Pharm Res (2015) 32:538–548
DOI 10.1007/s11095-014-1483-3

RESEARCH PAPER

Protective Effects of Calycosin Against CCl_4-Induced Liver Injury with Activation of FXR and STAT3 in Mice

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Received: 12 June 2014 / Accepted: 15 August 2014 / Published online: 21 August 2014

ABSTRACT

Purpose Investigating the hepatoprotective effect of calycosin against acute liver injury in association with FXR activation and STAT3 phosphorylation.

Methods The acute liver injury model was established by intraperitoneal injection of CCl_4 in C57BL/6 mice. Serum alanine aminotransferase, aspartate aminotransferase, HE staining and TUNEL assay were used to identify the amelioration of the liver histopathological changes and hepatocytes apoptosis after calycosin treatment. ELISA kit and 5-bromo-2-deoxyuridine immunohistochemistry were used to measure the liver bile acid concentration and hepatocyte mitotic rate *in vivo*. The relation between calycosin and activation of FXR and STAT3 was comfirmed using the Luciferase assay, Molecular docking, Real-time PCR and Western Blot *in vitro*.

Results The liver histopathological changes, hepatocytes apoptosis, liver bile acid overload and hepatocyte mitosis showed significant changes after calycosin treatment. Calycosin promoted the expression of FXR target genes such as FoxM1B and SHP but the effect was reversed by FXR suppressor guggulsterone. Molecular docking results indicated that calycosin could be embedded into the binding pocket of FXR, thereby increasing the expressions of STAT3 tyrosine phosphorylation and its target genes, Bcl-xl and SOCS3.

Conclusions Calycosin plays a critical role in hepatoprotection against liver injury in association with FXR activation and STAT3 phosphorylation.

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KEY WORDS acute liver injury · bile acid metabolism · cholesterol 7α-hydroxylase · farnesoid X receptor · STAT3

ABBREVIATIONS

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BA	Bile acid
Bcl-xl	B-cell lymphoma-extra large
BSEP	Bile salt export pump
CNTF	Ciliary neurotrophic factor
CYP450	Cytochrome P450
CYP7A1	Cytochrome P450 7A1
FoxM1B	Forkhead box M1B
FXR	Farmesoid X receptor
LIF	Leukemia inhibitory factor
SHP	Small heterodimer partner
SOCS3	Suppressor of cytokine signaling 3
STAT3	Signal transducer and activator of transcription 3
TCMs	Traditional Chinese medicines

INTRODUCTION

The liver represents the primary target for chemical toxic reaction due to its vital role in metabolism and in the excretion of heterogenous compounds. Drug overdose, ionizing radiations and environmental pollutants are capable of damaging hepatocytes' membrane and organelles (1). Due to drug-induced liver injury (DILI), the U.S. Food and Drug Administration (FDA) polled out some drugs from the market such as bromfenac, ebrotidine, and troglitazone. Certain hepatotoxic drugs such as risperidone, trovafloxacin, and nefazodone were assigned a "black box" warning (2,3). Carbon-tetrachloride (CCl_4), an hepatotoxin is used to induce hepatic steatosis in experiment model. Its biochemical and histological patterns are very similar to human liver cirrhosis. CCl4 is metabolized

by cytochrome P450 (CYP450) in the liver. CYP 450 enzymes have different isoforms that are involved in the metabolism of the exogenous toxicant (4) caused by the necrosis of parenchymal cells and hepatic fibrosis (5).

Many genes and pathways have been identified as the main targets to regulate liver repair. Farnesoid X receptor (FXR, NR1H4), initially cloned in 1995, belongs to the hormone nuclear receptor superfamily. It is expressed mostly in the liver, intestine, kidney and adrenal glands (6,7). FXR had been reported as a ligand-activated transcription factor regulating the process of liver regeneration (8). The FXR knockout mice produced a low rate of liver regeneration after 70% partial hepatectomy compared to the wild mice (8). FXR is adeqaute to accelerate hepatocytes proliferation in old-aged mice by directly activating FoxM1B transcription (9). Concisely, FXR acts as the primary bile acid (BA) sensor to release the stress of BA overload. It transforms BA into promotion force for liver repair (8,10). Several studies have demonstrated that a synthetic FXR agonist, GW4064, prevents liver injury by decreasing the endogenous synthesis of BA while increasing its biotransformation and excretion (11,12). When combined, these novel findings provide evidences for the critical role of FXR in liver regeneration, liver injury, BA metabolism and chemoprotection (7).

Signal transducers and activators of transcription 3 (STAT3) is a cytoplasmic signal transcription factor that belongs to the signal transducers and activators of transcription family (STATs) (13). STAT3 is activated by various cytokines and growth factors which are interleukin-6 (IL-6), leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) (14,15). Investigations revealed that STAT3 has positive properties of promoting liver regeneration, maintaining carbohydrate and lipid homeostasis (16,17). In a recent study, BA showed a specific effect on STAT3 activation after liver injury. The overload of BA represses the tyrosine phosphorylation of STAT3 which is the active form of STAT3 (8). Thus, attenuating BA overload and activating STAT3 phosphorylation may be beneficial strategies against the CCl_4 induced acute liver injury.

Many modern medicines such as N-Acetyl-L-cysteine and L-carnitine have been used to treat ALI (18). However, achieving therapeutic goals are difficult due to their side effects. On the contrary, Traditional Chinese medicines (TCMs) provide many potential biologically active substances that are more advantageous in the treatment of human diseases (19,20). Calycosin is a major active component in Radix Astragali which has demonstrated good clinical effects on liver protection such as antifibrotic properties. Its chemical structure is shown in Fig. 1 (21,22). A previous study revealed that an active constituent of Radix Astragali significantly inhibited the progression of hepatic fibrosis by scavenging free radicals induced by CCl_4 (23). Calycosin also inhibits apoptosis (24). However, the effect of Calycosin on acute liver injury induced by CCl_4 has never been reported. The present study was designed to investigate the hepatoprotective effect and the possible mechanisms of calycosin against CCl_4-induced acute liver injury.

