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REVIEW ARTICLE

Salvia miltiorrhiza compounds protect the liver from acute injury by regulation of p38 and NFκB signaling in Kupffer cells

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Abstract

Context: *Salvia miltiorrhiza* Bunge is a traditional Asian medicine used to treat cerebral and cardiac ischemia. However, the effects of the active compounds of *S. miltiorrhiza* on liver damage are unclear.

Objective: In this study, we tested the effects on acute liver injury of crude *S. miltiorrhiza* extracts from roots as well as neotanshinone B, dehydromiltirone, tanshinol A, tanshinone I, dihydrotanshinono I, neotanshinone A, cryptanshinono, tanshinone II A, and salvianolie acid B from purified *S. miltiorrhiza* extracts.

Materials and methods: Various compounds or ethanol extract of *S. miltiorrhiza* (50, 100, and 200 mg/kg, p.o.) were administered to rats for five consecutive days. After acute carbon tetrachloride (CCl_4)-induced liver injury by treatment of rats with a single dose of CCl_4 (0.75 mL/kg, p.o), rat liver function was tested by measuring serum biochemical parameters. Serum cytokine concentrations were assessed by enzyme-linked immunosorbent assay (ELISA). Expression of p38 and NFκB was evaluated by western blot.

Results: All *S. miltiorrhiza* components showed their effects on liver function from the dose from 50 to 200 mg/kg. At the dose of 200 mg/kg, they reduced serum levels of alkaline phosphatase (ALP) by 34–77%, alanine aminotransferase (ALT) by 30–57%, aspartate aminotransferase (AST) by 43–72%, creatine total bilirubin (BIL-T) by 33–81%, albumin (ALB) by 37–67%, indicating that *S. miltiorrhiza* extracts protected liver from CCl_4-induced damage. Moreover, *S. miltiorrhiza* extracts at 200 mg/kg reduced the increase in the proinflammatory cytokines tumor necrosis factor-α (TNF-α) by 25–82%, interleukin-1 (IL-1) by 42–74% and interleukin-6 (IL-6) by 67–83%, indicating an effect on alleviating liver inflammation. Furthermore, *in vitro*, *S. miltiorrhiza* extracts inhibited p38 and NFκB signaling in Kupffer cells. This effect could be a main mechanism by which *S. miltiorrhiza* protects against acute liver toxicity.

Discussion and conclusion: Active compounds of *S. miltiorrhiza* protected the liver from CCl_4-induced injury. Protection might have been due to inhibition of p38 and NFκB signaling in Kupffer cells, which subsequently reduced inflammation in the liver.

Keywords

Inflammation, interleukin, nuclear factor kappa B, tumor necrosis factor

History

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Introduction

The active components in extracts of *Salvia miltiorrhiza* Bunge (Danshen), a Chinese traditional herbal medicine, have been used to treat conditions such as cerebral and cardiac ischemia, menstrual disorders, miscarriage, edema and liver diseases (Fu et al., 2007; Gao et al., 2008; Lee et al., 2006; Sun et al., 2005). Recent findings for *S. miltiorrhiza* components indicate that cryptotanshinone inhibits human glioma cell proliferation (Lu et al., 2013), salvianolic acid A can be used to treat Alzheimer's disease (Cao et al., 2013), and tanshinone I and tanshinone IIA protect the liver from acute and chronic injury (Park et al., 2009). However, although more than 20 active components have been isolated from *S. miltiorrhiza*, the molecules with liver-protective effects are unclear.

Kupffer cells (KCs) are the main cells of the liver monocyte-macrophage system and function in anti-inflammation by removing bacteria and toxins. KCs are important in chronic inflammatory liver injury because they influence the functions of liver, hepatic stellate, and endothelia cells (Kolios et al., 2006; Tavares et al., 2013; You et al., 2008). Nuclear factor κB (NFκB) and p38 are involved in several

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inflammatory cytokine responses such as the pathways that mediate endotoxins that cause liver damage (Kolios et al., 2006; Luckey & Petersen, 2001). Blocking the NFκB or p38 signaling pathways inhibits a positive feedback loop that is mediated by proinflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α that attenuate inflammation (Wei et al., 2012; Yang et al., 2005). Crude extracts of *S. miltiorrhiza* enhance phagocytosis by KCs, reduce endotoxin secretion, and inhibit cytokine secretion during endotoxemia (Liang et al., 2009). However, no studies have investigated the influence of the active components of *S. miltiorrhiza* on p38 and NFκB pathway-related protein expression (Figure 1).

