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Protective effects of Asiaticoside on acute liver injury induced by lipopolysaccharide/D-galactosamine in mice

Li Zhang [a,b,1], Hong-zhong Li [a,c,1], Xia Gong [a,d], Fu-ling Luo [a,e], Bin Wang [f], Ning Hu [g], Chang-dong Wang [a], Zhuo Zhang [a], Jing-yuan Wan [a,*]

[a] Chongqing Key Laboratory of Biochemistry and Molecular Pharmacology, Chongqing Medical University, Chongqing 400016, People's Republic of China
[b] Department of Pathophysiology, Chongqing Medical University, Chongqing 400016, People's Republic of China
[c] Pharmacological and Toxicological Evaluation Center, Chongqing Pharmaceutical Research Institute, Chongqing 400061, People's Republic of China
[d] Department of Anatomy, Chongqing Medical University, Chongqing 400016, People's Republic of China
[e] Department of Pharmacy, The first Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China
[f] Department of Anesthesiology, The first Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China
[g] Department of Orthopedics, The first Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China

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ABSTRACT

Asiaticoside (AS), a triterpenoid product isolated from *Centella asiatica*, has been described to exhibit anti-inflammatory activities in several inflammatory models. However, the effects of AS on liver injury are poorly understood. The present study was undertaken to investigate whether AS is efficacious against Lipopolysaccharide (LPS) /D-galactosamine (D-GalN)-induced acute liver injury in mice and its potential mechanisms. AS (5, 10 and 20 mg/kg/d) was pretreated orally once daily for 3 days before LPS/D-GalN injected in mice. The mortality, hepatic tissue histology, plasma levels of Tumor necrosis factor-alpha (TNF-α) and alanine aminotransferase (ALT) and aspartate aminotransferase (AST), hepatic tissue TNF-α and caspase-3 activity were measured. Besides, western blotting analysis of phospho-p38 mitogen-activated protein kinase (phospho-p38 MAPK), phospho-c-jun N-terminal kinase (phospho-JNK) and phospho-extracellular signal regulated kinase (phospho-ERK) were determined. As a result, AS showed significant protection as evidenced by the decrease of elevated aminotransferases, hepatocytes apoptosis and caspase-3, alleviation of mortality and improvement of liver pathological injury in a dose-dependent manner. Further, we found that AS dose-dependently reduced the elevation of phospho-p38 MAPK, phospho-JNK, phospho-ERK protein and TNF-α mRNA expression in liver tissues and plasma TNF-α. These results suggest that AS has remarkable hepatoprotective effects on LPS/D-GalN-induced liver injury and the possible mechanism is related to inhibition of TNF-α and MAPKs.

Introduction

Acute hepatic failure characterized by hepatic encephalopathy, severe coagulopathy, jaundice, and hydroperitoneum is associated with high patient mortality, for which there is still no available therapy except liver transplantation limited by the chronic shortage of donor livers (Lee, 1994; Van Thiel et al., 2002). As the main pathogenic factor of Gram-negative bacteria, Lipopolysaccharide (LPS) inducing a systemic pro-inflammatory process which leads to multiple organ failure and death is implicated in the pathogenesis of liver injury (Jirillo et al., 2002).

LPS/D-GalN-induced liver injury in mice has been used frequently in the preparation of experimental animal models with endotoxemic shock and acute hepatic failure, which is similar to acute hepatic failure in clinic (Silverstein, 2004; Xiong et al., 1999). In this model, LPS exerts its effects by stimulating inflammatory cells and hepatic Kupffer cells to produce various pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12) and interferon-gamma (IFN-γ) (Batey and Wang, 2002; Malhi and Gores, 2008). Among these factors, TNF-α is the dominant mechanism of liver injury in this model. It induces activation of caspases and apoptosis in hepatocytes prior to secondary necrosis and release of transaminases by activation of TNF receptor-1 (de la Mata et al., 1990; Wullaert et al., 2007). With regard to the regulation of TNF-α, mitogen-activated protein kinases (MAPKs) family including p38 kinase, c-jun N-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) are important mediators. MAPKs serve to regulate diverse cellular responses to extracellular stimuli, and modulate various cellular activities including gene expression, mitosis, differentiation and cell survival/apoptosis (Pearson et al., 2001).

