% prevention of induction and 87% prevention of induction for ALT and AST, respectively in the LCA group. The concentrations of LCA in serum of each group are presented in Fig. 5C. As expected, LCA treated mice had markedly elevated the concentrations of LCA in serum compared with mice in the control group. Treatment with LE extract, glycyrrhizin or PCN prevented the increase in LCA caused by LCA treatment alone. The results suggest that glycyrrhizin can prevent toxic accumulation of bile acids and attenuate the hepatocellular damage caused by LCA.

The serological changes were further confirmed by histological analysis. As shown in Fig. 6B, histological alteration of the liver in hepatectomized LCA mice was characterized by large areas of focal necrosis, hepatic steatosis and inflammatory cell infiltration in the midzone and periportal regions of the liver. In contrast, LCA-induced hepatic necrosis, steatosis and inflammatory cell infiltration were markedly prevented by glycyrrhizin, LE aqueous extracts and PCN (Fig. 6C–G). Histological examination of liver tissues from these groups showed significant LCA-related alterations comparable to the control group. The results of this in vivo study indicated
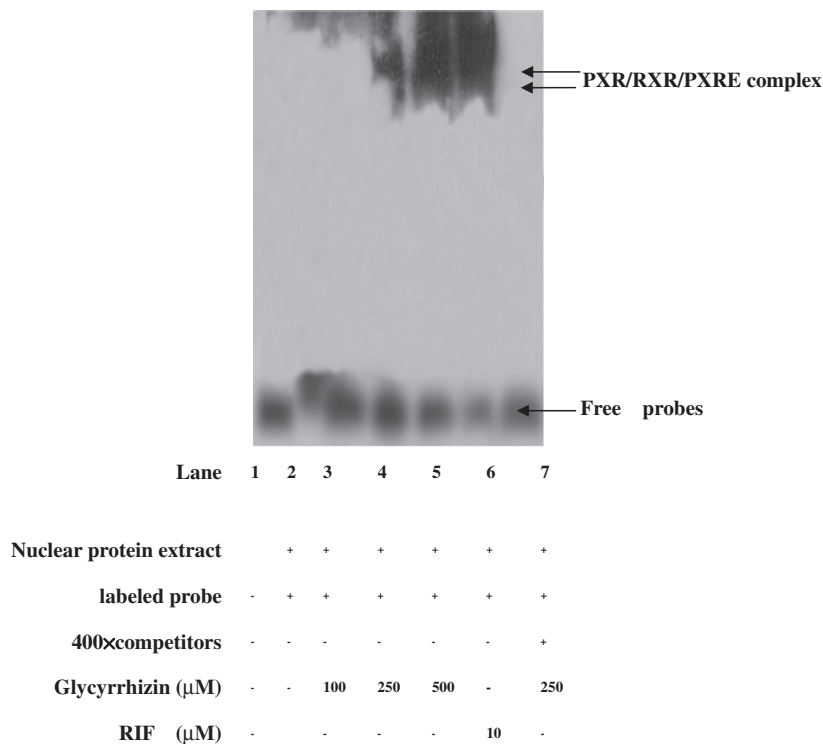
Fig. 4. Effect of glycyrrhizin and RIF on DNA-binding activity of nuclear pregnane X receptor. The double arrows indicate the specific binding of PXR complex to PXRE, which competes against in the presence of 400-fold excess of unlabelled PXRE (lane 7). The single arrow indicates the free probes. Only the labeled probes were added to the reaction system (lane 1). Results are the representative of two separate experiments.

that LE and its active component glycyrrhizin have hepatoprotective activity against LCA-induced liver toxicity.

3.6. Glycyrrhizin protects against LCA toxicity via induction of CYP3A11 and SHP and inhibition of CYP7A1

The known PXR target genes include a variety of Phase I- and Phase II-modifying enzymes and transporters. Some of these enzymes are associated with LCA formation and metabolism. For example CYP3A11, SHP and CYP7A1, have been reported to be the major genes responsible for bile acid formation and metabolism. Based on the results from the in vivo study discussed above, we further investigated the gene expressions of these enzymes in the liver tissues of mice with LCA-induced hepatic injury and mice treated with glycyrrhizin, LE or PCN to explore the possible mechanism for glycyrrhizin-induced hepatoprotection. Total liver RNA was isolated from these mice for RT-PCR analysis. Among the enzymes associated with LCA detoxification, the mRNA levels of CYP3A11 and SHP were significantly up-regulated by LCA, glycyrrhizin, LE aqueous extracts and PCN treatment, when compared to levels in saline-treated mice (Fig. 7A and B). In contrast, the CYP7A1 mRNA expression was markedly attenuated after 7 days in the same treatment groups (Fig. 7C).

