Saikosaponin-d alleviates carbon-tetrachloride induced acute hepatocellular injury by inhibiting oxidative stress and NLRP3 inflammasome activation in the HL-7702 cell line

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Abstract. Saikosaponin-d (SSd) the primary active component of triterpene saponin derived from Bupleurum falcatum L., possesses anti-inflammatory and antioxidant properties. The present study aimed to examine the potential therapeutic effects of SSd on carbon tetrachloride (CCl4)-induced acute hepatocellular injury in the HL-7702 cell line and its underlying mechanisms. HL-7702 cells were treated with SSd at different doses (0.5, 1 or 2 μmol/l). Cell viability was determined using an MTT assay. Injury was assessed by the levels of serum alanine aminotransferase (ALT) and aspartate transaminase (AST). Oxidative stress was assessed using malondialdehyde (MDA) content and total-superoxide dismutase (T-SOD) activity. The expression of nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3), apoptosis-associated speck-like protein (ASC), caspase-1 and high mobility group protein B1 (HMGB1) was assessed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. Interleukin (IL)-1β and IL-18 were determined by RT-qPCR and ELISA. SSd attenuated the inhibition of cell viability and the high AST and ALT levels induced by CCl4 in HL-7702 cells. Oxidative stress was induced in HL-7702 cells by CCl4, as demonstrated by the increase of MDA and the decrease of T-SOD activity. These changes were reversed by SSd. SSd significantly downregulated the mRNA and protein expression of NLRP3, ASC, caspase-1, IL-1β, IL-18 and HMGB1 induced by CCl4. In conclusion SSd alleviated CCl4-induced acute hepatocellular injury, possibly by inhibiting oxidative stress and NLRP3 inflammasome activation in the HL-7702 cell line.

Introduction

The liver is important for metabolic homeostasis, detoxification, immunity, and secretory functions (1). Sustained and progressive liver disorders will result in severe liver injury due to increasing cellular, tissue, and function disruption, and is associated with high mortality (2). Management of liver injury remains among the most challenging issues of contemporary medicine (3) and the development of new therapies for acute liver injury is needed.

Carbon tetrachloride (CCl4) is one of the most potent hepatotoxic compounds (4). Acute liver injury models produced using CCl4 are good models of acute chemical liver injury in humans. CCl4 result in the generation of trichloromethyl free radicals (•CCl3) by cytochrome P450. The •CCl3 causes lipid peroxidation and excessive production of reactive oxygen species (ROS), resulting in liver injury (5,6). The imbalance between ROS production and the antioxidant defense leads to oxidative stress, as shown by increased lipid peroxidation and decreased activities of antioxidant enzymes such as the superoxide dismutase (SOD), catalase, and glutathione peroxidase (7).

The nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome has been reported to be involved in the pathogenesis of acute liver injury (8). It is composed of three proteins: NLRP3, apoptosis-associated speck-like protein (ASC), and caspase-1. Once activated by stimuli, the NLRP3 binds to the adaptor
protein ASC, and in turn leads to oligomerization of caspase-1, subsequently promoting the activation of caspase-1. Active caspase-1 initiates the cleavage of pro-interleukin (IL)-1β and pro-IL-18 and then promotes the secretion of IL-1β, IL-18, and high mobility group protein B1 (HMGBl) (9). These inflammatory cytokines are critical in various liver diseases such as hepatic ischemia reperfusion injury, alcoholic steatohepatitis, non-alcoholic steatohepatitis, and drug-induced liver injury (10-13).

Many herbs exhibit anti-inflammatory and anti-oxidative capacity, and are being used as adjunctive therapy for liver injury. Some compounds extracted from traditional Chinese medicine herbs possess anti-inflammatory and antioxidants effects against CCl₄-induced oxidative stress, such as cardamomum, esculentoside A, Ocimum gratissimum, resveratrol, and quercetin (14-18). Among these herbs, Radix bupleuri is one of the most commonly used for many diseases such as viral hepatitis and chronic hepatic inflammation (19). Saikosaponin-d (SSd) is the major active component of Bupleurum falcatum L. and has been reported to have anti-inflammatory, antiviral, immunomodulatory, and anti-oxidant activity (20,21). SSd inhibits the proliferation of rat hepatic stellate cells via decreasing lipid peroxidation (22) and restraints the proliferation of activated T lymphocyte via the NF-AT, NF-xB, and AP-1 pathways (23). In addition, SSd alleviates ventilator-induced lung injury via inhibiting inflammatory responses and oxidative stress (24). Nevertheless, the hepatoprotective effects of SSd against CCl₄-induced acute hepatocellular injury remain unclear.

