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Full Paper

Hepatoprotective Effects of *Sedum sarmentosum* on D-Galactosamine/ Lipopolysaccharide–Induced Murine Fulminant Hepatic Failure

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Abstract. The hepatoprotective effects of sarmentosin-containing extracts of *Sedum sarmentosum* (SS) in D-galactosamine (D-GalN) / lipopolysaccharide (LPS)–induced fulminant hepatic failure mouse model. Pretreatment with SS markedly protected mice from lethal liver injury, which has known to be associated with an abrupt elevation of serum tumor necrosis factor (TNF)-α level. Indeed, SS significantly blocked the elevation of TNF-α and alanine aminotransferase and aspartate aminotransferase as well. SS also remarkably reduced number of apoptotic hepatocytes and DNA fragmentation in the liver, which correlated with blockade of caspase-3 activation. In addition, SS suppressed the increased expression of toll-like receptor 4 (TLR4). The activation of c-Jun NH_2-terminal kinase, extracellular signal-regulated kinase, and p38 induced by D-GalN/LPS was also significantly suppressed by SS treatment. Furthermore, SS significantly inhibited the activation of nuclear factor-κB. In RAW 264.7 cells stimulated with LPS, TNF-α release and TLR4 expression was suppressed by SS pretreatment, which was in line with in vivo results. These findings suggested that SS prevents D-GalN/LPS–induced fulminant hepatic failure, and this protection is likely associated with its anti-apoptotic activity and the down-regulation of mitogen activated protein kinase activity associated at least in part with suppressing the transcription of LPS receptors.

Keywords: *Sedum sarmentosum*, D-galactosamine (D-GalN) / lipopolysaccharide (LPS), toll-like receptor 4 (TLR4), tumor necrosis factor (TNF)-α, nuclear factor (NF)-κB

Introduction

Exposure of mice to D-galactosamine (D-GalN) and lipopolysaccharide (LPS) are widely used for an animal model of fulminant hepatic failure. A low dose of LPS in combination with D-GalN has been shown to induce experimental liver injury which is similar to acute hepatic failure in the clinical setting (1). D-GalN/LPS–induced fulminant hepatic failure in mice is a promising animal model for elucidating the mechanisms of clinical liver complaints and for evaluating the efficiency of hepatoprotective activity (2 – 4). It was found that dysfunction of liver microcirculation, metabolic changes, direct cytotoxicity, and macrophages-mediated liver injury occurred in LPS-treated mice (5). D-GalN, a specific hepatotoxic agent, was used to increase the sensitivity to the lethal effects of endotoxin (6).

It is known that Kupffer cells produce several kinds of biologically active mediators and seem to be the most important source of tumor necrosis factor (TNF)-α (7). As LPS is a potent stimulator of TNF-α released from Kupffer cells, TNF-α plays a critical role in the pathogenesis of D-GalN/LPS–induced fulminant hepatic failure in mice (8). TNF-α induces apoptosis in hepatocytes and neutrophil transmigration, a critical step in the necrosis of hepatocytes, that occurs at a later stage in this liver injury (9, 10). The hepatic lesions in this model resemble those of human hepatitis because the up-regulation of TNF-α expression and hepatic apoptosis have been reported as pathogenic symptoms in human hepatitis. Death receptors that have been assessed in purified liver

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cell preparations include Fas, toll-like receptors (TLRs), and TNF receptors (TNFR)-1 and -2, as well as TNF-related apoptosis-inducing ligand (TRAIL) receptors 1, 2, 3, and 4 (11). Therefore, blockade of death receptor pathways has been shown to ameliorate liver disease with various degrees. Nuclear factor (NF)-κB activation is a common pathway that mediates LPS-induced up-regulation of genes encoding inflammatory cytokines (12). It is also reported that administration of D-GalN/LPS caused activation of MAPK (mitogen activated protein kinase), which is composed of JNK (c-Jun NH_2-terminal kinase), ERK (extracellular signal-regulated kinase), and p38 (13). The MAPK cascades are activated by various cellular stresses and growth factors and involved in various biological responses such as cytokine production, differentiation, proliferation, and cell death (14).

Sedum sarmentosum BUNGE (*Crassulaceae*, SS) is a native plant that grows on Jeju island in Korea and reputed as the medicine "tollamul". The young leaves and stem are also used as a vegetable, raw or cooked, in Korea. Fang et al. (15) isolated the bioactive component sarmentosin from water extract of SS and indicated that sarmentosin has hepatoprotetive effects (16). And it was repoted that Yoshikawa et al. (17, 18) isolated 11 new megastigmane glycosides, sarmentoic acid, sarmentol A, sedumosides A1, A2, A3, B, C, and D, from the whole plant of SS, together with eight known megastigmane constituents; and these megastigmane constituents show hepatoprotective effect on D-GalN–induced cytotoxicity in primary cultured mouse hepatocytes. Even though SS have been used traditionally for the treatment of chronic viral hepatitis, few studies have investigated its related mechanism. We were intrigued to know whether SS could prevent the fulminant hepatic failure induced by D-GalN/LPS. We also investigated whether the protective effect of SS is associated with down-regulation of MAPK activation. Here, we report that sarmentosin-containing SS extracts have a significant preventive effect on D-GalN/LPS–induced fulminant hepatic failure. Our results suggest that the anti-apoptotic activity of SS is associated with down-regulation of MAPK activation exerted at least in part through the inhibition of the transcription of LPS receptors.

Materials and Methods

Reagents

LPS and D-GalN were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The TNF-α kit (Quantikine® Mouse TNF-α Immunoassay) was purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals used were of analytical grade. Silymarin, a reference drug for the in vivo experiments, was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA).

Plant material and sample preparation

SS was collected on JeJu island of South Korea in November 2003 and authenticated by Dr. Jung Joon Lee of Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). We deposited a voucher specimen (KRIBB002003) at the herbarium of Korea Research Institute of Bioscience and Biotechnology. Briefly, 10 volumes of distilled water was added to 500 g dried SS and boiled under reflux for 5 h and then percolated and concentrated in a rotary vacuum evaporator followed by lyophilization. The yield (w/w) was measured to be 35%. The freeze-dried extract was used in both chemical analysis and pharmacological studies. The water-soluble fraction of SS was analyzed by HPLC to confirm the presence of sarmentosin and trans-caffeoyl-malic acid (Fig. 1A). The content of sarmentosin was 1.16% in the SS extract. The extract was pre-solubilized in distilled saline for the in vivo studies.