4. Discussion

In this study, we demonstrated that PXR activation by glycyrrhizin, LE or PCN could result in protection of the liver against LCA hepatotoxicity via down-regulation of CYP7A1, an enzyme responsible for LCA formation, and up-regulation CYP3A11, an enzyme responsible for LCA metabolism (Fig. 8), which may reduce the accumulation of bile acids in the livers of LCA-treated mice and avoid the irreversible liver damage induced by LCA. PXR-mediated transcription regulation of bile acid associated enzymes by glycyr-

rhizin is one of the mechanisms resulting in hepatoprotective activity against LCA-induced injury. It is well recognized that CYP3A4 gene expression can be induced by numerous xenobiotics, resulting in altered drug metabolism and drug–drug interactions in addition to enhanced metabolism of endogenous substrates [23]. The nuclear hormone receptor PXR serves as a key regulator of CYP3A4 transcription and mediates transcriptional induction of CYP3A4 by many xenobiotics and endobiotics [24]. Therefore, PXR activation with CYP3A induction plays an important role in drug–drug interactions, and physiological homeostasis, such as bile acid homeostasis.

Glycyrrhizin, a natural compound extracted from the roots of licorice, has been used for more than two decades to treat hepatitis in Japan and no side effect or toxicity has been observed [25]. Early studies including our study (data not shown) revealed that licorice could induce CYP3A enzyme activity in the rat livers. However, the molecular mechanisms underlying the effects of licorice have not been explored thoroughly. Therefore, it is necessary to investigate whether the CYP3A induction by licorice and its major active component glycyrrhizin is mediated by PXR and to determine the correlation between the glycyrrhizin-induced CYP expression and its hepatoprotective effect. In the present study, we investigated the mechanism of CYP3A induction by glycyrrhizin in HepG2 cells, and the role of glycyrrhizin in regulating CYP genes involved in bile acid biosynthesis and metabolism was examined as a possible mechanism for hepatoprotection.

We began the investigation by examining the effects of glycyrrhizin treatment on CYP3A4 mRNA expression in HepG2 cells. RT-PCR analysis revealed that glycyrrhizin up-regulated CYP3A4 mRNA expression. In order to determine whether the induction of CYP3A4 mRNA expression was accompanied by a simultaneous change in CYP3A4 protein level, western blot analysis was performed. The results showed that CYP3A4 protein levels were also increased by glycyrrhizin in a dose dependent manner. These
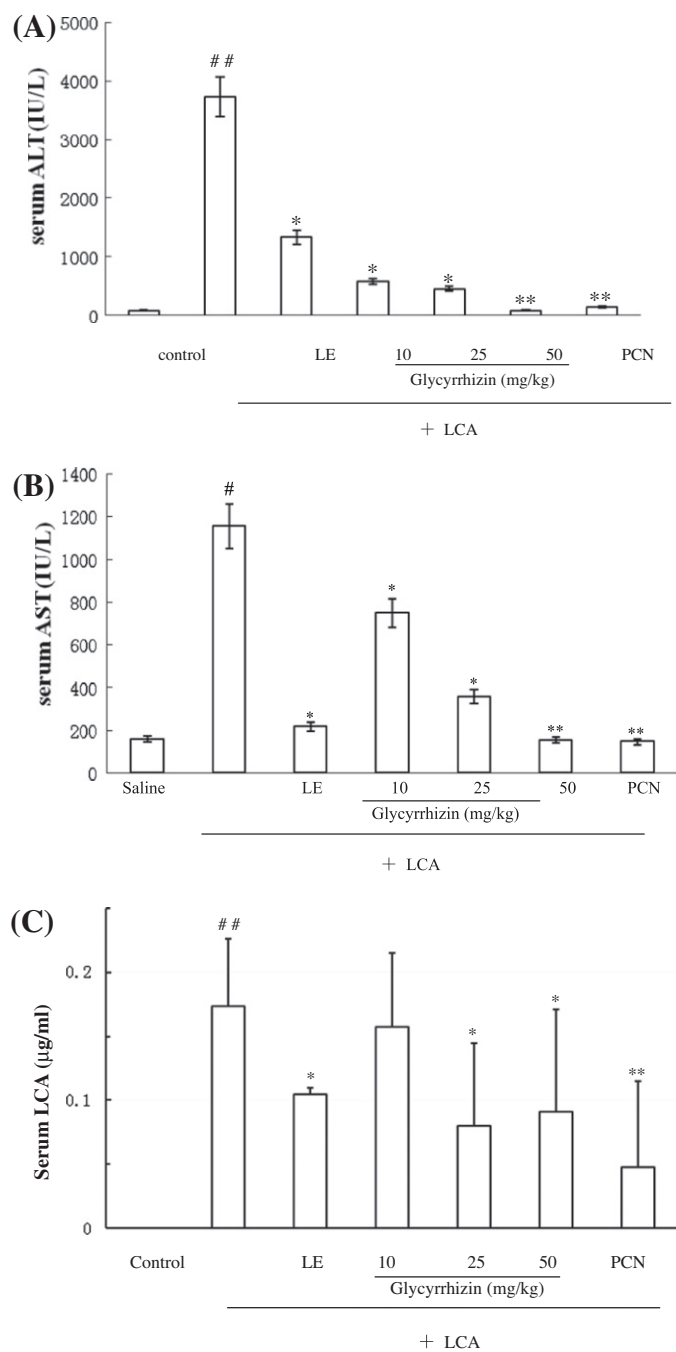
(A)