Based on the known effects of SSd, we hypothesized that SSd alleviates the effects of CCl₄ on hepatocytes by reducing inflammation and oxidative stress. Therefore, the aim of this study was to investigate the protective effects of SSd on CCl₄-induced acute hepatocellular injury in the HL-7702 cell line and whether the effects were related to oxidative stress and NLRP3 inflammasome activation.

Materials and methods

Cell culture. HL-7702 cells (the human normal liver cell line from FuDan IBS Cell Center, Shanghai, China) were routinely cultured in RPMI-1640 medium (Wuhan Boster Biological Technology, Ltd., Wuhan, China) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 5% CO₂ at 37°C. The cells were treated with CCl₄ (Beijing Chemical Reagent Company, Beijing, China), and N-acetyl-L-cysteine (NAC; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). SSd, CCl₄, and NAC were dissolved in dimethyl sulfoxide (DMSO) immediately before use (final concentration of DMSO: <0.1%). DMSO (<0.1%) alone was included as a control in all experiments and did not have any effect on the parameters measured.

MTT assay. Cell proliferation was analyzed by the MTT assay. HL-7702 cells were washed twice with phosphate-buffered saline solution (PBS) and counted. Then, 1x10⁴ cells were seeded on 96-well plates and cultured for 24 h. The cells were exposed to SSd at various concentrations for 24 h. Other cells were seeded onto 96-well plates, incubated for 24 h, and preincubated with NAC (100 µmol/l) or the indicated doses of SSd for 1 h. After that, CCl₄ was added at 10 mmol/l to induce acute injury for 24 h (25,26). Then, 100 µl of MTT solution (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) were added to each well and incubated for 4 h. The medium was discarded and 150 µl of DMSO was added for 24 h. The absorbance of each well was measured at 570 nm with an ELx800 universal microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The relative cell viability was calculated as: (absorbance of drug treated group)/(absorbance of untreated group) x 100 (%).

Alanine aminotransferase (ALT) and aspartate transaminase (AST) in supernatants. HL-7702 cells were seeded onto 6-well plates at 8x10⁵ cells/well and cultured in RPMI-1640 with 10% FBS for 24 h. After HL-7702 cells were pretreated with NAC or the indicated doses of SSd for 1 h, CCl₄ was added at 10 mmol/l to induce acute hepatic cellular injury for 24 h (25,26). The supernatants were collected and stored at -20°C. ALT and AST levels were measured using a Var10skan Flash fluorescence plate reader (Thermo Fisher Scientific, Inc.) using the ALT and AST assay kits (C009-1 and C010-1; Nanjing Jiancheng Institute of Biotechnology, Nanjing, China).

Total-superoxide dismutase (T-SOD) activity and malondialdehyde (MDA) levels. HL-7702 cells were seeded into 6-well plates and cultured in RPMI-1640 with 10% FBS for 24 h. The cells were pretreated with NAC or the indicated doses of SSd for 1 h, followed by CCl₄ treatment for another 24 h (25,26). The supernatants were collected and stored at -20°C. MDA was detected by the thioarbituric acid (TBA) assay using a MDA assay kit (A003-1; Nanjing Jiancheng Institute of Biotechnology). T-SOD activity was determined using the hydroxylamine method with the T-SOD assay kit (A001-1-1; Nanjing Jiancheng Institute of Biotechnology).