Animals and treatment

Male C57BL/6 mice (20 – 22 g) were purchased from Dae Han Laboratory Animal Research and Co. (Choongbuk, Korea), fed with a standard chow diet (Jae Il Chow, Daejeon, Korea), and given tap water ad libitum. Animals were housed in plastic cages and maintained at 22 ± 2°C and 50% – 60% relative humidity, with 12-h light–dark cycles throughout the experiment. Animal experiments were performed under the latest edition of "Guiding Principles in the Use of Animals in Toxicology" adopted by the Society of Toxicology (USA). The study was approved by the Korea Research Institute of Bioscience and Biotechnology animal care committee. SS (50 or 100 mg/kg body weight) or silymarin (100 mg/kg) was administered intraperitoneally to mice at 12 and 1 h before D-GalN/LPS administration. At 1 h after the last doses of SS, except the normal control group, mice were given an intraperitoneal injection of D-GalN (700 mg/kg body weight), immediately followed by intraperitoneal injection of LPS (10 μg/kg body weight), to induce fulminant hepatic failure. Animals were sacrificed; and blood was collected from the carotid artery at 1, 2, 4, or 6 h after administration of D-GalN/LPS. Blood samples were allowed to coagulate at 4°C for 30 min. Serum was then separated by centrifugation at 3000 rpm for 30 min at 4°C. The liver of each mouse was removed immediately and then was kept at −80°C until analyzed. There were at least 10 animals in each group.

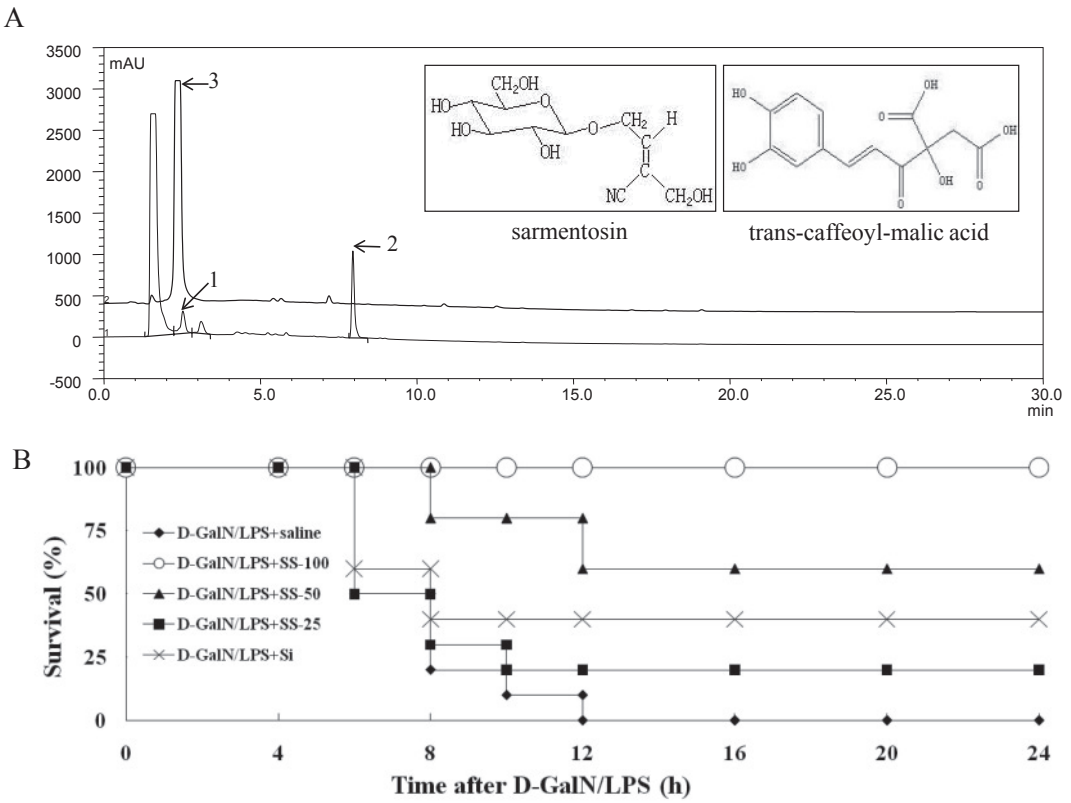


Fig. 1. HPLC chromatograms of SS and the survival curve. A: column, J'sphere ODS-H80 (150 × 4.6 mm); flow rate, 0.5 ml/min; mobile phase, started with a gradient of 5% – 100% acetonitrile/water (containing 0.05% trifluoroacetic acid) for 20 min followed by washing with 100% acetonitrile. Peaks: 1, sarmentosin; 2, trans-caffeoyl-malic acid; 3, standard sarmentosin. B: Effect of SS on the survival curve of mice after D-GalN/LPS injection. Mice were intraperitoneally injected with D-GalN (700 mg/kg body weight) / LPS (10 μg/kg body weight). SS (25, 50, or 100 mg/kg body weight) or silymarin (100 mg/kg) was intraperitoneally administered at 12 and 1 h before D-GalN/LPS injection. Each group consisted of 10 mice.

Cell cultures

RAW 264.7 murine macrophages (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml), all from Gibco, Stockholm, Sweden. The cells were grown for a maximum of 20 passages, and all experiments were performed at 70% cell confluence. Cells were treated with LPS (1 μg/ml) as described in detail in the figure legends.

Cell viability

The cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) assay. RAW 264.7 cells were plated at 5×10^4 cells in 96-well plates (Nunc, Roskilde, Denmark). After an overnight culture period, cells were washed with phosphate-buffered saline (PBS) and then incubated in DMEM medium containing various concentrations of SS (50, 100, or 200 μg/ml). After 24 h of incubation, medium was replaced with MTT solution (0.5 mg/ml in DMEM containing 10% FBS) and incubated for another 3 h at 37°C. The supernatant was discarded and 100 μl of DMSO was added to each cell. Reduction of MTT to formazan was measured spectrophotometrically at 540 nm. The optical density of MTT-formazan formed in normal (untreated) cells was taken as 100% viability.