In this study, we tested the effects of nine components of *S. miltiorrhiza* on acute liver injury. We used experimental liver injury induced by carbon tetrachloride (CCl_4) in rats as a model. High levels of ALB, ALT, AST, ALP, and BIL-T in blood serum indicate severe liver damage after CCl_4 treatment, so these parameters were used to evaluate the liver-protective efficacy of compounds from *S. miltiorrhiza*. We used lipopolysaccharide (LPS)-activated KCs as models for investigating the effect of each *S. miltiorrhiza* component on the p38 and NFκB signaling pathways. Our results provide theoretical support and experimental evidence for the clinical treatment of acute liver injury with *S. miltiorrhiza*.

Materials and methods

Reagents

LPS, PDTC, and SB239063 were from Sigma (St. Louis, MO). Primary antibodies against NFκBp65, p-NFκBp65, inhibitoryκB (IκB), p38, p-P38 and c-fos, and secondary antibody sheep anti-rat HRP were from Cell Signaling Company Ltd (Danvers, MA). Neotanshinone B (070706), dehydromiltirone (070625), tanshinol A (070120), tanshinone I (070315), dihydrotanshinono I (070315), neotanshinone A (070605), cryptanshinono (030806), tanshinone II A (040616), salvianolie acid B (060328), and ethanol extract of *S. miltiorrhiza* were from Xi'an Honson Biological Technology Company, China. All components were >98% pure. HPLC was used to determine the content above in the standardized fraction and in the ethanol extract (Park et al., 2009) and shown in Supplementary figure.

Acute liver injury

Neotanshinone B, dehydromiltirone, tanshinol A, tanshinone I, dihydrotanshinono I, neotanshinone A, cryptanshinono, tanshinone II A, salvianolie acid B, or ethanol extract of *S. miltiorrhiza* (50, 100, and 200 mg/kg, p.o.), were administered to rats for five consecutive days. To induce acute liver