Now in spite of an increasing need for agents to protect the liver from damage, modern medicine still lacks a reliable liver protective drug. Therefore numbers of natural substances have been studied to

* Corresponding author: Tel.: +86 023 6848 5038; fax: +86 023 8613 4172.
E-mail address: jywan@cqmu.edu.cn (J.-y. Wan).
[1] Contributed equally to this work.

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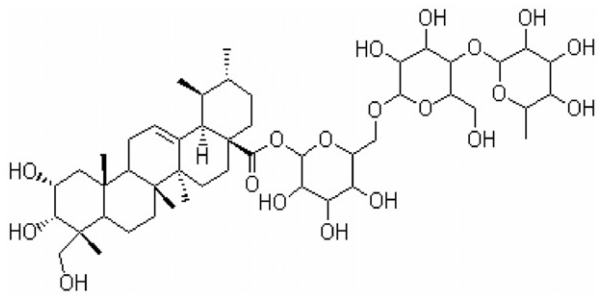

Fig. 1. Chemical structure of asiaticoside (AS).

evaluate the hepato-protective activity. The herb, *Centella asiatica*, a perennial creeper growing abundantly in moist areas and distributed widely in tropical and subtropical countries, has been used for centuries in Ayurvedic and traditional Chinese medicine to alleviate symptoms of wound, ulcer, arthritis, depression and anxiety (Brinkhaus et al., 2000; Jayathirtha and Mishra, 2004; Li et al., 2009). In South China, *C. asiatica* is even widely used as a dietary supplement and an ingredient of special tea to promote positive health and keep immunomodulation by establishing body equilibrium. Asiaticoside (AS) (Fig. 1), a major pentacyclic triterpenoid saponin component of *C. asiatica*, has been described to have wound healing, immunomodulatory and anti-inflammatory activities (Jia and Lu, 2008; Shukla et al., 1999). Our earlier study indicated that AS attenuated the inflammation on LPS-induced acute lung injury and collagen-induced arthritis in mice through reducing the level of cytokines such as TNF-α, IL-6 and PGE_2 (Li et al., 2007; Zhang et al., 2008). However, there are few reports on the effects and mechanisms of AS in treatment of acute liver injury.

In the present study, we utilized LPS/D-GalN model to evaluate the protective effects of AS on acute hepatic damage and then investigated whether TNF-α and MAPKs were involved in the potential mechanism.

Materials and methods

Animals

Balb/c mice (6–8 weeks old; weight range, 18-22 g) were obtained from the Laboratory Animal Center of Chongqing Medical University (Chongqing, PR China). All mice received human care according to the guidelines of the local institutes of health guide for the care and use of laboratory animals. They were maintained under controlled conditions (22°C, 55% humidity and 12 h day/night rhythm) and fed standard laboratory chow.

Reagents

LPS (Escherichia coli, 0111:B4), D-GalN and silymarin were purchased from Sigma (St. Louis, MO, USA). Asiaticoside ($C_{48}H_{78}O_{19}$, MW: 959.12, purity < 90%) determined by HPLC as previously described (Schaneberg et al., 2003) was purchased from Guangxi Changzhou Natural Products Development Co. Ltd. (Nanning, China). Rabbit anti-mouse phospho-p38 MAPK, phospho-JNK, phospho-ERK and Rabbit anti-mouse β-actin antibody was purchased from Cell Signaling Technology (Boston, MA, USA), and horseradish peroxidase-conjugated goat anti-rabbit antibody, bicinchoninic acid (BCA) protein assay kit and enhancer chemiluminescent (ECL) reagents were obtained from Pierce Biotechnology (Rockford, IL). alanine aminotransferase (ALT) and aspartate aminotransferase (AST) detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Hoechst 33342 fluorescent dye and caspase 3 activity assay kits were obtained from Beyotime Institute (Shanghai, China). Mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit was purchased from Bender Med Systems (Vienna, Austria).