Determination of lethality

SS extract was administered intraperitoneally at a dose of 25, 50, or 100 mg/kg to mice at 12 and 1 h before D-GalN/LPS injection. Control mice were given saline. The survival rate of mice was monitored for 24 h after D-GalN/LPS injection.

Blood biochemistry and TNF-α assay

Blood was collected at 1, 2, 4, or 6 h after D-GalN/LPS administration. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by the Autodry Chemistry Analyzer (SPOTCHEM™ SP4410; Arkray, Kyoto). Serum TNF-α was determined at 1, 2, 4, or 6 h after D-GalN/LPS injec-

tion by an enzyme-linked immunosorbent assay kit according to the manufacturer's protocol. The culture supernatant from RAW 264.7 cells at 24 h after LPS stimulation was used to determine the production of TNF-α according to the manufacturer's protocol.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

Apoptotic cells were detected by the TUNEL method using an in situ detection kit (Roche, Mannheim, Germany) for the detection and quantification of apoptosis at the single-cell level. Staining of tissue sections was performed according to the manufacturer's protocol, as follows: Paraffin-embedded sections were dewaxed in xylene and rehydrated by passing them through a graded series of ethanol solutions, ending with PBS. Sections were permeabilized with proteinase K (20 μg/ml in 10 mM Tris-HCl, pH 7.4 – 8.0) at 37°C for 15 min. After washing, sections were stained with fluorescent anti-TdT. Sections were viewed and photographed using standard fluorescent microscopic techniques.

DNA fragmentation analysis

Genomic DNA was extracted from liver tissue by a Wizard® genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Extracted DNA was subjected to electrophoresis on a 1.5% agarose gel containing 0.1 μg/ml ethidium bromide.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts from liver tissue were prepared by using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Nuclear extracts were incubated for 20 min at room temperature with a gel-shift binding buffer [5% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 50 μg/ml poly(dI-dC) poly(dI-dC)] and ^{32}P-labeled oligonucleotide. The DNA–protein complex formed was separated on 4% native polyacrylamide gels, and the gel was transferred to Whatman 3 MM paper, dried, and exposed to X-ray film.

Western blot analysis

Preparation of crude protein extracts from mouse liver was performed as previously described (19). Proteins from RAW 264.7 cells were prepared as follows: SS-pretreated RAW 264.7 cells were stimulated with LPS (1 μg/ml). Cells were rinsed twice with ice-cold PBS and harvested by scraping in 1 ml of ice-cold PBS. After centrifugation, cell pellets were lysed in Western and IP lysis buffer (Beyotime Biotechnology, Shanghai, China) for 30 min on ice. The lysates were centrifuged at 15,000 × g for 10 min and then the supernatants were immediately aliquoted and stored at −70°C until use. The protein concentration was determined by a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (50 μg) were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% skim milk in incubation buffer and incubated with antibodies against mouse JNK, p-JNK, p-ERK, ERK, p38, p-P38 (1:1000; Cell Signaling Technology, Beverly, MA, USA), caspase-3, and TLR4 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bound antibody was detected with horseradish peroxidase–conjugated secondary antibody, and the reacted bands were revealed by enhanced chemiluminescence using the WEST-ZOL™(plus) Western blot detection system (iNtRON Biotechnology Co., Seongnam, Korea). Loading accuracy was evaluated by membrane rehybridization with monoclonal antibodies against α-tubulin (Sigma-Aldrich, St. Louis, MO, USA). Densities of the immunoreactive bands were analyzed with Image Master 1D Elite software (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analyses

All values are expressed as the mean ± S.D. A comparison of the results was performed with one-way ANOVA and Tukey's multiple comparison tests. Statistically significant differences between groups were defined as P-values less than 0.05. Calculations were performed by the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA, USA).

Results

Effects of SS on lethality in mice with D-GalN/LPS–induced fulminant hepatic failure

To determine protective effects of sarmentosin-containing extracts of SS on lethality, SS or silymarin as a control were administered intraperitoneally to mice 12 and 1 h before D-GalN/LPS treatment. Mice began to die within 6 h of D-GalN/LPS injection, and at 24 h, the lethality rate was 100%. However, pretreatment with SS reduced the lethality in a dose-dependent manner. Briefly, 24 h after D-GalN/LPS administration, the lethality rate was 80%, 40%, and 0% (survival of 2, 6, and 10 of 10 mice) in the mice pretreated with 25, 50, and 100 mg/kg SS, respectively, as listed in Fig. 1B. Silymarin (100 mg/kg) showed significantly lower level of protection than SS. Therefore, we selected 50 and 100 mg/kg of SS for the following study.

Effects of SS on serum ALT and AST levels of mice with D-GalN/LPS–induced fulminant hepatic failure

To address the effect of SS on serum enzymes, we investigated whether SS regulates serum AST and ALT levels in D-GalN/LPS–induced liver failure. D-GalN/LPS co-injection induced fulminant hepatic failure with marked time-dependent increase in serum AST and ALT levels, which reached 3987 ± 342 and 2356 ± 256 IU/l at 6h after D-GalN/LPS injection, while vehicle-treated mice levels were 125 ± 19 and 22 ± 8 IU/l, respectively (Fig. 2, A and B). Intraperitoneal pretreatment with 50 or 100 mg/kg of the SS reduced serum AST levels to 7.3% ($P < 0.001$) and 3.4% ($P < 0.001$), respectively, of those in D-GalN/LPS–treated mice 6 h after D-GalN/LPS administration. ALT levels in the 50 or 100 mg/kg–dose groups also significantly decreased to 5.9% ($P < 0.001$) and 0.8% ($P < 0.001$), respectively, of those in the D-GalN/LPS–treated mice 6 h after D-GalN/LPS administration. However, mice pretreated with silymarin (100 mg/kg) prior to D-GalN/LPS showed a significantly higher ALT (28.9% of those in the D-GalN/LPS–treated mice) and AST (21.7% of those in the D-GalN/LPS–treated mice) levels than mice that received SS. There were no significant differences in the serum AST and ALT levels in mice treated with 100 mg/kg SS only when compared with those in the vehicle-treated normal mice.