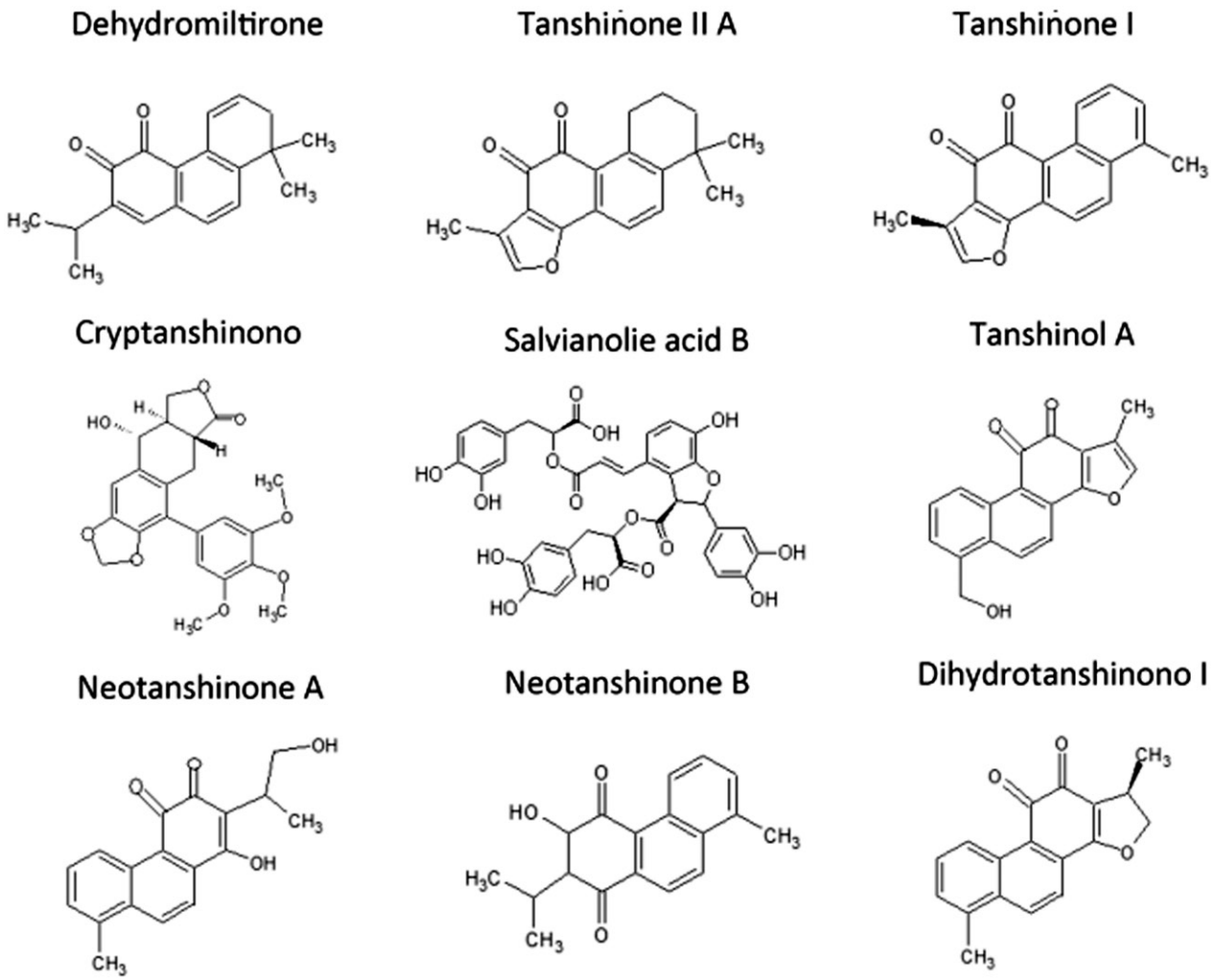

Figure 1. Chemical structures of active components of *S. miltiorrhiza*.

injury, rats were given a single dose of CCl_4 (0.75 mL/kg, p.o., diluted in corn oil), 1 h after the final dose of *S. miltiorrhiza* extract or purified component. Control rats received the same amount of corn oil alone.

Liver function tests

To test liver function, rats were euthanized 24 h after CCl_4 treatment by cardiac puncture under ether anesthesia and serum was obtained. Biochemical analysis of serum samples used an automatic chemistry analyzer (Roche Integra 400 Plus, Mannheim, Germany). Parameters measured were alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (BIL-T), and albumin (ALB).

Measurement of cytokine levels

Cytokine levels in serum were determined by enzyme linked immunosorbent assay (ELISA) using kits specific for rats (Biosource International, Camarillo, CA) and according to the manufacturer's instructions. Spectrophotometric measurements were at 450 nm (Wang et al., 2009). Cytokines evaluated were TNF-α, IL-1, and IL-6. Each sample was assayed in duplicate. All data were normalized for total protein and expressed as pg mg^{-1} of protein.

Primary KCs separation and culture

KCs were separated and cultured as described previously (Kitani et al., 2010). Briefly, DMEM/F12 containing 200 mL/L calf serum was used to precipitate cells by washing twice and centrifuging at 3000 rpm for 10 min. After testing cell viability with 4 g/L Trypan blue dye, the cell density was adjusted to 1×10^{11}/L. Cells were cultured at 37 °C in 5% CO_2.

Cells were seeded in flasks or dishes until 60–70% confluent then treated with PDTC at 10 mmol/L; SB239063 at 10 mmol/L; or neotanshinone B, dehydromiltirone, tanshinol A, tanshinone I, dihydrotanshinono I, neotanshinone A, cryptanshinono, tanshinone II A, salvianolie acid B, or crude *S. miltiorrhiza* ethanol extract at 100 μg/mL for 24 h. For control cells, equal volumes of DMSO were added. Except for the control group, LPS was added to all cells at 60 ng/mL.

Western blotting

Protein expression was detected by western blotting as described previously (Wang et al., 2009). KCs were washed twice with cold PBS. RAPI cell lysate was added, lysates were centrifuged, and supernatants were collected. After 10% SDS-PAGE, proteins were transfer onto nitrocellulose membranes and incubated with primary antibodies against NFκBp65, p-NFκBp65, IκB, p38, p-p38, or c-fos at 4 °C overnight. Horseradish peroxidase-labeled second antibody (1:5000) was added. Bands were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK) and quantified with Total Lab software (Sigma-Aldrich, St. Louis, MO). Each experiment was performed in triplicate.

Statistical analysis

SPSS 14.0 (SPSS Inc., Chicago, IL) was used for analysis, and all data are expressed as $\bar{x} \pm s$. Differences between means of two samples were assessed by *t*-test. Means of several groups were compared by random design analysis of variance. $p < 0.05$ was regarded as statistically significant.

Results

Effects of *S. miltiorrhiza* on CCl_4-induced alteration in hepatic function and inflammation

The effects of the active components of *S. miltiorrhiza* on CCl_4-induced acute liver injury in rats were tested by measuring serum biochemical markers (Figure 2). The results indicated that, at 100 or 200 mg/kg, all components of *S. miltiorrhiza* Bunge significantly prevented CCl_4-induced increases in ALB, ALT, AST, ALP, and BIL-T levels ($p < 0.01$). At the dose of 200 mg/kg, they reduced serum levels of ALP by 34–77%, ALT by 30–57%, AST by 43–72%, BIL-T by 33–81%, and ALB by 37–67%, suggesting these compounds had protective effects on the liver.