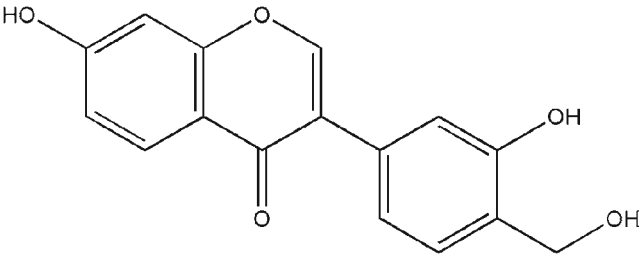

Fig. 1 The chemical structure of calycosin.

MATERIALS AND METHODS

Animals

Male (28–32 g) C57BL/6 mice used in this study were purchased from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number: SCXK 2008–0002). The mice were housed in laboratory animal facilities under a standard 12-h light/dark cycle, the mice were provided with food and tap water. Normal group and calycosin-only group were injected with 10 mL/g of olive oil while the other groups were injected with a single intraperitoneal injection of CCl_4 (10 mL/kg, 0.1% (v/v) in olive oil). Calycosin groups were pretreated with calycosin (Must Bio-technology, Chengdu, China, two times a day, 12.5 mg/kg, 25 mg/kg and 50 mg/kg each time) orally for 48 h and subsequently injected with CCl_4 intraperitoneally. Administered calycosin orally at each timed intervals. Pentobarbital sodium (65 mg/kg, i.p.) was used to anesthetize the mice. At timed intervals (24, 48 and 72 h after CCl_4 injection), animals from each treatment group were killed ($n=6$/group). Blood was collected and processed to obtain the serum. Their livers were harvested and either in formalin or frozen in liquid nitrogen. Experiments were performed according to the local institutional guidelines on the care and use of laboratory animals.

Cell Culture

HepG-2 human hepatocellular carcinoma cell line were purchased from KeyGEN BioTECH (Nanjing, china) and were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Carlsbad, CA), supplemented with 10% fetal bovine serum (Gibco BRL, Carlsbad, CA), 100 U/ml penicillin (Sigma, St. Louis, MO, USA) and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere composed of 5% CO2.

Liver Histology

The specimens from different lobes of the livers were fixed in 10% formaldehyde (neutral buffered formalin), dehydrated and embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin & eosin (H&E) as well as 5-bromo-2-deoxyuridine for morphological and morphometric analyses. Liver injury detected by H&E was the basis of morphological criteria as previously described (25). For BrdU staining, animals were injected intraperitoneally with 50 mg/kg BrdU (BrdU; B5002, Sigma-Aldrich, Zwijndrecht, Netherlands) 2 h prior to the killings. Immunohistochemistry of incorporated BrdU was performed as previously described (26). The total numbers of BrdU-positive cells were counted in at least 10 randomly selected fields for each tissue section.

Hepatic Bile Acids and Serum Biochemistry Measurements

In regards to the bile acids (BAs) measurement in the liver, tissues specimens (50 mg) were mixed with PBS (0.01 mol/L, pH 7.0) and then centrifuged at 12,000 g per min at 4°C. Total BAs in supernatant were analyzed by total bile acids using ELISA kit (Roche, Indianapolis, Indiana, USA) according to the manufacturer's protocol. Serum ALT and AST levels were determined using the plasma collected from suborbital veins. Results obtained were the mean of ten different animal's liver per time point.

Protein Isolation and Western Blot

Liver tissues were homogenized in protein lysis buffer containing 1 mM PMSF. The protein concentration was determined by following by the bicinchoninic acid procedure that uses bovine serum albumin as the standard (BCA; Solarbio, China). Proteins were denatured with an equal volume of 2× Loading buffer in boiling water for 5 min. Equal amount of proteins were loaded and separated in sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) by electrophoresis and transferred onto PVDF membranes by electroblotting. These proteins were incubated overnight at 4°C with primary antibodies (Table I). All secondary antibodies were purchased from Bioworld Technology (Minnesota, USA). The results were detected by an enhanced chemiluminescence (ECL) method using Bio-Spectrum Gel Imaging System (UVP, USA).

Quantitative Real-Time PCR

Quantitative real-time PCR was used to confirm the mRNA expression levels. The sequence of the primers in mice is shown in Table II. Total liver RNA was extracted with RNAiso Plus® Reagent Kit (Takara Biotechnology, Dalian, China). cDNA was produced from RNA using PrimeScript® RT Reagent Kit with gDNA Eraser (Takara Biotechnology, Dalian, China), and was amplified by SYBR® Premix Ex Taq™ Kit (Takara Biotechnology, Dalian, China). Thermal cycling was performed in the ABI PRISM® 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The change of fold for the genes relative to the control was calculated using the $2^{-\Delta\Delta CT}$ method with β-actin as the normalization controls.

Table I The Information of the Antibodies Used in the Present Work

Antibody	Source	Dilutions	Company
β-actin	Rat	1:1000	Proteintech group, chicago, USA
Cyclin D1	Rat	1:1000	Proteintech group, chicago, USA
Cyclin B1	Rabbit	1:1000	Proteintech group, chicago, USA
FoxM1B	Rabbit	1:1000	Proteintech group, chicago, USA
STAT3	Rabbit	1:1000	Bioworld Technology, USA
p-STAT3	Rabbit	1:500	Bioworld Technology, USA

TUNEL Assay

Liver tissue samples embedded in paraffin were prepared, and used for TUNEL assay. We utilized the apoptosis detection kit according to the manufacturer's instructions (*In Situ* Cell Death Detection Kit, Fluorescein, Roche, Basle, Sweden). 10 microscopic fields within the view were randomly selected, and the positive cells (green spots) were counted. The extent of damage was evaluated using the average number of positive cells.