Effects of SS on serum TNF-α level of mice with D-GalN/LPS–induced fulminant hepatic failure

It is known that TNF-α is not only the first released inflammatory cytokine, but also is an important cytokine involved in hepatocyte apoptosis in mice with D-GalN/LPS–induced fulminant hepatic failure. Therefore, TNF-α plays a crucial role in the lethal activity of D-GalN/LPS and induces hepatocyte apoptosis in the early stages of live injury in D-GalN–sensitized mice. Furthermore, TNF-α–induced neutrophil transmigration occurs in the later stage of liver injury and causes massive hepatocyte necrosis (9, 10). In our study, TNF-α induction by D-GalN/LPS peaked at 1 h and decreased at 4 h after D-GalN/LPS co-injection. We found that the increase in serum level of TNF-α induced by D-GalN/LPS was down-regulated by SS pretreatment. Intraperitoneal pretreatment with 50 or 100 mg/kg of SS reduced serum TNF-α levels to 6.9% ($P < 0.001$) and 3.4% ($P < 0.001$), respectively, of those in the D-GalN/LPS–treated mice 1 h after D-GalN/LPS administration (Fig. 2C). However, mice that received 100 mg/kg silymarin showed markedly higher TNF-α (34.7% of those in the D-GalN/LPS–treated mice) level than those with SS.

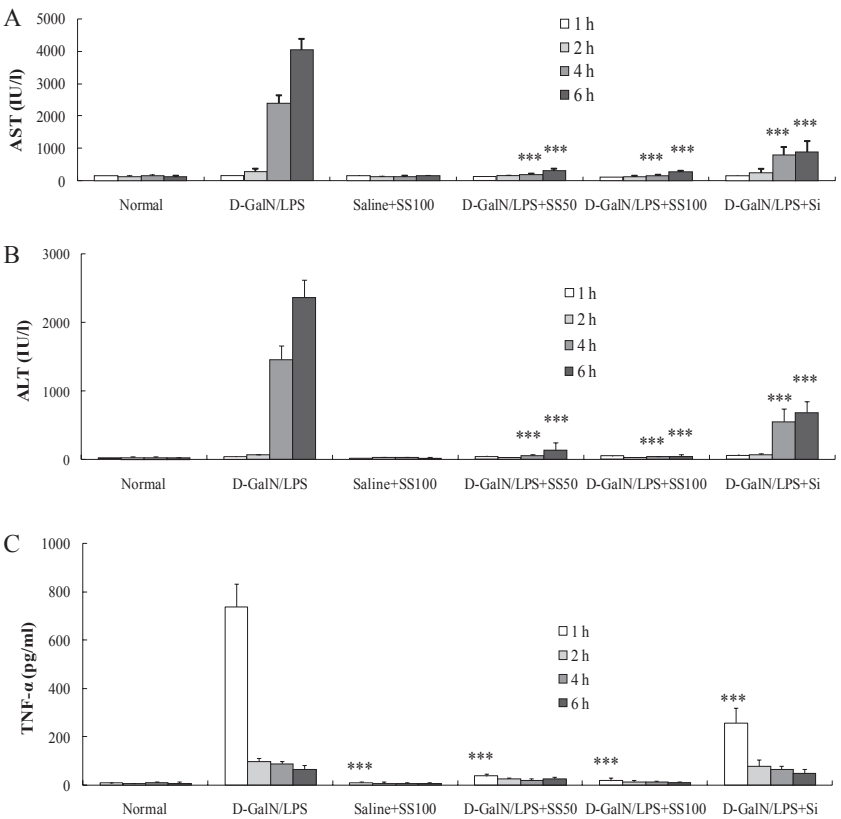


Fig. 2. Hepatoprotective effect of SS on serum biochemical parameters after D-GalN/LPS administration. Mice were intraperitoneally injected with D-GalN (700 mg/kg body weight) / LPS(10 μg/kg body weight). SS (50 or 100 mg/kg body weight) or silymarin (100 mg/kg) was intraperitoneally administered at 12 and 1 h before D-GalN/LPS injection. Serum AST (A), ALT (B), and TNF-α (C) was measured at several time points, and each value is expressed as the mean ± S.D. (n = 10). ***$P < 0.001$, significantly different when compared with D-GalN/LPS alone group.

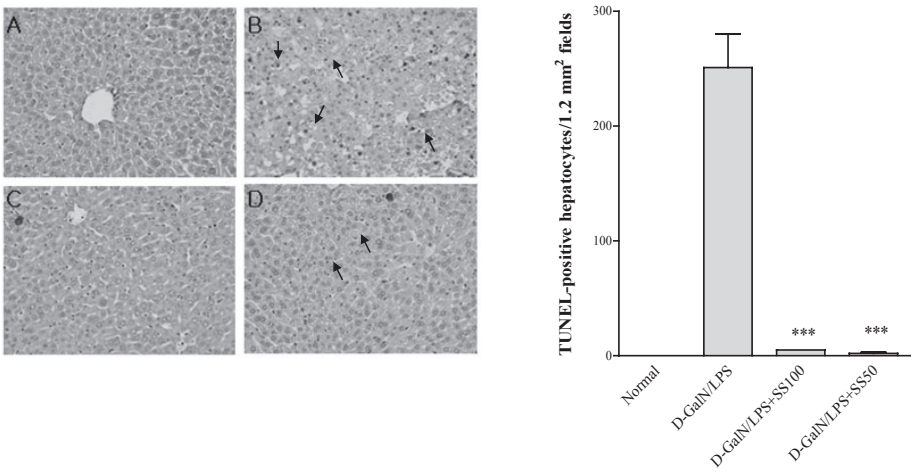


Fig. 3. TUNEL assay and quantitative analysis of apoptotic hepatocytes. Photos represent the liver of control mice or SS-treated mice at 6 h after D-GalN/LPS co-injection (original magnification ×100). TUNEL-positive hepatocytes were counted in ten 1.2-mm^2 fields. The average number of total hepatocytes per 1.2-mm^2 fields in the normal liver was about 180, and the approximate percentage of apoptotic hepatocytes can be estimated according to this number. Each value represents the mean ± S.D. (n = 5). ***$P < 0.001$, significantly different when compared with the D-GalN/LPS–alone group. Black arrows indicate TUNEL-positive hepatocytes. A: Normal, B: D-GalN/LPS, C: D-GalN/LPS + SS100, D: D-GalN/LPS + SS50.