Western blotting. NLRP3, caspase-1, ASC, and HMGBl proteins were measured by western blot. Whole cellular proteins were extracted using the M-PER lysis buffer (Thermo Fisher Scientific, Inc.). Lysates were centrifuged at 12,000 x g for 15 min at 4°C to remove debris. Proteins (50 µg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon-P; EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk and probed using NLRP3 (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), ASC (1:500; Santa Cruz Biotechnology, Inc.,) pro-caspase-1 (1:1,000; Abcam, Cambridge, MA, USA), caspase-1 (1:1,000; Abcam), HMGB1 (1:1,000; Abcam), or β-actin (1:2,000; Santa Cruz Biotechnology, Inc.) antibodies overnight at 4°C. Primary antibodies were detected with the corresponding horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) and visualized using an enhanced chemiluminescence (ECL) kit (EMD Millipore) western blotting detection system (Amersham, GE Healthcare, Waukesha, WI, USA). The band intensity was quantified using a Bio-Rad GS-690 Scanner (Bio-Rad Laboratories, Inc.). The relative expression level of each protein was normalized to β-actin or pro-caspase-1.
Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RNA (3 μg) was reverse-transcribed to cDNA with the ReverTra Ace-α® RT kit (Toyobo, Inc., Tokyo, Japan), according to the manufacturer's protocols. RT-qPCR was performed using the SYBR-Green PCR Master Mix (Toyobo, Inc.) in a PCR detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers were: NLRP3 forward 5'TTGACTCCGCAAGGACCTCGG-3' and reverse 5'GGCCCGGGTTATGCTGCTGTG-3'; ASC forward 5'GGCTGCTGGATGCTGTGA-3' and reverse 5'AGGCTGTTGTGAAACTGAAGA-3'; caspase-1 forward 5'CAGACAAGGAGTCTGCAAACAA-3' and reverse 5'TCAGAATAACCGAGTCAATCA-3'; IL-1β forward 5'TGGCAATGAGGATGACTTGTG-3' and reverse 5'TGGTGCTCGGAGATTGT-3'; IL-18 forward 5'GACCTCCAGATCGCTTCCTC-3' and reverse 5'GATGCAATTGTCTTCTACTGGTC-3' and HMGB1 forward 5'TATTCGCCCAAAATCAAGG-3' and reverse 5'TAAGGCTGTTGTACATGC-3'. The amplification process was the same for all genes and was 35 cycles of denaturation at 95°C for 45 sec, annealing at 50°C for 45 sec, and elongation at 72°C for 45 sec. β-actin was used as internal control and the primers were: forward 5'CCTCCACCTGGCCTCCTG-3' and reverse 5'GCTGTACCTTCCAGCTTCC-3'. The 2^ΔΔCq method was used to represent the relative mRNA expression of the target genes.

Enzyme-linked immunosorbent assay (ELISA). The levels of IL-1α and IL-1β in the supernatants of HL-7702 cells were measured by ELISA (BMS224HS and BMS267-2; eBioscience, San Diego, CA, USA), according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was conducted using SPSS 21.0 (IBM Corp., Armonk, NY, USA). Results were expressed as mean ± standard deviation. The differences among groups were analyzed using one way analysis of variance (ANOVA) with the Student-Newman-Keuls test for post hoc analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of SSd on the viability of HL-7702 cells. The MTT cell viability assay was performed to examine whether SSd produced cytotoxic effects on HL-7702 cell line. The treatment of HL-7702 cells with SSd at 0.5-2 μmol/l for 24 h did not affect cell viability (Fig. 1), while cell viability was decreased using 2-24 μmol/l (Fig. 1). Therefore, SSd at 0.5, 1, and 2 μmol/l were selected as the low-dose, moderate-dose, and high-dose groups in the subsequent experiments.

SSd alleviates CCl4-induced acute hepatocellular injury. The MTT assay was used to investigate the effects of SSd on CCl4-induced acute hepatocellular injury. As shown in Fig. 2A, CCl4 alone significantly decreased cell viability and SSd reversed the phenomenon in a dose-dependent manner (all P<0.01; Fig. 2A). NAC, as a positive control, also attenuated the decreased cell viability induced by CCl4 (P<0.01; Fig. 2A).

The effects of SSd on CCl4-induced serum AST and ALT levels were examined. CCl4 significantly increased the levels of serum ALT and AST. SSd treatment prevented CCl4-induced increase of AST level in a dose-dependent manner (P<0.05 for low-dose and P<0.01 for the other doses, Fig. 2B). The levels of ALT in the SSd groups were decreased significantly at the moderate and high doses compared to the CCl4 group (P>0.05 for low-dose and P<0.01 for the other doses, Fig. 2C). NAC almost completely reversed the increased levels of AST and ALT induced by CCl4 (P<0.01; Fig. 2B-C).

SSd attenuates CCl4-induced oxidative stress. To explore the mechanisms by which SSd alleviates CCl4-induced acute hepatocellular injury, the anti-oxidative effects of SSd on CCl4-induced acute hepatocellular injury in HL-7702 cells were explored. We examined the levels of T-SOD and MDA in the cell culture supernatants. The decreased T-SOD induced by CCl4 was attenuated in a dose-dependent manner by SSd (all P<0.01; Fig. 3A), while MDA showed opposite changes (P<0.05 for low-dose and P<0.01 for the other doses, Fig. 3B). CCl4-induced oxidative stress was blocked by NAC (P<0.01; Fig. 3A and B). Thus, the suppressive effect of SSd on oxidative stress could be related to the increase of T-SOD and the reduction of MDA.