(B)

(C)

Fig. 5. Inhibitory effects of Glycyrrhizin and Licorice on the LCA-induced elevation of serum transaminase (A) ALT level. (B) AST level. (C) Serum LCA concentration. All animals were pretreated with testing inducers (Licorice, 10 g/kg/d; Glycyrrhizin, 10, 25 and 50 mg/kg; PCN, 42 mg/kg) or vehicle for the first 3 days via i.p. injection. Starting on day 4, LCA (125 mg/kg) treatment was initiated with i.p. injections co-administrated with the inducers twice daily for the remaining 4 days. Data represent the mean ± S.D., $n = 6$. $*P < 0.05$, $**P < 0.01$ vs. LCA group; $\#P < 0.05$, $\#\#P < 0.01$ vs. control group.

Fig. 6. The PXR mediated protective properties of glycyrrhizin and licorice against LCA-induced hepatotoxicity. Mice were treated with vehicle alone (A), LCA (B), LCA and LE (C), LCA and glycyrrhizin (D, E and F) or LCA and PCN (G). Pictures are representative of pathology for treated groups with a minimum of two animals per group. 20× magnification. Liver sections were prepared for histology and stained with hematoxylin and eosin. Areas of sever necrosis in panels C, D and E are indicated with arrows.

observations indicate that glycyrrhizin interacts with the CYP3A enzyme at the transcriptional level in HepG2 cells and presumably also in the normal human liver.

It is well-established that PXR is a key regulator of xenobiotic-induced CYP3A gene expression [24] Transfection studies in HepG2 cells were carried out based on the above noted CYP transcription findings to determine activation of human PXR by glycyrrhizin. A clear concentration- and time-response were observed between glycyrrhizin t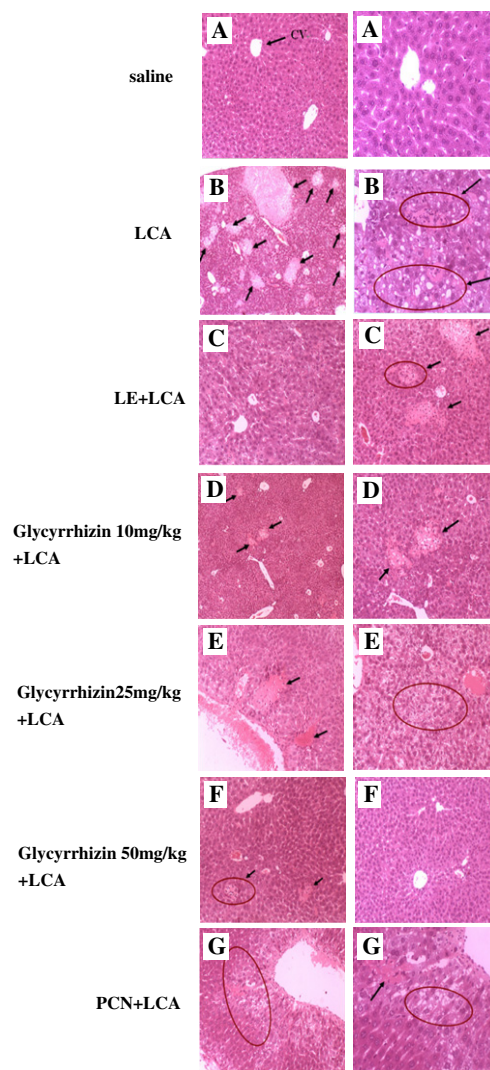reatment and activity of the reporter gene. The activation of PXR by glycyrrhizin at the highest concentrations examined was comparable to that of 10 μM rifampicin, a well known PXR ligand, indicating that glycyrrhizin is effective in activating human PXR in HepG2 cells.