SS inhibits hepatocyte apoptosis in mice with D-GalN/LPS–induced fulminant hepatic failure

SS suppressed lethality and serum enzymes in mice with D-GalN/LPS–induced fulminant hepatic failure and these findings prompted us to investigate whether SS also inhibited hepatocyte apoptosis. Indeed, a few TUNEL-positive hepatocytes were found in the livers from animals pretreated with SS. In contrast, a large number of TUNEL-positive hepatocytes were seen in the livers of D-GalN/LPS–treated mice 6 h after injection (Fig. 3).

To confirm the suppressive effect of the SS on hepatocyte apoptosis, genomic DNA fragmentation was assayed. Genomic DNA fragmentation was observed in the livers of mice treated with D-GalN/LPS alone 6 h after D-GalN/LPS injection, while very little DNA fragmentation was observed in the livers of mice pretreated with 50 or 100 mg/kg of the SS (Fig. 4). Next, we investigated whether SS regulated activation of caspase-3. Caspases are crucial mediators of apoptosis, and among them, caspase-3 is one of the key executioners of apoptosis, being responsible either totally or partially for the proteolytic cleavage of many key proteins. The proteolytic activation of caspase-3 was measured in mice with D-GalN/LPS–induced hepatic failure, with or without SS treatment. Caspase-3 was proteolytically processed to the active p17 fragment 4 h after D-GalN/LPS treatment in mice (Fig. 5A). However, SS pretreatment prior to D-GalN/LPS injection significantly inhibited caspase-3 activation.

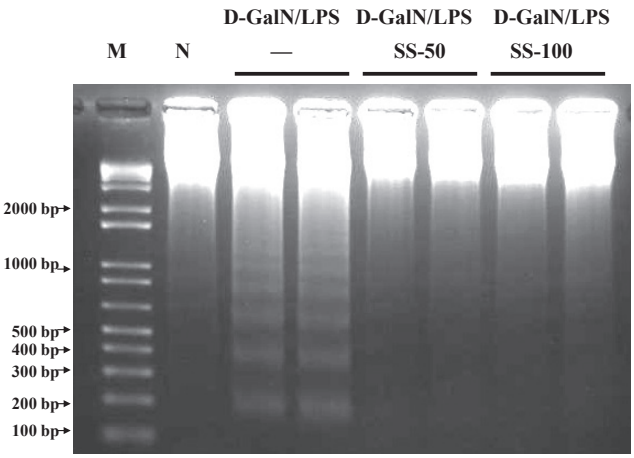


Fig. 4. The hepatoprotective effect of SS on the DNA fragmentation in D-GalN/LPS–induced fulminant hepatic failure. SS (50 and 100 mg/kg) or saline was intraperitoneally administered at 12 and 1 h before D-GalN/LPS injection. DNA was extracted from liver tissues of mice at 6 h after D-GalN/LPS injection.

SS suppressed the transcription of LPS receptors

TLR4, as the critical component of the LPS receptor complex, were determined in D-GalN/LPS intoxicated mice. Expression of TLR4 in liver was significantly elevated 6 h after D-GalN/LPS treatment. Pretreatment with SS inhibited the over-expression of TLR4 induced by D-GalN/LPS (Fig. 5B).

We examined the activation of the transcription factor NF-κB in vivo. As shown in Fig. 6, DNA binding activity of NF-κB was determined 1 h after D-GalN/LPS administration. SS pretreatment attenuated DNA-binding of NF-κB in D-GalN/LPS–challenged mice compared with controls receiving saline before D-GalN/LPS treatment.

SS inhibits MAPK activation in mice with D-GalN/LPS–induced fulminant hepatic failure

It has been well established that MAPKs such as JNK are redox-sensitive and involved in apoptosis (20 – 22).