SSd inhibits CCl4-induced NLRP3 inflammasome activation. The NLRP3 inflammasome is composed of the NLRP3, caspase-1, and ASC (27,28). To verify whether SSd may affect the NLRP3 inflammasome, NLRP3, ASC, caspase-1, and HMGB1 were assessed by RT-qPCR and western blotting. As shown in Fig. 4A, CCl4 significantly increased NLRP3, caspase-1, ASC, and HMGB1 mRNA levels. Treatment with SSd decreased CCl4-induced NLRP3, ASC, and HMGB1 mRNA expression in a dose-dependent manner (all P<0.01; Fig. 4A), and decreased the level of caspase-1 mRNA at the moderate and high doses (P<0.05 for low-dose, P<0.05 for the moderate dose, and P<0.01 for the high dose; Fig. 4C).

![Figure 1. Effects of SSd on the viability of HL-7702 cells. HL-7702 cells were treated with various doses of SSd (0.1-24 μmol/l). The cell viability was assessed by an MTT assay. Data are shown as mean ± standard deviation for at least three independent experiments. **P<0.01 vs. the untreated group. SSd, saikosaponin-d.](image-url)
The effects of CCl₄ injury were blocked by the positive control drug NAC (P<0.01; Fig. 4A).

Similar to the qPCR results, western blotting showed that the expressions of NLRP3, ASC, caspase‑1 and HMGB1 were increased by CCl₄, but these effects were blocked by NAC (P<0.01; Fig. 4B). The protein expressions of NLRP3, ASC, caspase‑1, and HMGB1 were gradually reduced as the dosage of SSd increased (P<0.05 for the low‑dose group for ASC, and all other P<0.01; Fig. 4B). Therefore, the anti‑inflammatory effects of SSd could be associated with the inhibition of the NLRP3 inflammasome.

SSd inhibits the secretion of proinflammatory cytokines. The production of inflammatory cytokines IL‑1β and IL‑18 in culture supernatant was examined by qPCR and ELISA. CCl₄ significantly increased IL‑1β and IL‑18 expression, which could be attenuated by NAC. More importantly, treatment with SSd gradually attenuated the expressions of IL‑1β and IL‑18 as the dosage of SSd increased (all P<0.01; Fig. 5).

Discussion

The CCl₄‑induced acute liver injury model is frequently used to examine the efficacy of liver protective agents. SSd has been found to attenuate CCl₄‑induced hepatic injury in rats by inhibiting lipid peroxidation (22). Nevertheless, the downstream molecular mechanism remains unclear. Therefore, this study aimed to examine the effects of SSd on CCl₄‑induced acute injury in HL‑7702 cells and whether the mechanisms could be related to oxidative stress and NLRP3 inflammasome activation. The results suggest that SSd attenuated CCl₄‑induced acute injury by inhibiting oxidative stress and NLRP3 inflammasome activation in the HL‑7702 cell line.

In the present study, SSd at 0.5-2 µmol/l was protective against CCl₄‑induced injury, but SSd was toxic at doses >2 µmol/l. Chen et al showed that mitochondrial apoptosis was the main toxic effect of SSd at high concentration (21). Li et al found that saikosaponins at 200 µg/ml induced hepatotoxicity through oxidative stress and lipid metabolism dysregulation (29). On the other hand, Zhao et al found that SSd at 45-60 µmol/l could protect renal tubular epithelial cell against high-glucose-induced injury by regulation of SIRT3 (30). Yao et al found that SSd inhibited the proliferation of prostate cancer cells at 50 µM (31). These results suggest that SSd is toxic at high concentrations, but protective at lower doses.