Plasmid Construction, Transient Transfection and Reporter Luciferase Assay

Human FXR expression plasmid was constructed by cloning genes encoding human FXR into pCI-neo Mammalian Expression Vector (Promega, Madison, WI, USA). Human bile salt export pump (BSEP) promoter was constructed by cloning a genomic DNA fragment upstream of the transcription start site (−1000 to +85) into the luciferase vector pGL4.14 [luc2/Hygro] (Promega, Madison, WI, USA) as previously described (27). For vitro transfection study, HepG2 cells were seeded in 24-well plates at a density of 5×10^5 cells/well in DMEM media supplemented with 10% FBS and cultured for 24 h. Then, the cells were cultured in serum-free DMEM medium for 4 h, and were transiently transfected with 100 ng of FXR expression plasmid, 100 ng of BSEP promoter luciferase reporter vector and 10 ng of the null-Renilla luciferase plasmid as an internal control. 24 h after transfection, drugs of different concentrations were added and incubated for another 24 h. Finally, following the manufacturer's protocol for luciferase assay (Promega, Madison, WI, Technical Bylletin), the luciferase activities were determined with a Dual-

Table II The Primer Sequences Used for Real-Time PCR Assay in Mice

Gene	GenBank accession	Forward primer (5′-3′)	Reverse primer (5′-3′)
Cyclin D1	NM_007631.2	TACCGCACAACGCACTTTC	AAGGGCTTCAATCTGTTCCTG
Cyclin B1	NM_172301.3	CACCCCTGGVATCTTCTCCTT	AGCGTCTTCAGAGACAGCCAG
FoxM1B	NM_008021.4	ACCTGGAGCAGAATCGGGTTA	CAGTGCTGTTGATGGCAAACTGTA
SHP	NM_011850.2	GTCTTTCTGGAGCCTTGAGCTG	GTAGAGGCCATGAGGAGGATTC
CYP7A1	NM_007824.2	CAAGAACCTGTACATGAGGGAC	CACTTCTTCAGAGGCTGCTTTC
BSEP	NM_021022.3	AGCAGGCTCAGCTGCATGAC	AATGGCCCGAGCAATAGCAA
Bcl-xl	NM_009743.4	GAGAGGCAGGCGATGAGTTT	CGATGCGACCCCAGTTTACT
SOCS3	NM_007707.3	GCGGGCACCTTTCTTATCC	GGAACTGGCTGCGTGCTT
β-actin	NM_007393.3	TATTGGCAACGAGCGGTTC	ATGCCACAGGATTCCATACCC

Luciferase Reporter assay system as described previously by Berthold Technologies microplate luminometer (PerkinElmer Life and Analytical Sciences, Boston, MA) (28).

Molecular Docking

In order to better understand the interaction of calycosin with FXR, molecular docking with Autodock 4.1 (The Scripps Research Institute, La Jolla, CA) was performed. The crystal structure of human FXR LBD (Protein Data Bank identification code 1OSH) was used as a receptor template (29). To find the best ligand conformer with the least binding energy, 3D structures of calycosin were built and optimized using ChemDraw Ultra 11 (CambridgeSoft Corporation, Cambridge, MA). Figures were created with Pymol (DeLano Scientific, San Carlos, USA). Repeated dockings were carried out until there was no further refinement in clustering or binding energy of conformer. Based on the population size and the binding energy, the best dock conformation was chosen for further analysis of the protein-ligand interaction.

Statistic Analysis

Data are expressed as mean ± SD. Statistical analyses between two groups were performed using Student's *t* test while multiple comparisons were performed using one-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Calycosin Protects Against Liver Injury Induced by CCl_4

As shown in Fig. 2a, the higher levels of ALT and AST, which are considered as the main markers of liver function, were measured in the plasma of CCl_4-treated mice. After a 3-day administration of calycosin (12.5, 25, 50 mg/kg, p.o., $n=6$), we observed significant reduction in plasma concentration of ALT and AST. Liver sections stained by H&E showed the liver histopathological changes which are severe congestion in the portal area, sinusoids, numerous vacuoles and necrosis being significantly ameliorated by calycosin treatment (Fig. 2b-c). Compared to the normal group, the number of apoptotic cells stained by TUNEL in model group was significantly increased, indicating an increase in apoptotic degeneration of hepatocytes as a result from CCl_4 intoxication (Fig. 2d). After 48 h of calycosin treatment, there was a significant decrease in TUNEL-positive cells (Fig. 2e). Therefrore, Fig. 2 data showed calycosin improved the CCl_4-induced liver injury in mice. However, we didn't observe any difference between the normal group and the calycosin only group, indicating that calycosin may not impair the liver.

Calycosin Decreases the Levels of Bile Acids in the Liver after CCl_4 Treatment

Disorder of bile acid metabolism and high intrahepatic bile acid level still led to BA overload despite CCl_4 injection. This overload caused hepatocellular apoptosis and necrosis. To further determine the effect of calycosin on increased BA level, we measured the intrahepatic BA concentration using Elisa assay ($n=6$). As shown in Fig. 3a, the high level of hepatic BA occurred after 24 h of CCl_4 treatment, and the level of BA in the model group was elevated more sharply than that of the calycosin group (12.5, 25, 50 mg/kg, p.o., $n=6$). The high dose calycosin significantly reduced BA to a normal level after 3 days of treatment. Compared to the model group, the expression of the rate-limiting enzyme of BA synthesis, CYP7A1 was significantly decreased in calycosin group (Fig. 3b). Small heterodimer partner (SHP) suppressed CYP7A1 expression and FXR regulated SHP directly. We observed calycosin induced SHP expression as shown in Fig. 3c. The expression of BSEP, a crucial transporter in the delivery of taurocholate and other cholate conjugates from hepatocytes to bile, was increased in the mice after calycosin treatment (Fig. 3d).