To further ascertain whether transcriptional activation of the CYP3A4 gene by glycyrrhizin was caused by activation of the PXR-PXRE complex in the nucleus, we performed an EMSA assay to test the interaction between glycyrrhizin and PXR complexes. The results confirmed that glycyrrhizin caused an obvious activation of human PXR. These data, in conjunction with the observed increases in CYP3A4 mRNA expression, protein expression and reporter activity suggest that the induction of CYP3A expression by glycyrrhizin or licorice is most likely due to their abilities to interact directly with the receptor and function as PXR ligands. Numerous herbal remedies including St. John's wort, coleus forskohlii, guggulsterone and many others that contain constituents can activate PXR [26]. However, ligand binding assays would be necessary to confirm direct competitive binding to the receptor in future studies. Agonist activity of glycyrrhizin, even at the highest
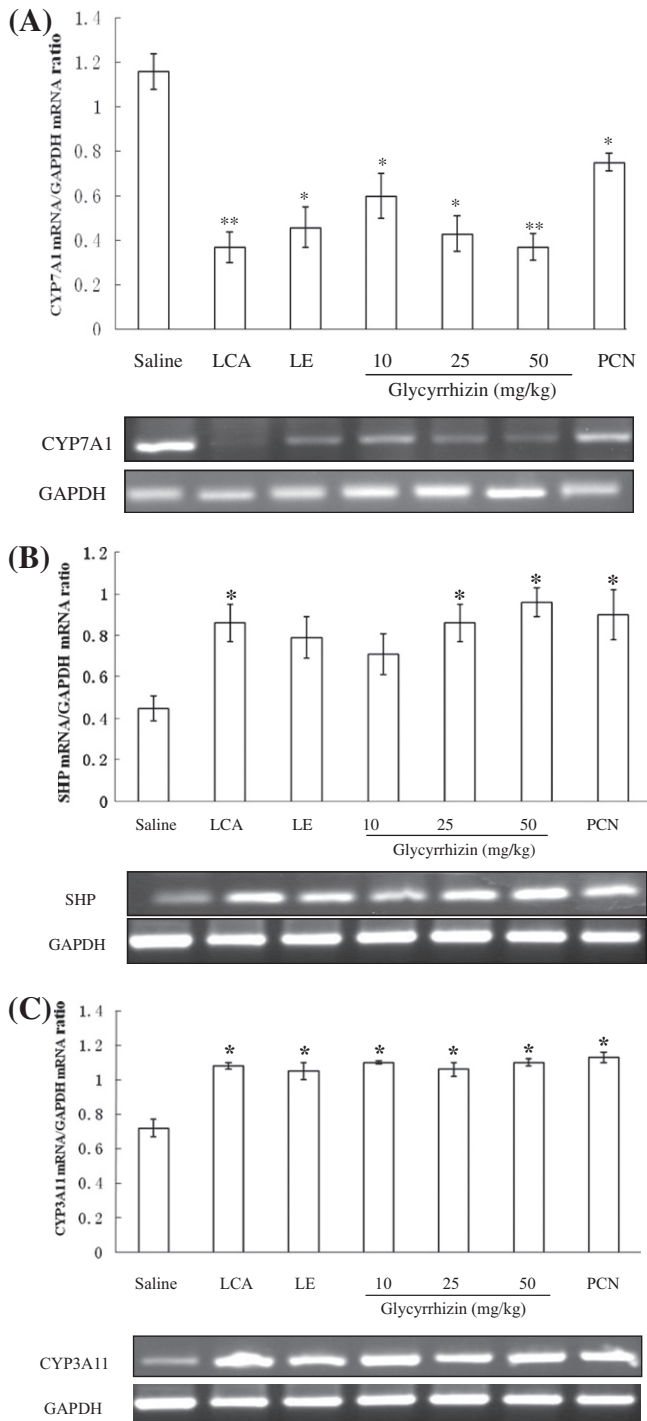
(A)