Oxidative stress is known to be related to the pathogenesis of acute liver injury (32‑34). CCl₄ is widely used to induce...
Figure 4. Effects of SSd on CCl₄-induced NLRP3, ASC, caspase-1 and HMGB1 expression. HL-7702 cells were pretreated with 100 µmol/l NAC or different doses of SSd for 1 h and then treated with 10 mmol/l CCl₄ for 24 h. (A) The mRNA levels of NLRP3, ASC, caspase-1, and HMGB1 were detected by reverse transcription-quantitative polymerase chain reaction. (B) The protein levels of NLRP3, ASC, caspase-1 and HMGB1 were determined by western blotting. Data are shown as mean ± standard deviation for at least three independent experiments. *P<0.05, **P<0.01 vs. the CCl₄ only group. CCl₄, carbon tetrachloride; SSd, saikosaponin-d; NAC, N-acetyl-L-cysteine; L, low dose, 0.5 µmol/l; M, moderate dose, 1 µmol/l; H, high dose, 2 µmol/l; NLRP3, nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; HMGB1, high mobility group protein B1; ASC, apoptosis-associated speck-like protein.
acute hepatic injury in animals, has strong hepatotoxic effects that leads to the excessive generation of free radicals associated with oxidative stress, and ultimately results in acute liver injury with functional impairment. Serum AST and ALT are widely used as makers of acute hepatic injury. Decreased levels of AST and ALT associated with fewer necrotic lesions and lipid peroxidation could imply protection against CCl\textsubscript{4}-induced injury (35). Therefore, we first verified that SSd could attenuate CCl\textsubscript{4}-induced AST and ALT increases. MDA and T-SOD are indicators of CCl\textsubscript{4}-induced oxidative stress (36). The increase of MDA, a product of lipid peroxidation, is considered to be a direct indicator of abnormal peroxidation and impaired antioxidant defenses (36). As an antioxidant enzyme, T-SOD catalyzes the dismutation of superoxide anions into hydrogen peroxide and oxygen. Studies showed that CCl\textsubscript{4} could lead to excessive generation of free radicals and oxidative stress in the liver by increasing the levels of MDA and decreasing the levels of T-SOD, ultimately leading to acute liver injury (37-39). In the present study, SSd played an antioxidant role by inhibiting the production of MDA and improving T-SOD levels in CCl\textsubscript{4}-induced acute injury in liver cells.

The activation of the NLRP3 inflammasome results in the maturation of the inflammatory cytokines IL-1\textbeta and IL-18, and also leads to the release of HMGB1 (9). These potent proinflammatory cytokines further aggravate the inflammatory progress initiated by oxidative stress. IL-1\textbeta and IL-18 are members of the IL-1 superfamily and contribute to inflammation (40,41). HMGB1 is a nuclear protein and proinflammatory mediator, and promotes inflammation and necrotic cell death. IL-1\textbeta, IL-18, and HMGB1 may be involved in acute hepatocellular injury (42-46). The effect of the NLRP3 inflammasome has been explored in many liver conditions such as drug-induced liver injury, non-alcoholic steatohepatitis, alcoholic steatohepatitis, hepatic ischemia reperfusion injury, and fibrosis (10-13,47,48). Kim et al (49) showed that NLRP3 inflammasome activation play a central role in GalN/LPS-induced inflammatory responses and the development of hepatic injury. Gong et al (50) showed that the activation of the NLRP3 inflammasome contributes to the induction of inflammation, which might be associated with BDL-induced fibrosis in non-alcoholic and alcoholic steatohepatitis. In the present study, SSd decreased the mRNA and protein expression of the NLRP3 inflammasome components after induction by CCl\textsubscript{4}, which is consistent with the role of the NLRP3 inflammasome in liver injury. In addition, the levels of IL-1\textbeta and IL-18 in the culture supernatants were reduced.

Oxidative stress induced by the overproduction of ROS is an important cause of organ and tissue injury in various inflammatory diseases (33,34). In addition, ROS play crucial roles in the activation of the NLRP3 inflammasome (51,52). Recent evidence also indicate that some herb extracts have anti-inflammatory effects by suppressing ROS
production (53). Moreover, it has been confirmed that oxidative stress and inflammatory cytokines are frequently identified as the main factors contributing to acute liver injury, and anti-inflammatory and antioxidant compounds are thought to prevent acute hepatocellular injury (54-56). Dang et al (57) showed that SSD attenuated CCl4-induced liver fibrosis in rats through the downregulation of TNF-α, IL-6, and NF-κBp65, and the upregulation of 1-kBα. Wu et al (55) also showed the beneficial effects of SSD against liver fibrosis in CCl4 rats models. Taken together, ROS-induced NLRP3 inflammasome activation may involve in CCl4-induced acute hepatocellular injury. Furthermore, the anti-inflammatory effect of SSD may depend on the modulation the inflammation through the NLRP3 inflammasome. Nevertheless, ROS and factors such as TNF-α, IL-6, NF-κBp65, and 1-κBα were not evaluated in this study, and further experiments are needed to confirm those mechanisms. In addition, NLRP3 inhibitors should be used to confirm the role of the NLRP3 inflammasome in CCl4-induced injury and the beneficial effects of SSD.

In conclusion, this study suggests that the suppressive effects of SSD on CCl4-induced acute hepatocellular injury may depend on the inhibition of oxidative stress and NLRP3 inflammasome activation. This study suggests new evidence for the potential efficacy of SSD for the treatment of acute hepatocellular injury.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
LL made substantial contributions to the acquisition of the data, data analysis and interpretation of the data and writing the manuscript. RQ and YL participated in the conception and design of the study and the revision of the manuscript. LL, YS, YC and NY performed the experiments. YL was primarily responsible for the revision of the manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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