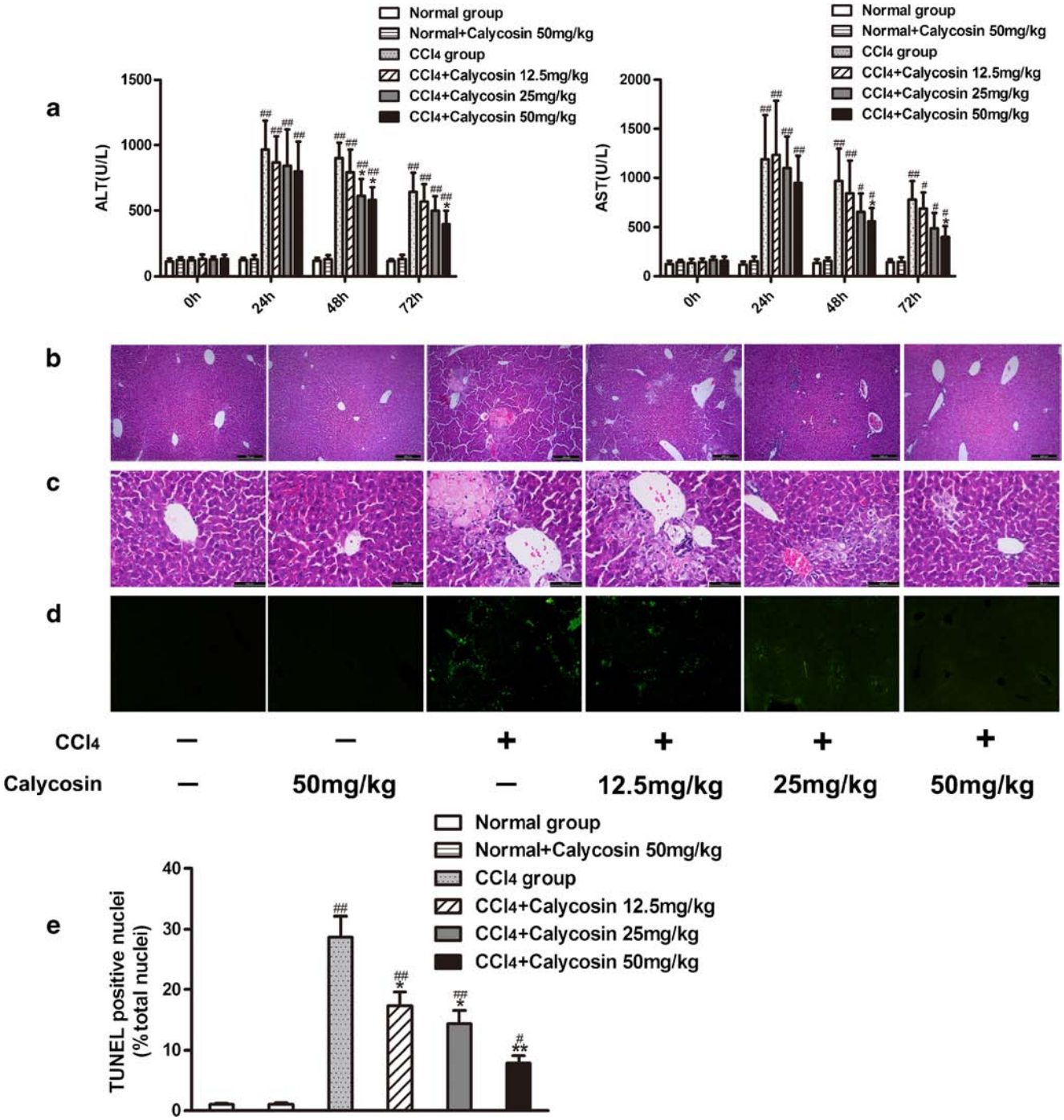

Fig. 2 Calycosin treatment (12.5, 25, 50 mg/kg, p.o.) improved acute liver injury induced by CCl_4. (**a**) Effect of calycosin on serum ALT and AST levels in mice. (**b**) Effect of calycosin on liver histopathological examination by hematoxylin and eosin (H&E) in mice (×100, magnification). (**c**) Effect of calycosin on liver histopathological examination by hematoxylin and eosin (H&E) in mice (×400, magnification). (**d**) The protective effect of calycosin against CCl_4-induced hepatocyte apoptosis though TUNEL method (×100, magnification). Green fluorescence indicates apoptotic cells. (**e**) Statistic analysis of TUNEL fluorescent images in mice. Data are presented as mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with CCl_4 group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with control group ($n = 6$).

Calycosin Promotes the Hepatocyte Mitosis after CCl_4 Treatment

The regeneration rate of hepatic parenchymal cells regeneration and mitosis also played a key role in the process of liver repair after toxicant injury. As shown in Fig. 4a, b, more 5-bromo-2'-deoxy-uridine (BrdU) positive hepatocytes were observed in model and calycosin group. It has been reported that restoration of damaged liver is associated with increased expressions of Cyclin D1, Cyclin B1 and FoxM1B, the two

