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

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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.



## 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

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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