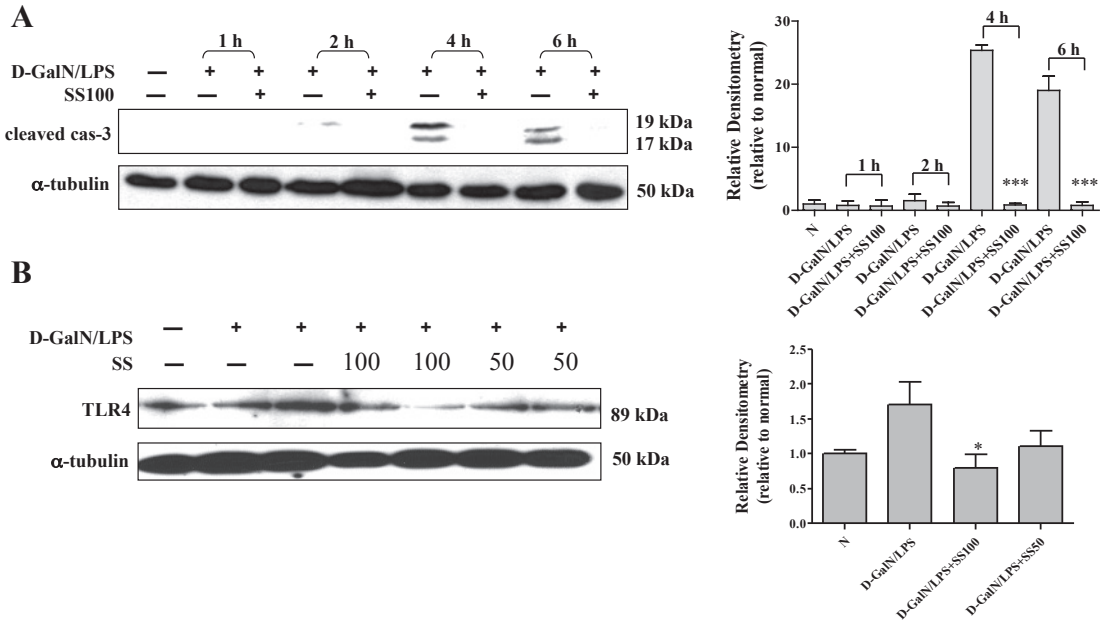


Fig. 5. Western blot analysis of the caspase-3 cleavage and TLR4 expression. SS (50 or 100 mg/kg) or saline was intraperitoneally administered at 12 and 1 h before D-GalN/LPS injection. The active form of caspase-3 (A) was detected at the indicated time points and TLR4 (B) was detected 6 h after D-GalN/LPS treatment. Each immunoreactive band was digitized and expressed as a ratio of α-tubulin levels. The ratio of the normal group band was set to 1.00. α-Tubulin was used as an internal control. Values of densitometric analysis were each the mean ± S.D. of three independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$, significantly different when compared with the D-GalN/LPS–alone group at different time points after D-GalN/LPS injection.

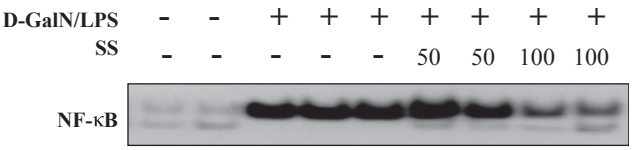


Fig. 6. SS inhibits NF-κB activation induced by D-GalN/LPS. Mice were intraperitoneally injected with D-GalN (700 mg/kg) / LPS (10 μg/kg). SS (50 or 100 mg/kg) or saline was intraperitoneally administered at 12 and 1 h before D-GalN/LPS injection. Control animals received saline alone. At 1 h after D-GalN/LPS treatment, nuclear extracts were prepared from liver tissue. DNA binding capacity of NF-κB was determined by EMSA. The results shown are representative of three independent experiments.

We examined whether the MAPK pathways were involved in the protective effect of SS on D-GalN/LPS–induced fulminant hepatic failure. Phosphorylated ERK, p38, and JNK significantly increased after treatment with D-GalN/LPS, while their protein levels were almost the same as those of the control from 1 to 6 h after D-GalN/LPS treatment (Fig. 7). Phosphorylated ERK and p38 was significantly increased after 2 h and maintained at a significantly high level for 2 – 6 h after D-GalN/LPS injection, while the levels of ERK and p38 proteins were almost the same as those of the control after D-GalN/LPS treatment. As far as phosphorylated JNK is concerned, phosphorylated JNK of 54 kDa was more significantly increased. However, pretreatment with 100 mg/kg of SS decreased the levels of phospho-ERK, -p38, and -JNK. In contrast, pretreatment with 100 mg/kg SS dramatically abolished the increase of phosphorylated ERK and p38, while the levels of ERK and p38 protein were almost the same as those of the control from 1 to 6 h after D-GalN/LPS treatment (Fig. 7). Phosphorylated JNK of 54 kDa was also significantly down-regulated by SS pretreatment. These results indicated that SS markedly suppressed the D-GalN/LPS–induced phosphorylation of JNK, ERK, and p38.

Effects of SS on LPS-stimulated RAW 264.7 mouse macrophages

TNF-α, an important factor in mediating D-GalN/LPS–induced liver damage, is rapidly produced by macrophages in response to tissue damage. Herein we applied the RAW 264.7 cell line to confirm whether SS inhibited TNF-α release induced by LPS in vitro. As shown in Fig. 8A, no statistically significant difference was observed on the viability of mouse macrophages with or without SS (50, 100, or 200 μg/ml). The MTT results indicated that SS had no cytotoxic effects. Under the stimulation by LPS, macrophages secret TNF-α, and meanwhile,