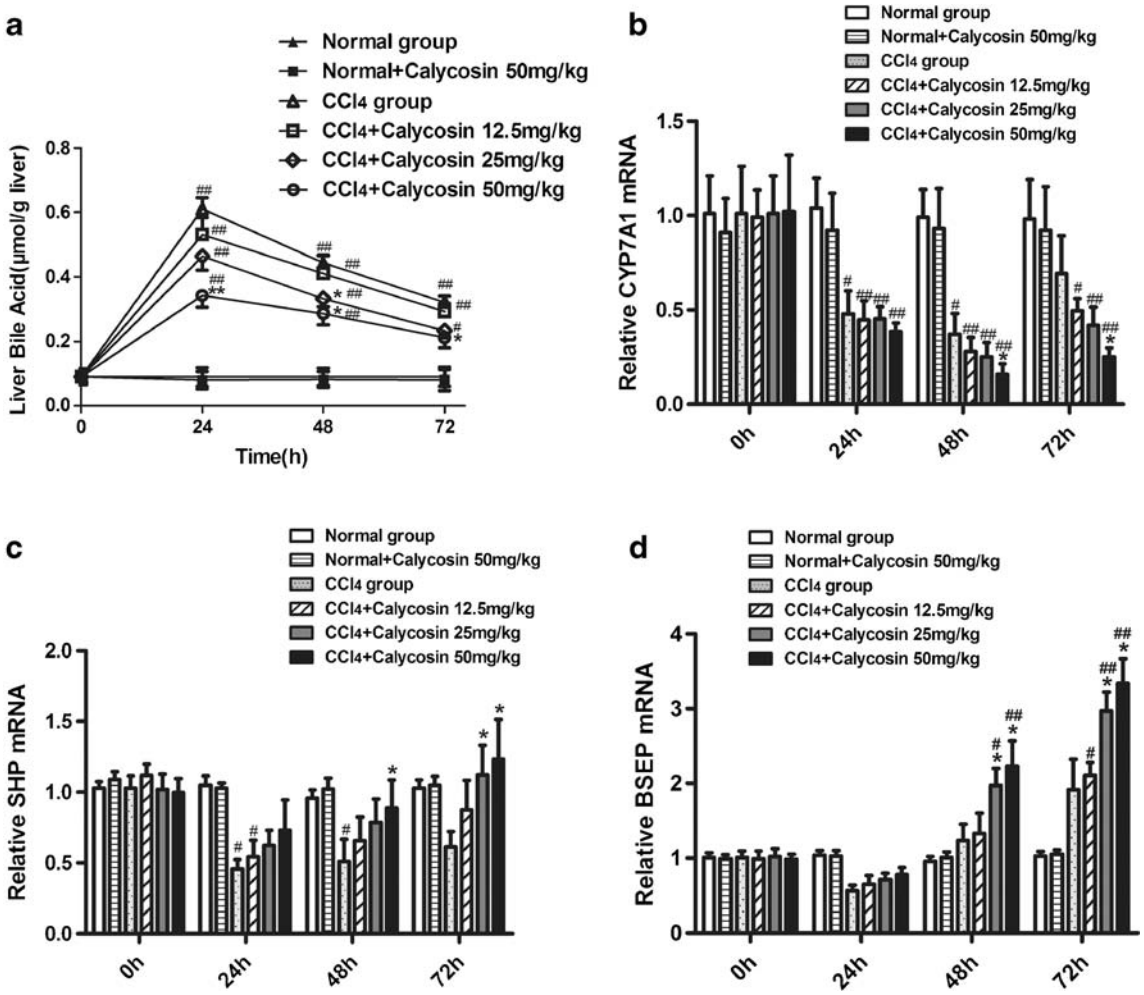

Fig. 3 Calycosin treatment (12.5, 25, 50 mg/kg, p.o.) reduces BA overstress after CCl_4-induced liver injury. (**a**) Effect of calycosin on hepatic BA levels in mice. (**b**) Effect of calycosin on expression of CYP7A1 in mice. (**c**) Effect of calycosin on expression of SHP in mice. (**d**) Effect of calycosin on expression of BSEP in mice. Data are presented as mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with CCl_4 group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with control group ($n = 6$).

major cell cycle regulatory genes and their up-stream regulator. As shown in Fig. 4c, the increased mRNA levels of Cyclin D1, Cyclin B1 and FoxM1B detected by quantitative real-time PCR were observed in model and calycosin group (12.5, 25, 50 mg/kg, p.o.). Calycosin significantly up-regulated the protein expressions of Cyclin D1, Cyclin B1 and FoxM1B. (Fig. 4d)

Calycosin Hepatoprotective Activity may be Associated with FXR but the Effect may be Inhibited by Guggulsterone Administration

In this present study, the expression levels of direct target genes of FXR, SHP and FoxM1B were significantly increased after calycosin treatment (Figs. 3c and 4c, d). To further investigate the effect of calycosin on FXR, transient transfection assay in HepG2 cells was performed. As shown in Fig. 5a, calycosin markedly stimulated FXR-dependent BSEP reporter activity by about 3-fold in the high dose group. The expressions of FXR target gene FoxM1B, Cyclin D1 and Cyclin B1 were assessed by Western blot. Calycosin showed a significant effect on the up-regulation of these genes, which were then strongly inhibited after the oral treatment with Guggulsterone (50 mg/kg/day), a highly efficient FXR antagonist (Fig. 5b). We used molecular docking studies to predict the interaction between calycosin and FXR. As shown in Fig. 5c, the docking results indicated that calycosin could be embedded into the binding pocket of FXR. This creates a favorable interaction between the oxygen of calycosin and Thr288 of FXR. This result suggested good compatibility between the two structures.

Calycosin Activates STAT3 after CCl_4 Treatment

In order to evaluate the effects of calycosin on the activation of STAT3, the expression of STAT3 tyrosine phosphorylation was assessed by Western blotting. Result indicated a higher