CCl_4-induced hepatotoxicity was accompanied by a significant rise in the levels of proinflammatory cytokines TNF-α, IL-1, and IL-6 compared with controls. Pretreatment with all the *S. miltiorrhiza* active components except salvianolie acid B significantly reduced serum cytokine levels (Figure 3). At 200 mg/kg, components reduced the increase in the proinflammatory cytokines TNF-α by 25–82%, IL-1 by 42–74%, and IL-6 by 67–83%, suggesting that *S. miltiorrhiza* Bunge components had an anti-inflammatory effect.

Influence of *S. miltiorrhiza* on proteins in the p38, NFκB, and c-fos pathways in KCs

Compared with KCs treated with LPS alone, LPS-treated KCs treated with PDTC, an NFκB inhibitor, or SB239063, a p38 inhibitor, significantly decreased p-p38 levels ($p < 0.05$), but PDTC showed no effect on total p38 expression. All compounds except neotanshinone B, tanshinone II A and salvianolie acid B reduced p38 expression. All active components from *S. miltiorrhiza* except salvianolie acid B decreased p-p38 to varying degrees ($p < 0.05$) (Figure 4).

Compared with KCs treated with the LPS alone, PDTC and SB239063 slightly elevated IκB protein levels and decreased p-NFκBp65 protein. Tanshinol IIA and salvianolie acid B also showed no effect on NFκBp65 expression but significantly increased IκB levels. The other *S. miltiorrhiza* components and the crude *S. miltiorrhiza* ethanol extract significantly reduced NFκBp65 and IκB levels. All tested active components and ethanol extract significantly reduced the increase in p-NFκB65 in KCs that was induced by LPS (Figure 5).

Compared with KC cells treated with LPS alone, SB239063 significantly decreased c-fos protein expression, whereas PDTC had no effect. All tested *S. miltiorrhiza* components and extracts decreased c-fos protein expression to varying degrees (Figure 6).