(B)

(C)

Fig. 7. Expression of bile acid synthesis and metabolism-related genes. (A) CYP7A1 expression in LCA-induced liver injury mice or Licorice, glycyrrhizin and PCN treatment. (B) SHP expression in LCA-induced liver injury mice or Licorice, glycyrrhizin and PCN treatment. (C) CYP3A11expression in LCA-induced liver injury mice or Licorice, glycyrrhizin and PCN treatment. RT-PCR was used to determine the effects of LCA, Licorice, glycyrrhizin and PCN treatment on the levels of CYP7A1, SHP and CYP3A11 mRNA in liver tissues of mice relative to the housekeeping gene GAPDH. Data represent the mean ± S.D., $n = 6$. $^*P < 0.05$, $^{**}P < 0.01$ vs. control group.

Fig. 8. Schematic illustration of the action of the positive and negative regulatory relationship between the genes CYP7A1, SHP and CYP3A11 in response to Glycyrrhizin and LCA in the pathway from cholesterol to bile acid secretion.

humans as indicated by the pharmacokinetics of the model substrate midazolam, which caused a significant reduction in midazolam area under curve (AUC) (increase in oral midazolam clearance) [27]. The inductive effect of the licorice extract is likely mediated by the activation of PXR.

PXR also play an important role in many essential physiological functions. For example, PXR is involved in regulation of the biosynthesis, transport, and metabolism of bile acids. Licorice is known to have hepatoprotective and/or general detoxifying properties [28]. Early studies suggested that glycyrrhizin also has hepatoprotective properties in liver injury models [29,30]. To reveal glycyrrhizin-mediated protective mechanisms for bile acid-induced hepatotoxicity, we used LCA-treated mice as a hepatotoxicity model, because LCA concentrations of 5–10 μM have been detected in the liver tissue of cholestatic patients and in rat models of biliary cholestasis [31]. As expected, treatment of mice with LCA resulted in severe liver damage manifested by the appearance of severe necrosis and high serum levels of ALT, AST and LCA concentration. The detrimental effects of LCA on mouse livers were significantly prevented by the co-treatment with glycyrrhizin or licorice extract. Thus, glycyrrhizin or LE can protect the liver tissue in mice from the harmful effects of LCA. The doses of glycyrrhizin used in vivo (10–50 mg/kg/d) [29] did not elicit any signs of toxicity in the present study including body weight, serum biochemical indices and blood parameters (data not shown). The dose (10 mg/kg/d) used in animals is 5-fold higher than compared to those under normal human exposure from licorice.

Previous study showed that toxic bile acids activate PXR and regulate transcription of genes involved in biosynthesis and metabolism of bile acids, such as CYP7A1 and CYP3A genes [32,33]. CPY7A1 is the hepatic enzyme for the conversion of cholesterol to bile acid in the neutral way and also plays a key role in cholesterol/bile acid homeostasis [34]. SHP interacts with liver receptor homologue-1 and/or HNF-4, which leads to transcriptional suppression of CYP7A1. Lower levels of CYP7A1 result in reduced bile acid production by decreasing the conversion of cholesterol into bile acids. When the excretion of bile acids is disrupted by disease, bile acids accumulate in hepatocytes, resulting in cholestasis [35]. CYP3A4 (CYP3A11 rodent homolog) participates in bile acid detoxification via 6α-hydroxylation of LCA [36]. The addition of this hydroxyl group makes the molecule more hydrophilic, which promotes elimination. Changes in bile acid biosynthesis and metabolism appear to be important in protecting against cholestatic liver injury. The aim is to elucidate whether the mechanism of glycyrrhizin-mediated hepatoprotection in cholestasis correlates with PXR-mediated regulation of bile acid