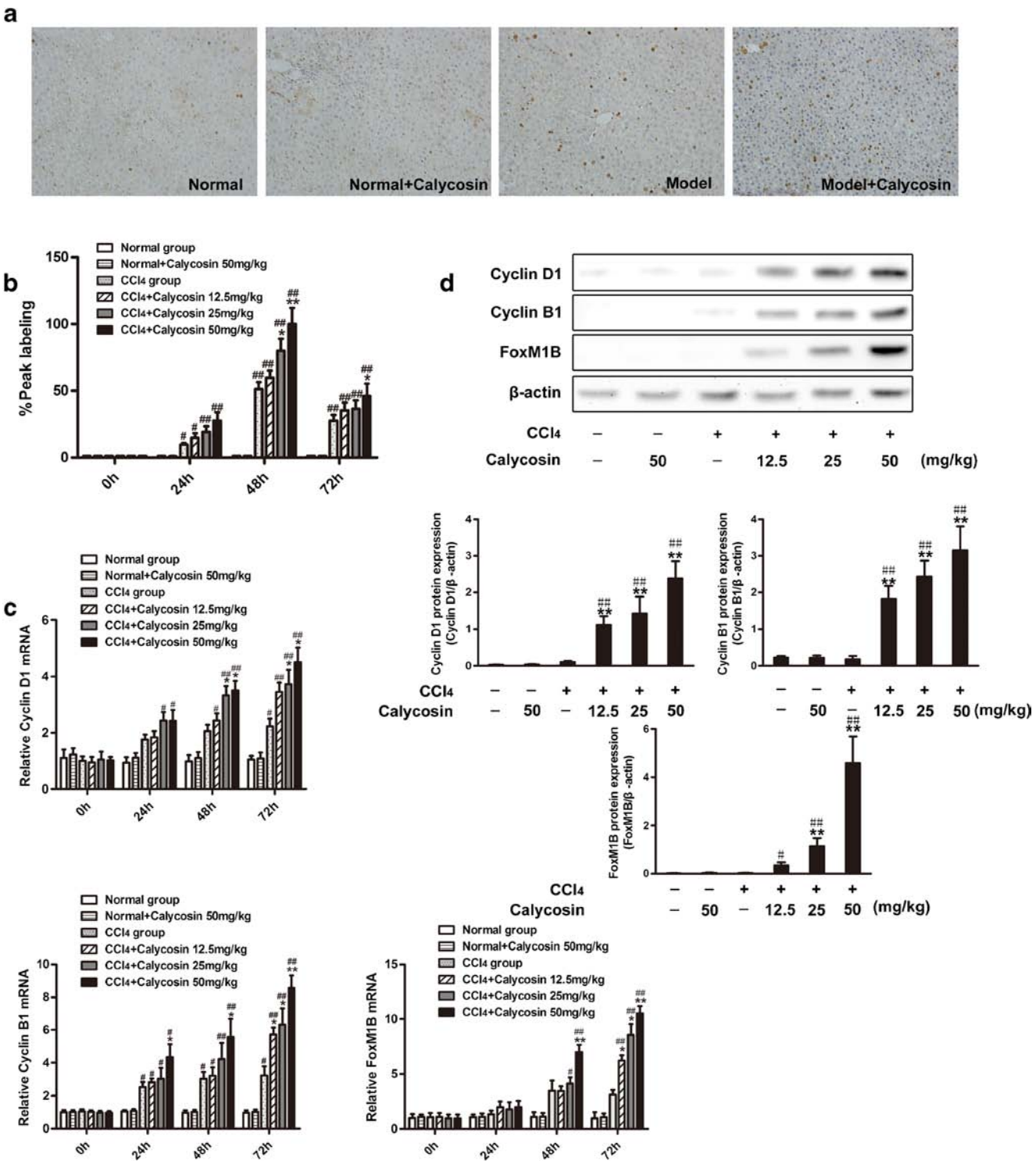

Fig. 4 Calycosin treatment (12.5, 25, 50 mg/kg, p.o.) promotes hepatocyte mitosis after CCl_4-induced liver injury. (**a**) Effect of calysosin on mitotic nuclei in liver cells as determined by BrdU staining (×100, magnification) in mice. (**b**) Statistic analysis of BrdU staining images in mice. (**c**) Effect of calycosin on transcriptional expression of Cyclin D1, Cyclin B1 and FoxM1B in mice. (**d**) Effects of calycosin on the protein expression of Cyclin D1, Cyclin B1 and FoxM1B in mice and statistic analysis of Cyclin D1, Cyclin B1 and FoxM1B Western blot images in mice. Data are presented as mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with CCl_4 group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with control group ($n = 6$).

level of STAT3 tyrosine phsphorylation after calycosin treatment with a constant total STAT3 protein expression (Fig. 6a). To further confirm the positive role that calycosin plays in STAT3 activation, we measured the expressions of the two main STAT3 target genes, Bcl-xl and SOCS3, after CCl_4 treatment. Compared to the model group, the