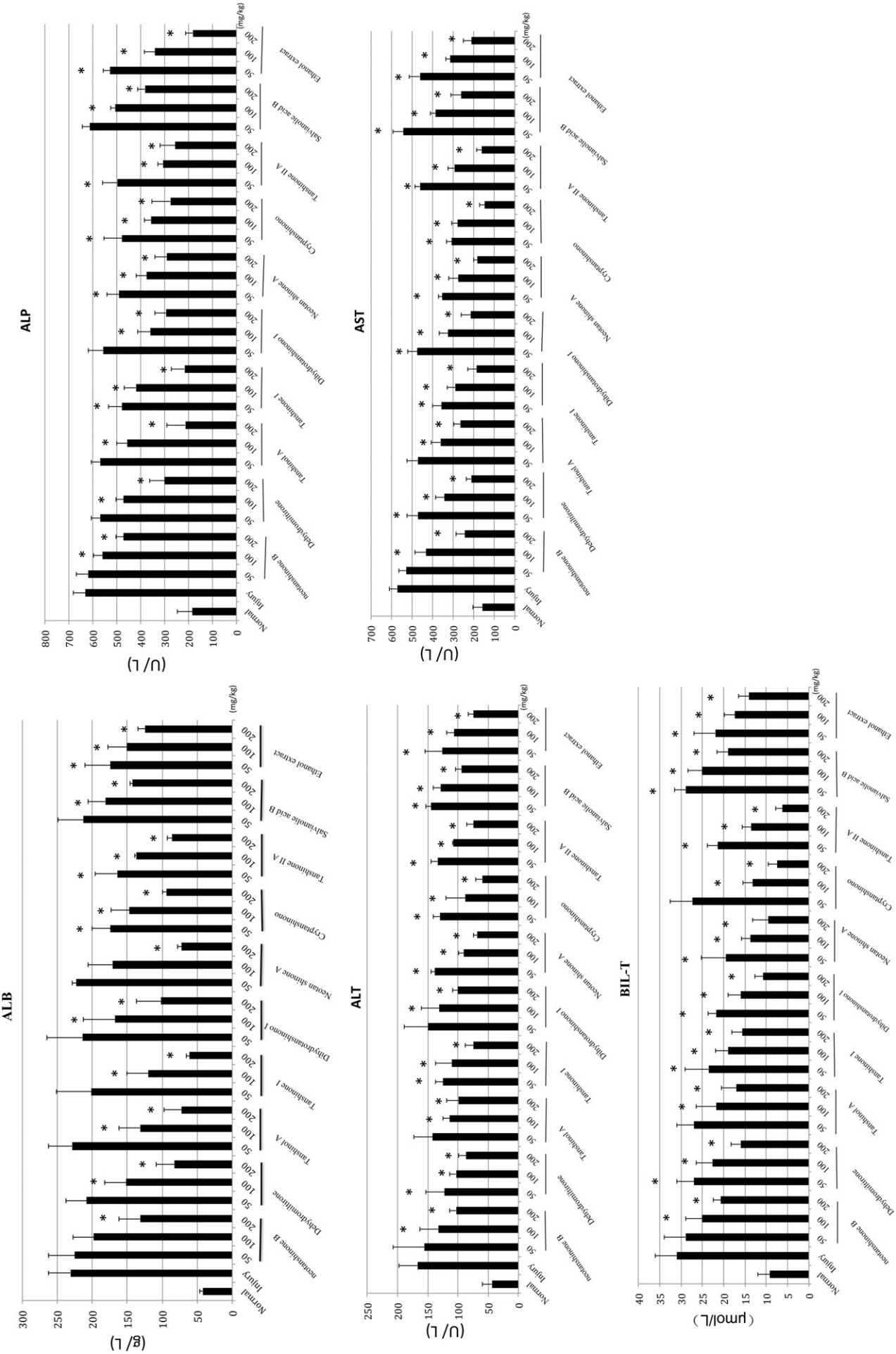

Figure 2. Effect of *S. miltiorrhiza* active components or ethanol extracts on liver function of rats suffering acute liver injury. Indicated compounds or extracts were administered to rats for five consecutive days. Acute liver injury was induced by carbon tetrachloride (CCl_4; 0.75 mL/kg, p.o.) given once after the last dose of the indicated agent. ALB, ALP, ALT, AST, and BIL-T levels in serum were tested. Values are means ± SD. Each group contained at least 10 rats. $^*p < 0.05$ indicates a significant difference from rats treated with CCl_4 alone.