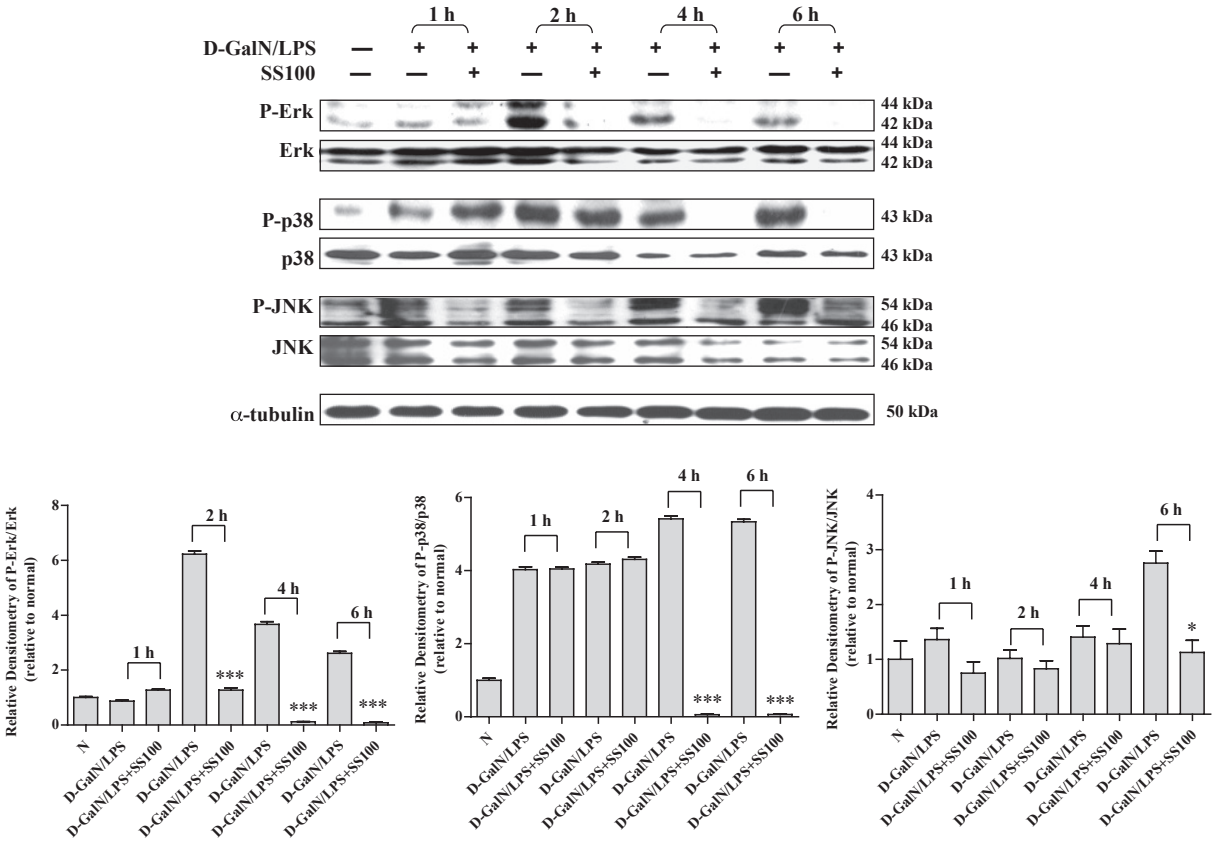


Fig. 7. Western blot analysis of MAPKs. SS (100 mg/kg) or saline was intraperitoneally administered at 12 and 1 h before D-GalN/LPS injection. Phosphorylation of JNK, ERK, and p38 were detected by Western blotting. Densitometric scanning data of phospho-JNK, phospho-ERK, or phospho-p38 levels were expressed as the ratio of JNK, ERK, or p38, respectively. The ratio of the normal group band was set to 1.00. Values of densitometric analysis were each the mean ± S.D. of three independent experiments. *$P < 0.05$, ***$P < 0.001$, significantly different when compared with the D-GalN/LPS–alone group at different time points after D-GalN/LPS injection.

TLR4 recognizes LPS and mediates macrophage activation and pro-inflammatory cytokine release. As expected, SS inhibited the transcription of TNF-α and TLR4 to a certain extent in vitro (Fig. 8, B and C).

Discussion

SS has been used as a traditional herbal medicine (23). Pharmaceutical granules of this plant have been used in clinics since 1971, while a water-soluble sarmentosin and trans-caffeoyl-malic acid isolated from this plant is considered as the active constituent of SS (23). It has been reported that the crude alkaloid fraction of SS may improve survival of hepatoma patients via the inhibition of excessive growth of human hepatoma cell lines (24). The ethyl acetate extract of SS was found to exhibit distinct angiotensin-converting enzyme (ACE) inhibitory activity (25). However, the protective effect of SS against fulminant hepatic failure has not been well studied. This study demonstrated the hepatoprotective effect of SS on fulminant liver failure.

To evaluate the hepatoprotective potential of SS in vivo, D-GalN/LPS was administered to mice to induce liver injury. D-GalN is a liver-specific toxin that increases the sensitivity of animals to the hepatotoxic and lethal effects of LPS more than 1000-fold. Co-administration of D-GalN/LPS produces typical hepatic apoptosis, followed by fulminant hepatic failure (6, 26, 27). In this study, mice in the D-GalN/LPS–treated group started to die at 6 h, and the mortality rate reached 100% at 24 h. At 6 h after the D-GalN/LPS injection, severe necrosis occurred as a result of D-GalN/LPS–induced fulminant hepatic failure. This was indicated by the significant increase in the serum ALT and AST levels. Our data showed that the ALT and AST began to increase at 4 h and reached a maximum at 6 h after the D-GalN/LPS injection (Fig. 2, A and B). Moreover, SS treatment prior to D-GalN/LPS markedly decreased ALT and AST levels. Therefore, the decrease in mortality and serum ALT and AST levels by SS in this study is probably due to its