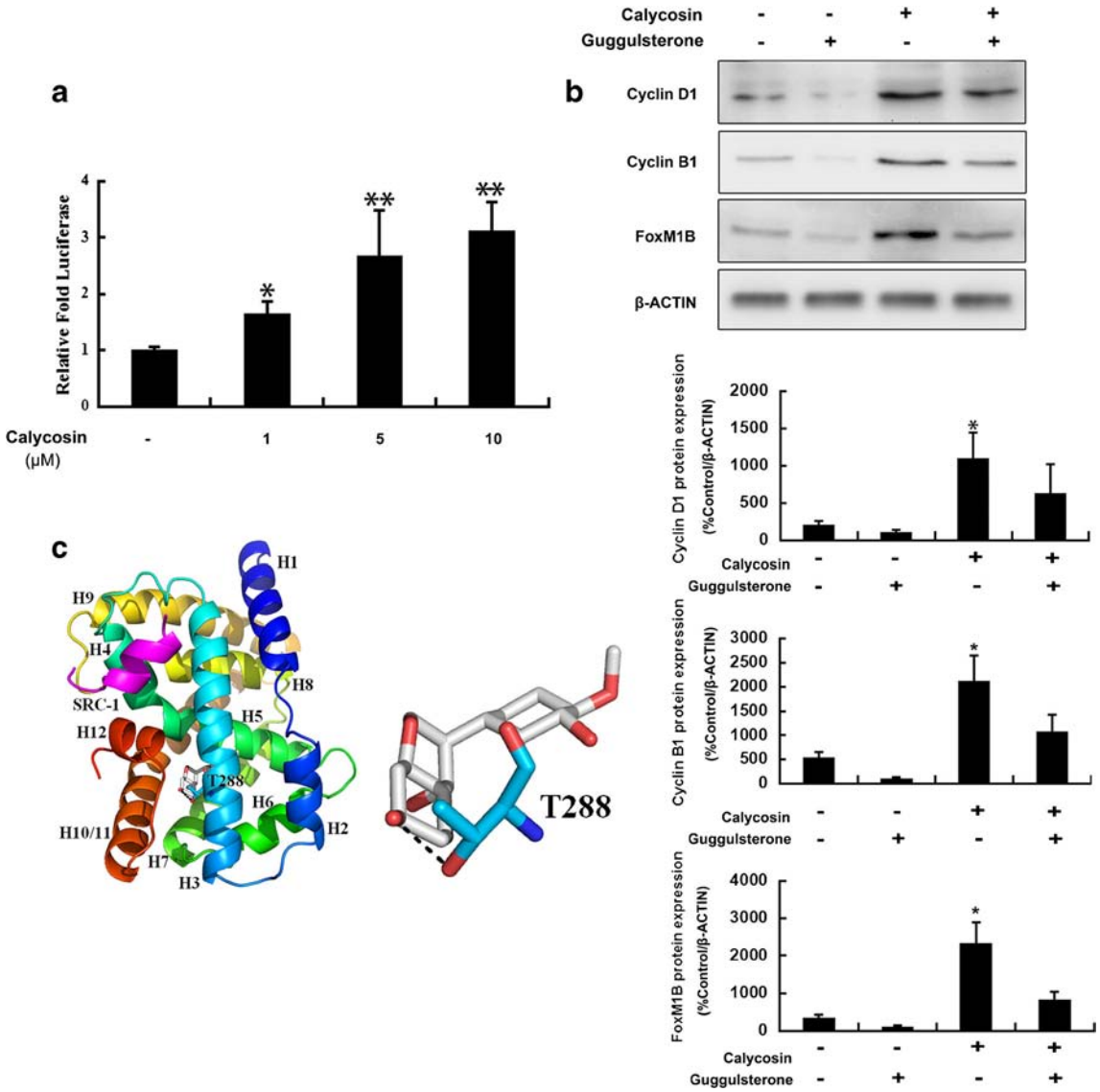

Fig. 5 Calycosin (12.5, 25, 50 mg/kg, p.o.) activates FXR directly. (**a**) The positive effect of calycosin on the activation of FXR-dependent BSEP responsive reporter that was determined by a dual-luciferase reporter assay system. (**b**) Guggulsterone inhibits the effects of calycosin on the protein expression of Cyclin D1, Cyclin B1and FoxM1B in mice. (**c**) Docking solutions of calycosin and FXR LBD. The secondary structural conformation of calycosin with FXR LBD is highlighted. Data are presented as mean ± SD. *$p < 0.05$ and **$p < 0.01$ compared with CCl_4 group ($n = 6$).

expressions of Bcl-xl and SOCS3 in mice were significantly up-regulated by the high dose of calycosin (Fig. 6b, c).

DISCUSSION

Liver is well known as the chief organ in the metabolism of toxic chemicals and drugs (22). It is also the most vulnerable organ to most toxic chemicals, usually resulting in its damage. This therefore poses a major health problem to people's well being (30). Previous researches demonstrated that various chemical substances such as CCl_4, 2, 4, 6- trinitrotoluene (TNT) and acrolein could cause severe hepatic injury (8,31). CCl_4-induced liver injury is a popular animal experiment model that is used to screen hepatoprotective agents (5, 32). As shown in Fig. 2a-c, CCl_4 administration caused acute liver damage in mice, which was then supported by the elevated levels of serum ALT, AST and classic histopathological changes (5). The aim of the present study was to identify the critical role played by calycosin in the hepotoprotective effects on the CCl_4-induced acute liver injury in mice.

CCl_4 induced liver injury caused impaired BA enterohepatic circulation and the accumulation of BA in the liver. These negative effects resulted in hepatocellular apoptosis, necrosis, and consequently aggravating liver fibrosis and cirrhosis (33). In the present study, we observed increased BA level after CCl_4 injection and a significant reduction of the

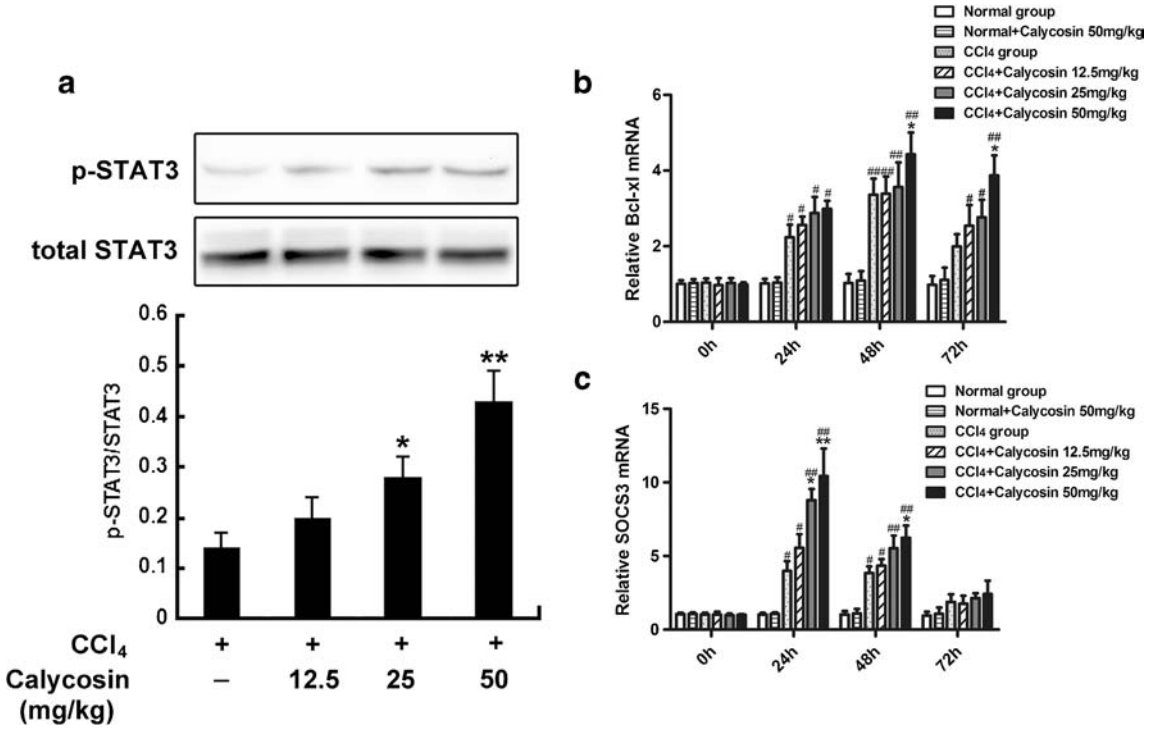

Fig. 6 Calycosin treatment (12.5, 25, 50 mg/kg, p.o.) activates STAT3 phosphorylation. (**a**) Effect of calycosin on expression of p-STAT3 in mice. (**b**) Effect of calycosin on expression of Bcl-xl in mice. (**c**) Effect of calycosin on expression of SOCS3 in mice. Data are presented as mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with CCl_4 group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with control group ($n = 6$).