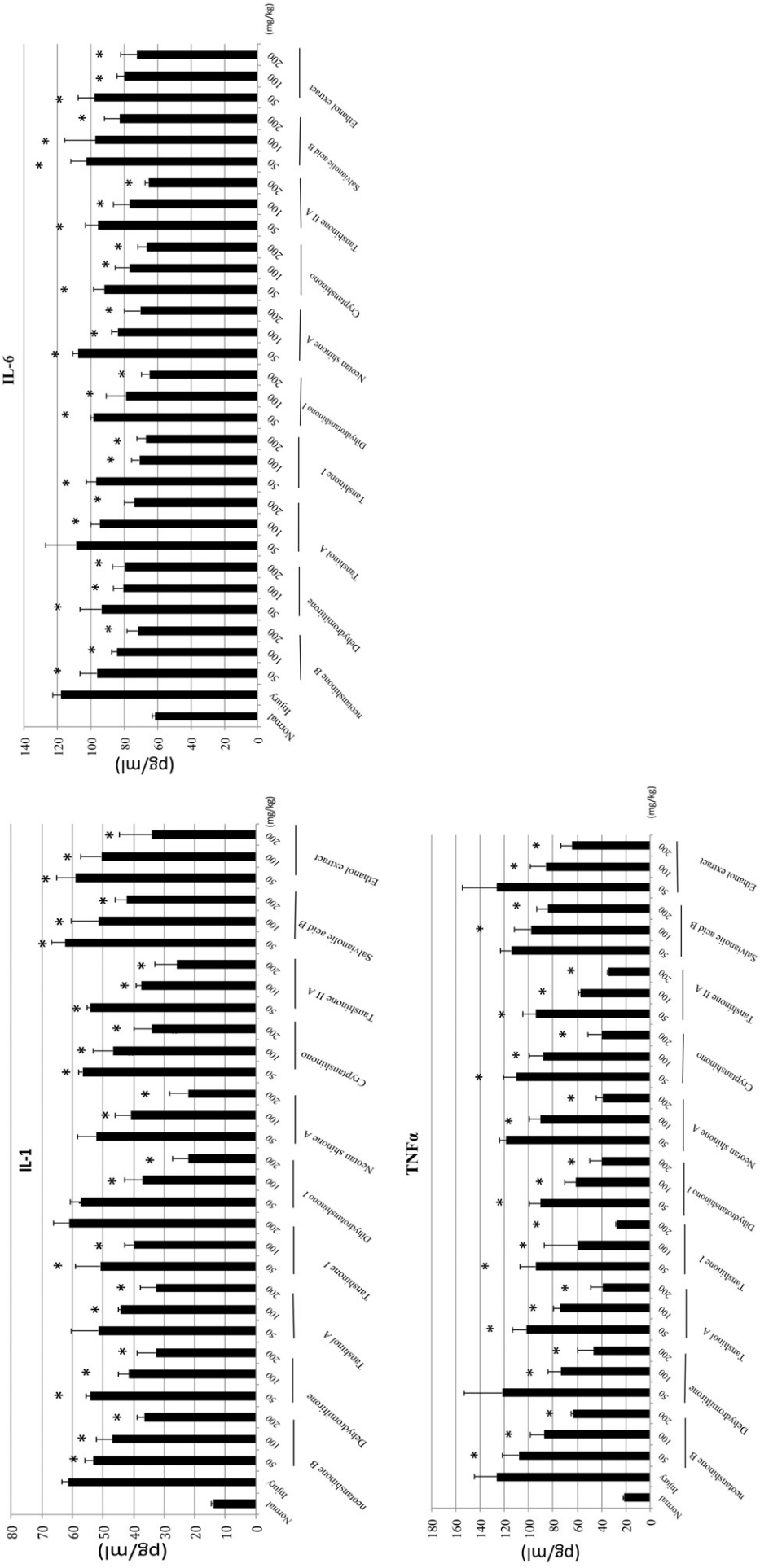

Figure 3. Effect of *S. miltiorrhiza* active components or ethanol extract on serum cytokine levels in rats suffering acute liver injury. Indicated drugs or extracts were administered to rats for five consecutive days. Acute liver injury was induced by carbon tetrachloride (CCl_4; 0.75 mL/kg, p.o.) given once after the last dose of the indicated agent. IL1, IL6, and TNF concentration in serum are expressed as means ± SD. Each group contained at least 10 rats. $^*p<0.05$ indicates a significant difference from rats treated with CCl_4 alone.

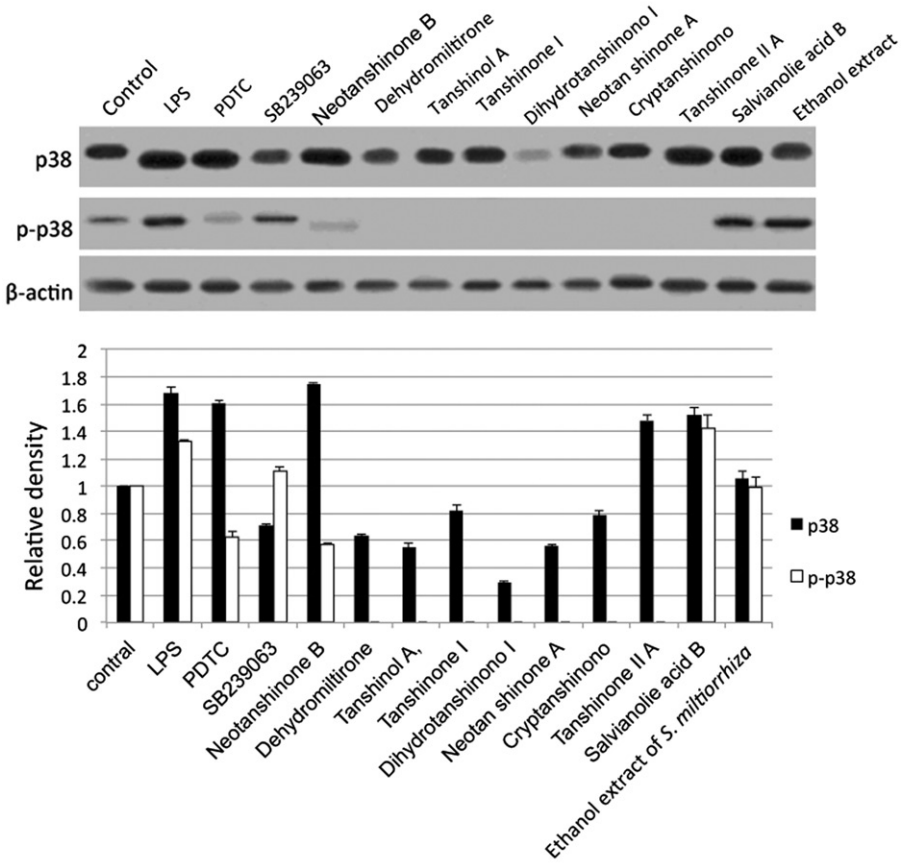

Figure 4. Effect of *S. miltiorrhiza* active components or ethanol extracts on the p38 pathway in Kupffer cells. Kupffer cells were treated with the indicated compounds or extracts at the indicated concentrations for 24 h as described in Materials and methods. Western blots were used to test p38 and p-p38 expression. The band density was quantified with Total lab software. $*p < 0.05$, compared with cells treated with LPS alone.