concentration treated, was much less than that of RIF. This suggests that glycyrrhizin possesses weak PXR agonist activity. Our previous study showed that the whole licorice extract increased CYP3A enzyme activity in rats in a concentration-dependent manner (data not shown). Recent studies have shown that administration of glycyrrhizin resulted in a modest induction of CYP3A in
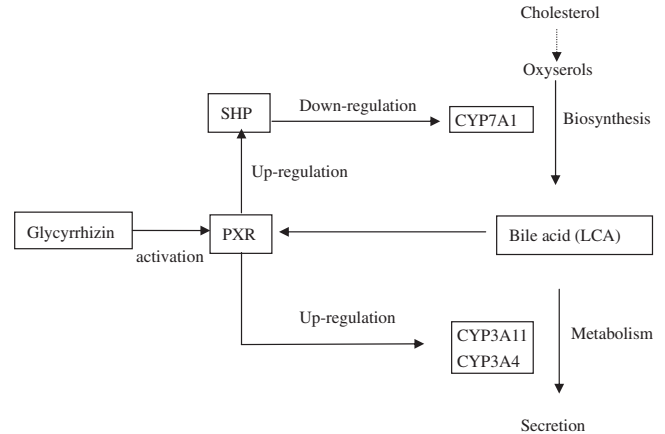
biosynthesis and metabolism. The present study revealed that CYP3A11 and SHP mRNA expression were significantly up-regulated by LCA, glycyrrhizin and LE extracts. As expected, CYP7A1 mRNA expression was markedly attenuated by LCA, glycyrrhizin and LE extracts. The results presented in Fig. 7 show that LCA and glycyrrhizin exhibit effects in the same direction. PXR is activated by LCA and coordinately regulates genes involved in the biosynthesis, transport and metabolism of LCA. LCA has been proven to be sensor of PXR that protects against liver toxicity. Excess concentration of LCA induces expression of CYP3A11 and decreases expression of CYP7A1 in the present study, which is consistent with results from Staudinger [17]. In vivo LCA treatment induces expression of CYP3A11 and reduces expression of CYP7A1, which is a type of negative feedback regulation of bile acid in protecting the liver against pathophysiological levels of LCA. We propose that the activation of PXR by glycyrrhizin results in inhibition CYP7A1 expression, which blocks bile acid biosynthesis. At the same time, the activation of PXR by glycyrrhizin induced expression of CYP3A11, which may promote the metabolism of LCA. These results indicated that the hepatoprotective function of glycyrrhizin against LCA-induced hepatotoxicity was due to the decrease in LCA concentration. The mechanism was mediated by PXR-regulated transcription of genes responsible for biosynthesis and metabolism of bile acids, such as CYP7A1 and CYP3A.

In conclusion, our study showed that glycyrrhizin can activate PXR and bind to CYP3A4 transcriptional region to induce CYP3A4 gene expression at mRNA and protein levels. This is the first report showing that the hepatoprotective and anti-hepatotoxicity effects of LE or glycyrrhizin are due to PXR-mediated CYP3A11 and SHP up-regulation and CYP7A1 gene down-regulation. The resultant regulation of drug-metabolizing enzymes may account for increased LCA metabolism and reduce its biosynthesis in LCA-induced liver injury in mice. The expression of many genes involved in xenobiotic/drug metabolism and transport is regulated by at least three nuclear receptors or xenosensors: aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and PXR. These receptors establish a crosstalk with other nuclear receptors or transcription factors controlling signaling pathways that regulate the homeostasis of bile acids, lipids, glucose, inflammation, vitamins, hormones, and others [37]. Further investigations will focus on whether other nuclear receptors contribute to the glycyrrhizin-mediated hepatoprotection through the regulation of the CYP3A gene.

References

[1] R.A. Isbrucker, G.A. Burdock, Risk and safety assessment on the consumption of Licorice root (Glycyrrhiza sp.) its extract and powder as a food ingredient with emphasis on the pharmacology and toxicology of glycyrrhizin, Regul. Toxicol. Pharmacol. 46 (2006) 167–192.

[2] D. Armanini, C. Fiore, M.J. Mattarello, J. Bielenberg, M. Palermo, History of the endocrine effects of licorice, Exp. Clin. Endocrinol. Diabetes 110 (2002) 257–261.

[3] Z.Y. Wang, D.W. Nixon, Licorice and cancer, Nutr. Cancer 39 (2001) 1–11.

[4] T. Okamoto, The protective effect of glycyrrhizin on anti-Fas antibody-induced hepatitis in mice, Eur. J. Pharmacol. 387 (2000) 229–232.

[5] T. Okamoto, T. Kanda, Glycyrrhizin protects mice from concanavalin A-induced hepatitis without affecting cytokine expression, Int. J. Mol. Med. 4 (1999) 149–152.

[6] J. Yin, D. Li, W. Hu, Q. Meng, Effects of glycyrrhizic acid on cocklebur-induced hepatotoxicity in rat and human hepatocytes, Phytother. Res. 22 (2008) 395–400.