overload after 3 days of calycosin treatment. As the rate-limiting enzyme of bile acid biosynthesis, CYP7A1 converts cholesterol to bile acids. (34) FXR represses CYP7A1 expression by up-regulating the expression of the small heterodimeric partner (SHP, NR0B2), an orphan nuclear hormone receptor that inhibits the activity of liver receptor homologue 1 (LRH-1) known to regulate CYP7A1 expression positively (35). BSEP, a member of the ATP-binding cassette superfamily, act as the major transporter to mediate canalicular BA excretion (36). FXR activation induced the expression of BSEP by binding specifically to the highly conserved IR-1 sequence of BSEP promoter (37). Calycosin significantly elevated the levels of SHP and BSEP while decreased the expression of CYP7A1. This indicated that calycosin protected the liver partially by reducing the BA biosynthesis and increasing the excretion.

Hepatocytic parenchymal cells that regenerated were substituted with the necrotic or apoptotic hepatocytes after the acute liver injury (38). The rates of liver regeneration and hepatocytes mitosis played a key role in the liver repair process. The Forkhead Box (Fox) transcription factors, consisting of more than 50 mammalian proteins, belonged to an extensive family that is essential for the regulation of cellular proliferation, apoptosis, and metabolic homeostasis (39,40). FoxM1B, mostly expressed at the G1/S transition, is a member of Fox transcription factors family. It also expresses throughout the period of proliferation (40). FoxM1B stimulated hepatocyte DNA replication and mitosis by increasing the expressions of numerous cell cycle regulatory genes such as the S-phase promoting Cyclin D1, Cyclin A2 and M-phase promoting Cyclin B1, Cyclin F (40). Cyclin D1 facilitated hepatocytes entry and progression through S phase while Cyclin B1 mediated cell cycle progression from the G2 phase into mitosis by combining with cyclin-dependent kinase 1(cdk1) (41). Further study revealed that FoxM1B decreased the levels of the S-phase inhibitor p21 and p27 protein and promoted the activation of Cyclin D1, Cyclin E and Cyclin B1 following accelerated hepatocytes proliferation (40). FXR activated FoxM1B by binding to the inverted repeat (IR-0) of FXR response element (FXRE) which served as an enhancer of intron 3 of the FoxM1B gene (40). Our results suggested that calycosin significantly improved the transcription and expression levels of FoxM1B, Cyclin D1 and Cyclin B1. This effect may contribute to parenchymal cells regeneration, necrotic hepatocytes substitution and normal hepatic restructuring.

FXR was investigated as a key factor of chemoprotection. GW4064, a FXR agonist, partly protected human hepetocytes against cisplatin-induced toxicity (7). The cisplatin IC50 increased 5.8-fold in Alexander cells when they were transfected with FXR+RXR and no change observed when transfected with RXR alone. And cisplatin up-regulated BSEP, a target gene of FXR, only when FXR is expressed (7). In our study, we observed a hepatoprotective effect of calycosin against CCl4-induced liver injury and up-regulated

the BSEP expression. These findings implied a association between calycosin and FXR. It was firstly showed by the augmentation of BSEP luciferase reporter gene expression after calycosin treatment. Secondly, guggulsterone is an active agent extracted from the resin of the guggul tree and acts as a highly efficient antagonist of FXR. The blocking effect of guggulsterone on expressions of Cyclin D1, Cyclin B1 and FoxM1B suggested an antagonistic relationship between guggulsterone and calycosin (42). Thirdly, the molecular docking results predicted a strong interation between calycosin and Thr288 of FXR LBD. Altogether, these results strongly suggested that calycosin hepatoprotective effect may be connected with FXR activation and this effect can disappear when the liver is not damaged.

Multiple researches described STAT3 as a crucial hepatoprotective transcription factor against toxicant injury. It is required for survival after 70% partial hepatectomy. It is also involved in mitigating exaggerated inflammatory reaction after hepatocyte necrosis (43). Our study showed that calycosin promoted STAT3 phosphorylation and activated the expressions of Bcl-xl and SOCS3, the two main target genes of STAT3. Bcl-xl inhibited hepatic caspase-3-like activity, therefore reduced hepatocyte apoptosis and liver fibrotic responses (44). SOCS3 protected liver by preventing liver apoptosis and hemorrhagic necrosis (45). These effects contributed to calycosin activity against hepatocytes apoptosis (Fig. 2d, e). However, the evidence of a direct effect of FXR on STAT3 phosphorylation had never been observed (8). Instead, BA intrahepatic accumulation led to a low level of phosphorylation of STAT3 (8). This suggests that calycosin activates STAT3 phosphorylation by relieving the stress of BA overload in the liver.

In summary, this study demonstrated the hepatoprotective effects of calycosin against CCl_4-induced liver injury. The mechanism of action is reduction of BA overload and promotion of the hepatocyte mitosis. These effects are in association with FXR activation and STAT3 phosphorylation. The protective and healing properties of calycosin need further investigation. It will be meaningful to develop calycosin as a new natural medicine for treatment of acute liver injury.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by a grant from the National Natural Science Foundation of The People's Republic of China (No. 81273580, 81302826).

The authors declare that there are no conflicts of interest.

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