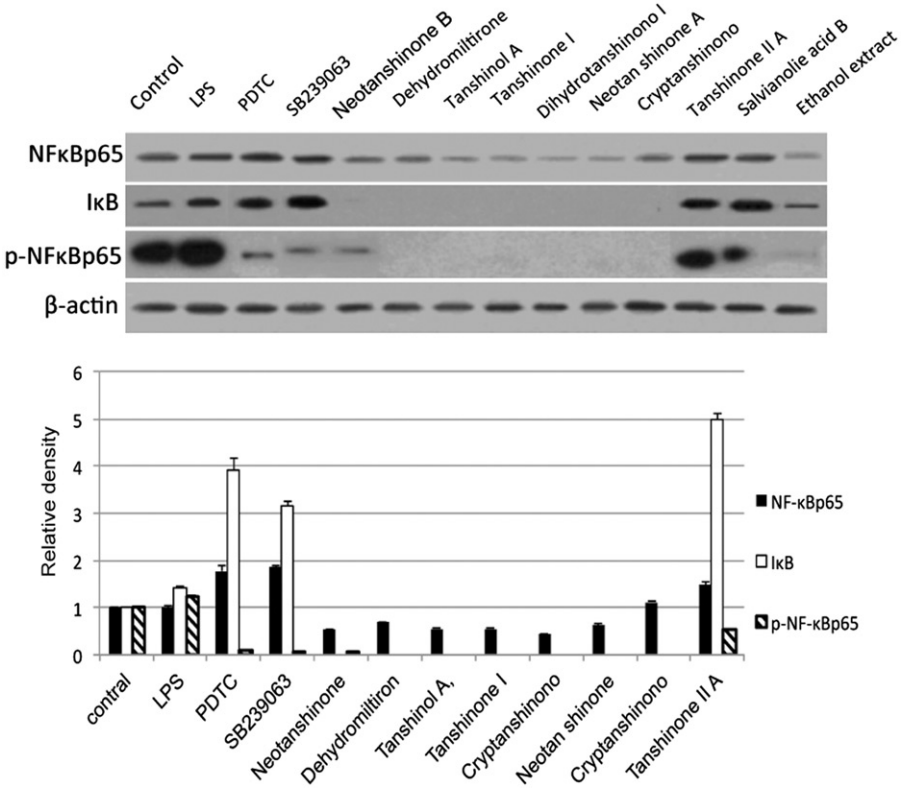

Figure 5. Effect of *S. miltiorrhiza* active components or ethanol extracts on the NFκB pathway in Kupffer cells. Kupffer cells were treated with the indicated compounds or extracts at the indicated concentrations for 24 h as described in Materials and methods. Western blot was used to test NFκBp65, IκB, and p-NFκBp65 expression. The band density was quantified as for Figure 4. $*p < 0.05$, compared with cells treated with LPS alone.

Discussion

Herbal extracts are widely used to alleviate chemical-induced liver injury (Su et al., 2013; Wu et al., 2007). Although neotanshinone B, dehydromiltirone, tanshinol A, tanshinone I, dihydrotanshinono I, neotanshinone A, cryptanshinono, tanshinone II A, and salvianolie acid B from *S. miltiorrhiza* have different structures, the administration of all these components significantly prevented hepatocyte injury and decreased levels of ALB, ALT, AST, ALP, and BIL-T.

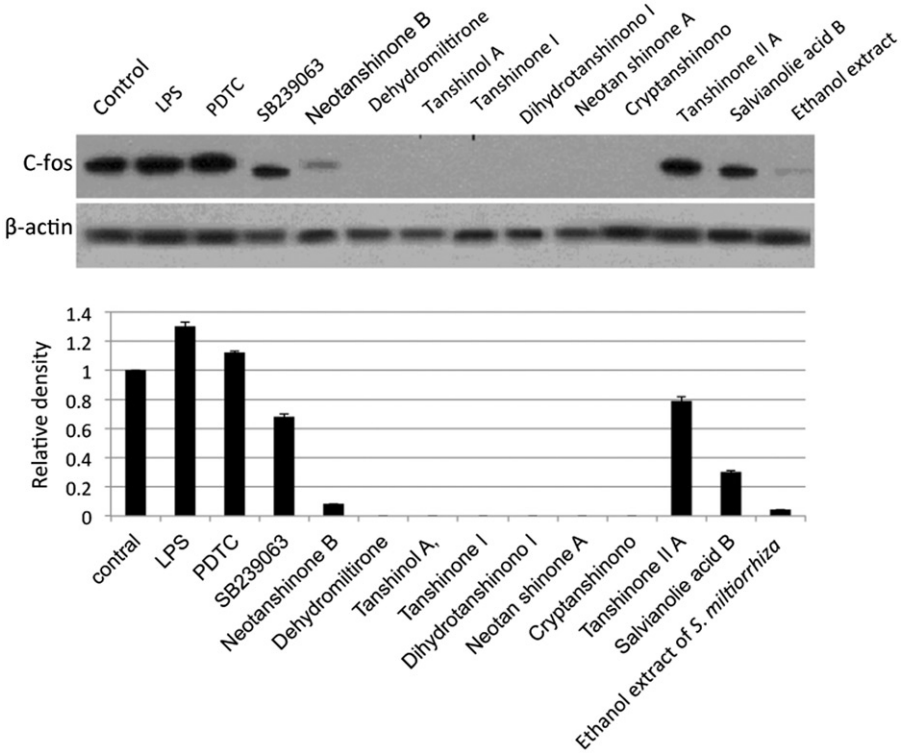

Figure 6. Effect of *S. miltiorrhiza* active components or ethanol extracts on c-fos expression in Kupffer cells. Kupffer cells were treated as for Figures 4 and 5. Western blot was used to test c-fos expression. The band density of bands was quantified as for Figure 4. $*p < 0.05$, compared with cells treated with LPS alone.