[7] B. Tang, H. Qiao, F. Meng, X. Sun, Glycyrrhizin attenuates endotoxin induced acute liver injury after partial hepatectomy in rats, Braz. J. Med. Biol. Res 40 (2007) 1637–1646.

[8] C. Yu, S. Ye, H. Sun, Y. Liu, L. Gao, C. Shen, S. Chen, S. Zeng, PXR-mediated transcriptional activation of CYP3A4 by cryptotanshinone and tanshinone IIA, Chem. Biol. Interact. 177 (2009) 58–64.

[9] K. Lichti-Kaiser, J.L. Staudinger, The traditional chinese herbal remedy tian xian activates pregnane X receptor and induces CYP3A gene expression in hepatocytes, Drug Metab. Dispos. 36 (2008) 1538–1545.

[10] L. Li, J.D. Stanton, A.H. Tolson, Y. Luo, H. Wang, Bioactive terpenoids and flavonoids from Ginkgo biloba extract induce the expression of hepatic drug-metabolizing enzymes through pregnane X receptor constitutive androstane receptor and aryl hydrocarbon receptor-mediated pathways, Pharm. Res. 26 (2009) 872–882.

[11] D.G. Teotico, J.J. Bischof, L. Peng, S.A. Kliewer, M.R. Redinbo, Structural basis of human pregnane X receptor activation by the hops constituent colupulone, Mol. Pharmacol. 74 (2008) 1512–1520.

[12] E.L. LeCluyse, Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics, Chem. Biol. Interact. 134 (2001) 283–289.

[13] J.M. Lehmann, D.D. McKee, M.A. Watson, T.M. Willson, J.T. Moore, S.A. Kliewer, The human orphan nuclear receptor PXR is activatedby compounds that regulate CYP3A4 gene expression and cause drug interactions, J. Clin. Invest. 102 (1998) 1016–1023.

[14] M. Paolini, L. Pozzetti, A. Sapone, G. Cantelli-Forti, Effect of licorice and glycyrrhizin on murine liver CYP-dependent monooxygenases, Life Sci. 62 (1998) 71–582.

[15] D. Jung, D.J. Mangelsdorf, U.A. Meyer, Pregnane X Receptor is a target of farnesoid X Receptor, J. Biol. Chem. 281 (2006) 19081–19091.

[16] J. Trottier, P. Milkiewicz, J. Kaeding, M. Verreault, O. Barbier, Coordinate regulation of hepatic bile acid oxidation and conjugation by nuclear receptors, Mol. Pharm. 3 (2006) 212–222.

[17] J.L. Staudinger, B. Goodwin, S.A. Jones, D. Hawkins-Brown, K.I. MacKenzie, A. LaTour, Y. Liu, C.D. Klaassen, K.K. Brown, J. Reinhard, T.M. Willson, B.H. Koller, S.A. Kliewer, The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity, Proc. Natl. Acad. Sci. USA 98 (2001) 3369–3374.

[18] W. Xie, A. Radominska-Pandya, Y. Shi, C.M. Simon, M.C. Nelson, E.S. Ong, D.J. Waxman, R.M. Evans, An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids, Proc. Natl. Acad. Sci. USA 98 (2001) 3375–3380.

[19] B. Goodwin, E. Hodgson, C. Liddle, The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module, Mol. Pharm. 56 (1999) 1329–1339.

[20] L.D. Beilke, L.M. Aleksunes, E.R. Olson, D.G. Besselsen, C.D. Klaassen, K. Dvorak, N.J. Cherrington, Decreased apoptosis during CAR-mediated hepatoprotection against lithocholic acid-induced liver injury in mice, Toxicol. Lett. 188 (2009) 38–44.

[21] D.X. Xu, W. Wei, M.F. Sun, C.Y. Wu, J.P. Wang, L.Z. Wei, C.F. Zhou, Kupffer cells and reactive oxygen species partially mediate lipopolysaccharide-induced downregulation of nuclear receptor pregnane x receptor and its target gene CYP3a in mouse liver, Free Radic. Biol. Med. 37 (2004) 10–22.

[22] X.F. Huang, L.W. Chen, N. Liu, W.D. Huang, The expression of bile acid metabolism related genes Cyp7A1 and SHP in the early stage of liver regeneration in mice, J. Fujian Med. Univ. 141 (2007) 495–497.