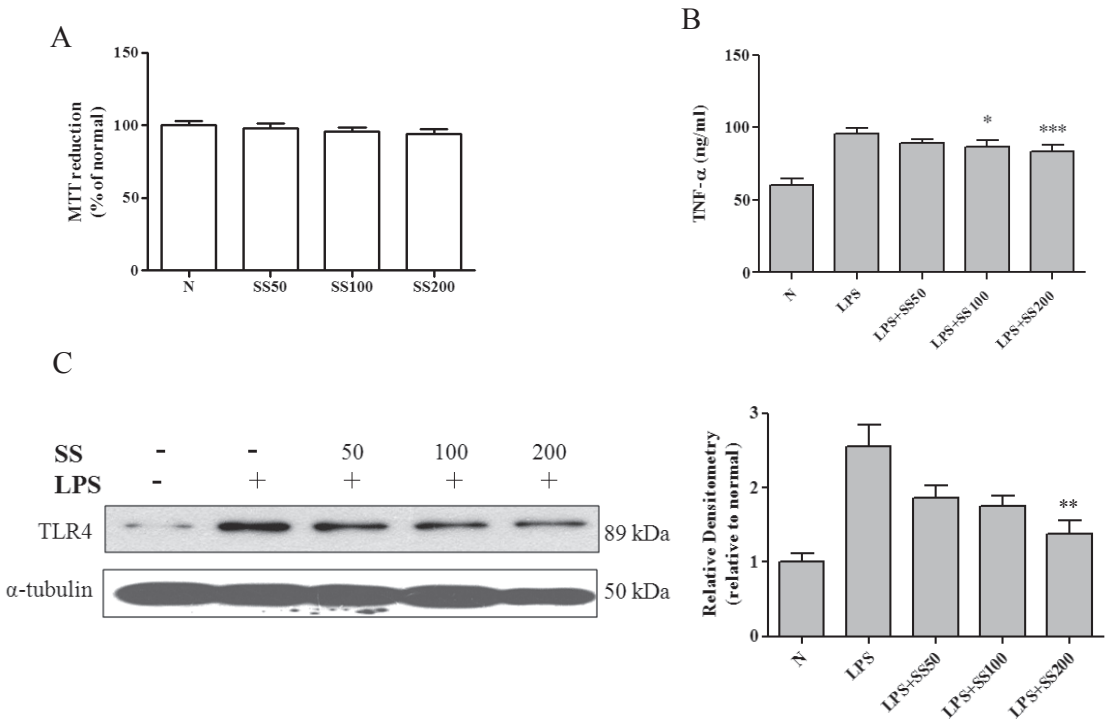


Fig. 8. Effects of SS on LPS-stimulated RAW 264.7 mouse macrophages. A: Effect of SS on cell viability of RAW 246.7 cells. Cells (n = 6) were treated with various concentrations of SS for 24 h prior to cytotoxicity being measured. B: Effects of SS on LPS-induced TNF-α release. RAW 264.7 cells were pretreated with the indicated concentrations of SS for 1 h prior to incubation of LPS (1 μg/ml) for 24 h. Levels of TNF-α in the culture medium of RAW 264.7 cells were quantified by ELISA. C: Effects of SS on TLR4 expression. RAW 264.7 cells were pretreated with the indicated concentrations of SS for 1 h prior to incubation with LPS (1 μg/ml) for 24 h. TLR4 was determined by Western blotting. Each immunoreactive band was digitized and expressed as a ratio of α-tubulin levels. The ratio of the normal group band was set to 1.00. Values of densitometric analysis were each the mean ± S.D. of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different when compared with LPS-stimulated normal cells.

protection against D-GalN/LPS–induced fulminant hepatic failure. Furthermore, mice that received 100 mg/kg silymarin had markedly higher ALT and AST levels than those that received SS. On the other hand, treatment of normal mice with SS at a dose of up to 10 g/kg body weight did not result in any signs of liver injury, such as elevation of serum ALT and AST (data not shown). These results encouraged us to examine the protective effect of SS in vivo.

D-GalN/LPS–induced murine hepatic failure, a well-established model, was initiated by a processes typical of hepatocyte apoptosis (28). Hepatocyte apoptosis plays an important role in the development of fulminant hepatic failure and the regulation of undesired hepatocyte apoptosis is one approach for treating fulminant hepatic failure. D-GalN/LPS–induced liver injury is characterized by massive apoptosis of hepatocytes. Therefore, TUNEL staining, genomic DNA fragmentation assay, and activation of caspase-3 were performed to confirm the suppressive effect of SS on hepatocyte apoptosis. Interestingly, D-GalN/LPS–induced hepatocyte apoptosis are significantly inhibited by pretreatment of SS. Moreover, SS pretreatment inhibited the apoptotic process occurring in D-GalN/LPS injury, which translates to a protective effect.

Under the stimulation by LPS, macrophages secret various pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α (29). Among these, TNF-α is a terminal mediator for hepatic apoptosis, subsequently leading to hepatic necrosis (30). TNF-α is essential for this severe liver injury because it directly activates the caspase-8–dependent apoptotic signals by binding to the TNF receptor on the surface of hepatocytes, and then caspase-8 triggers the activation of caspase-3, which is a downstream cysteine proteinase, through multiple apoptosis signaling pathways (8, 14). Kupffer cells, resident macrophages of the liver, have the transmembrane protein TLR4, which recognizes endotoxin (LPS) or the LPS–CD14 complex and mediates macrophage activation and pro-inflammatory cytokine release (31, 32). Upregulated TLR4 expression and function was observed in various situations of experimental liver injury such as partial

hepatectomy and acute hepatic failure (33, 34). In the current study, we found that the TNF-α level and TLR4 expression were enhanced after D-GalN/LPS injection, which was in line with the previous studies (35 – 37). SS administration inhibited the transcription of TNF-α and TLR4 to a certain extent, which would partially contribute to liver protection (Fig. 5B). In addition, in RAW 264.7 cells stimulated with LPS, TNF-α release and TLR4 expression were suppressed by SS pretreatment, which is consistent with the in vivo results (Fig. 8). Moreover, our study confirmed that an injection of D-GalN/LPS increases the activity of caspase-3 as well as the number of apoptotic hepatocytes, leading to the death of the animal. Thus, it provides a practical tool for the evaluation of drugs that interfere with hepatic apoptosis.

TLR4 signaling initiates systemic inflammation by producing several cytokines. Moreover, it is well known that expression of these molecules is controlled by transcription factors including NF-κB (38). D-GalN blocks the transcriptional activity of NF-κB, which is concomitantly activated by TNF-α, and suppresses the apoptotic effects of the latter (39). In this study, activation of NF-κB in liver was induced by D-GalN/LPS administration and suppressed by SS pretreatment. NF-κB was activated at 1 h after D-GalN/LPS injection, consistent with changes in the time course of TNF-α levels in serum and illustrating the involvement of NF-κB activation in TNF-α release. It is possible that SS pretreatment reduces the production of TNF-α via inhibiting the transcription of LPS receptors.

In addition to TLR4-signaling, D-GalN/LPS also activate MAPKs, which mediate a distinct signaling cascade activated by a variety of environmental stresses and have been proposed to play an important role in the regulation of apoptosis. At present three major groups of MAPKs are known: classic ERK, JNK, and p38 MAPK. JNK activation is an important component of the stress response in cells, but when JNK activation is sustained, it is believed to promote cell injury and death. Inhibition of MAPKs induces the decrease in endotoxin-induced JNK activation and apoptosis (40, 41). Moreover, JNK was considered to be important for TNF-α toxicity in both hepatocytes and nonhepatocytes. Sustained JNK phosphorylation is correlated with TNF-α toxicity (42). The ERK signaling pathway appeared to play a significant role in mediating LPS-provoked inflammatory conditions (43). As reported, ERK was transiently activated in the D-GalN/LPS–treated hepatocytes and Kupffer cells cocultured system (44). Proliferation of many inflammatory cells was reported to be mediated by the ERK signaling pathway, particularly by phosphorylation of ERK proteins (44). SS pretreatment significantly inhibited D-GalN/LPS–induced activation of JNK and ERK (Fig. 8). Furthermore, we firstly found that D-GalN/LPS coinjection also activated p-38 phosphorylation, and SS significantly inhibited p-38 phosphorylation.

Overall, it appears that SS prevents the D-GalN/LPS–induced fulminant hepatic failure, and this protection may correlate with attenuation of apoptosis and downregulating MAPKs phosphorylation associated at least in part with suppressing the transcription of LPS receptors. Thus, SS may provide an alternative for the prevention of fulminant hepatic failure. However, further investigations are required to determine the exact protective mechanism and to discover the active candidates.

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