Dehydromiltirone, tanshinol A, tanshinone I, dihydrotanshinono I, neotanshinone A and tanshinone II A, which are all diterpenoids containing *o*-quinone in their structures, showed stronger effects than neotanshinone B, which contains *p*-quinone, when administered at equivalent doses. The phenolic acid compound salvianolic acid B and the lignan cryptanshinono also showed liver-protecting effects.

KCs are important in liver injury, including injury caused by CCl_4. In chemically induced liver damage models, toxicity is induced by proinflammatory cytokines such as TNF-α, IL-1, and IL-6 from KCs (Badger et al., 1996). These cytokines activate NFκB, leading to necrosis and apoptosis of liver cells (Sun et al., 2001). In our study, CCl_4 treatment increased the levels of TNF-α, IL-1, and IL-6. This increase was significantly reduced by pretreatment with the active components of *S. miltiorrhiza*. These findings indicated that the anti-inflammatory properties of *S. miltiorrhiza* extracts might prevent hepatocyte injury.

The p38 is signaling pathway is an important mediator of inflammation. The activated p38 pathway promotes expression of inflammatory cytokines, and participates in the cellular inflammatory response and apoptosis under stress conditions (Ding et al., 2013). Pretreatment with SB203580 reduces IL-1α mRNA levels (Takeda & Ichijo, 2002). P38 is also involved in TNF-α induction after LPS treatment (Lin et al., 2013). NFκB is a nucleoprotein that specifically binds promoters and is involved in the inflammatory response through the regulating the expression of cytokines and adhesion molecules (Zhang et al., 2005). Therefore, NFκB is a potential target for inhibiting inflammation through *S. miltiorrhiza* treatment. The effect of *S. miltiorrhiza* extracts on LPS-activated NFκB signaling was investigated using consistent doses and parallel experiments. In KCs stimulated by LPS, both an NFκB inhibitor and a p38 inhibitor suppressed p-NFκBp65, but increased the expression of IκB. This result suggested that PDTC and SB239063 inhibited the NFκB signaling pathway by elevating IκB, thereby increasing the formation of the NFκB–IκB complex, which blocks NFκB translocation into the nucleus and activation. SB239063 blocked p-NFκBp65, indicating that the p38 pathway regulated the NFκB-signaling pathway. PDTC inhibited both the levels of p38 levels and p-p38, suggesting crosstalk between the NFκB and p38 pathways. In KCs, different *S. miltiorrhiza* compounds had different levels of impact on the activity of the NFκB signaling pathway after activation by LPS such as the regulation of inflammatory cytokines and effects on proliferation, differentiation, and apoptosis. Tanshinone II A and salvianolie acid B enhanced expression of IκB, and decreased expression of p-NFκBp65. The other *S. miltiorrhiza* components decreased levels of NFκBp65 and affected levels of p-NFκBp65 and IκB, indicating strong inhibition of the NFκB pathway.

C-fos is important in modulating cellular and intracellular signaling and secretion. C-fos couples extracellular signals with transcription of target genes (Hossaini et al., 2011). Inhibition of c-fos blocks transcription and translation of inflammatory cytokines, leading to a decrease in inflammation (Li et al., 2012). PDTC did not decrease c-fos levels. However, SB239063 decreased c-fos, consistent with a previous study showing that c-fos was downstream of p38 (Lee & Lim, 2009). Except for salvianolie acid B, all other tested components of *S. miltiorrhiza* and a crude ethanol extract of *S. miltiorrhiza* decreased p-p38 and c-fos. All components of *S. miltiorrhiza* markedly decreased expression of c-fos, possibly by inhibition of p38. The diterpenoids containing *o*-quinone in their structures also had stronger effects on c-fos levels, possibly by inhibiting p38, than compounds containing *p*-quinone.

In conclusion, extracts of *S. miltiorrhiza* protected the liver from acute injury by CCl_4, possibly through inhibition of p38 and NFκB signaling in KCs.

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Declaration of interest

There is no conflict of interest. This work was supported by National Nature Science Foundation of China (No. 81172291).

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