[23] B.W. Cooper, T.M. Cho, P.M. Thompson, A.D. Wallace, Phthalate induction of CYP3A4 is dependent on glucocorticoid regulation of PXR expression, Toxicol. Sci. 103 (2008) 268–277.

[24] S.A. Kliewer, B. Goodwin, T.M. Willson, The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism, Endocr. Rev. 23 (2002) 687–702.

[25] T.G. van Rossum, A.G. Vulto, W.C. Hop, S.W. Schalm, Chronic hepatitis C and portal hypertension: diagnosis treatment with interferon glycyrrhizin or propranolo, J. Gastroenterol. Hepatol. 16 (2001) 789–795.

[26] J.L. Staudinger, X. Ding, K. Lichti, Pregnane X receptor and natural products: beyond drug–drug interactions, Expert. Opin. Drug Metab. Toxicol. 2 (2006) 847–857.

[27] J.H. Tu, Y.J. He, Y. Chen, L. Fan, W. Zhang, Z.R. Tan, Y.F. Huang, D. Guo, D.L. Hu, D. Wang, H.H. Zhou, Effect of glycyrrhizin on the activity of CYP3A enzyme in human, Eur. J. Clin. Pharmacol. 66 (2010) 805–810.

[28] Y. Mu, J. Zhang, S. Zhang, H.H. Zhou, D. Toma, S. Ren, L. Huang, M. Yaramus, A. Baum, R. Venkataramanan, W. Xie, Traditional Chinese medicines Wu Wei Zi (Schisandra chinensis Baill) and Gan Cao (Glycyrrhiza uralensis Fisch) activate pregnane X receptor and increase warfarin clearance in rats, J. Pharmacol. Exp. Ther 316 (2006) 1369–1377.

[29] T. Yoshida, K. Abe, T. Ikeda, T. Matsushita, K. Wake, T. Sato, H. Inoue, Inhibitory effect of glycyrrhizin on lipopolysaccharide and d-galactosamine-induced mouse liver injury, Eur. J. Pharmacol. 576 (2007) 136–142.

[30] N. Tsuruoka, K. Abe, K. Wake, M. Takata, A. Hatta, T. Sato, H. Inoue, Hepatic protection by glycyrrhizin and inhibition of iNOS expression in concanavalin A-induced liver injury in mice, Inflamm. Res. 58 (2009) 593–599.

[31] K.D. Setchell, C.M. Rodrigues, C. Clerici, A. Solinas, A. Morelli, C. Gartung, J. Boyer, Bile acid concentrations in human and rat liver tissue and in hepatocyte nuclei, Gastroenterology 112 (1997) 226–235.

[32] B. Goodwin, K.C. Gauthier, M. Umetani, M.A. Watson, M.I. Lochansky, J.L. Collins, E. Leitersdorf, D.J. Mangelsdorf, S.A. Kliewer, J.J. Repa, Identification of bile acid precursors as endogenous ligands for the nuclear xenobiotic pregnane X receptor, Proc. Natl. Acad. Sci. USA 100 (2003) 223–228.

[33] E.G. Schuetz, S. Strom, K. Yasuda, V. Lecureur, M. Assem, C. Brimer, J. Lamba, R.B. Kim, V. Ramachandran, B.J. Komoroski, R. Venkataramanan, H. Cai, C.J. Sinal, F.J. Gonzalez, J.D. Schuetz, Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters and cytochrome P450, J. Biol. Chem. 276 (2001) 39411–39418.

[34] J.Y. Chiang, Bile acid regulation of gene expression: roles of nuclear hormone receptors, Endocr. Rev. 23 (2002) 443–463.

[35] L.D. Beilke, L.M. Aleksunes, R.D. Holland, D.G. Besselsen, R.D. Beger, C.D. Klaassen, N.J. Cherrington, Constitutive androstane receptor-mediated changes in bile acid composition contributes to hepatoprotection from lithocholic acid-induced liver injury in mice, Drug Metab. Dispos. 37 (2009) 1035–1045.

[36] Z. Araya, K. Wikvall, 6alpha-hydroxylation of taurochenodeoxycholic acid and lithocholic acid by CYP3A4 in human liver microsomes, Biochim. Biophys. Acta 1438 (1999) 47–54.

[37] J.M. Pascussi, S. Gerbal-Chaloin, C. Duret, M. Daujat-Chavanieu, M.J. Vilarem, P. Maurel, The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences, Annu. Rev. Pharmacol. Toxicol. 48 (2008) 1–32.