Experimental protocols

Mice were administered with AS (5, 10 and 20 mg/kg) dissolved in 0.5% sodium carboxymethyl cellulose or silymarin (50 mg/kg) or equal volume phosphate-buffered saline (PBS) once daily for 3 days prior to challenge experimentation. Mice were challenged intraperitoneally (i.p.) with a total volume of a combination of LPS (50 μg/kg) and D-GalN (800 mg/kg) dissolved in PBS. The doses of AS alone did not induce liver injury as determined by evaluating liver enzymes, cytokines, and liver histology (data not shown). Lethality was evaluated within 48 h after LPS/D-GalN administration. Liver samples for MAPKs, TNF-α analysis and histology were obtained at 0.5, 1.5 and 6 h after LPS/D-GalN administration, respectively.

Analysis of liver enzymes

Hepatocyte damage was assessed 6 h after LPS/D-GalN administration by measuring ALT and AST activities in serum and caspase-3 activity in hepatic tissue using corresponding detection kits according to the manufacturer's instructions.

Measurement of TNF-α Levels

Mouse serum samples were assayed for murine TNF-α by enzyme-linked immunosorbent assay as described by the manufacturer, while TNF-α in hepatic tissue was analyzed by were measured at 1.5 h after the LPS/D-GalN challenge (Endo et al., 1999).

Histologic analysis

Liver tissue was fixed in 10% neutral-buffered formalin and subsequently embedded in paraffin. Sections were stained with hematoxylin and eosin using a standard protocol and analyzed by light microscopy.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from hepatic samples using Trizol reagent according to the manufacturer's protocol. First-strand

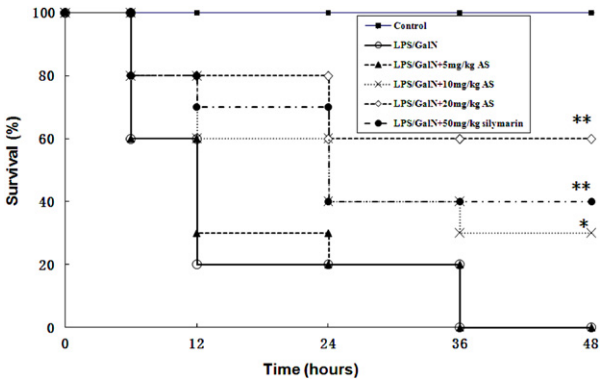

Fig. 2. Lethal toxicity induced by LPS/D-GalN in mice (n=10). Balb/c mice were pretreated orally with AS (5, 10 and 20 mg/kg) or silymarin (50 mg/kg) or equal volume phosphate-buffered saline (PBS) once daily for 3 days before LPS/ D-GalN administration. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with LPS/ D-GalN administration group.

complementary DNA (cDNA) was synthesized. The cDNA samples were then incubated at 90 °C for 7 min to stop the reaction. For amplification of TNF-α cDNA, the sequences of primers were 5′-GGC AGG TCT ACT TTG GAG TCA TTG C-3 (sense) and 5′-ACA TTC GAG GCT CCA GTG AAT TCG G-3′ (antisense). The primers used for amplification of β-actin cDNA as an internal standard were 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ (sense) and 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′ (antisense). The PCR products of TNF-α and β-actin were 300 and 349 base pairs (bp) in length, respectively. cDNA was amplified in 25 μl reaction system. PCR reactions were initiated at 94 °C for 5 min, followed by 34 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s) with a final primer extension at 72 °C for 7 min. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The intensity of each TNF-α mRNA band was quantified by the Kodak molecular imaging system (Kodak Gel Logic 1500, Kodak MI software, USA) and normalized to values for β-actin.

Western blotting

Total proteins from frozen hepatic samples were prepared according to the method described by the protein extract kit (Active Motif Commany, Carlsbad, U.S.A.). Protein concentrations were determined by BCA protein assay kit. Protein extracts were fractionated on 12% polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to nitrocellulose membrane. The membrane was blocked with 5% (w/v) fat-free milk in Tris-buffered saline (TBS) containing 0.05% tween-20, followed by incubation with a rabbit anti-phospho-p38 MAPK (1:5000), anti-phospho-JNK (1:5000) and anti-phospho-ERK (1:5000) polyclonal

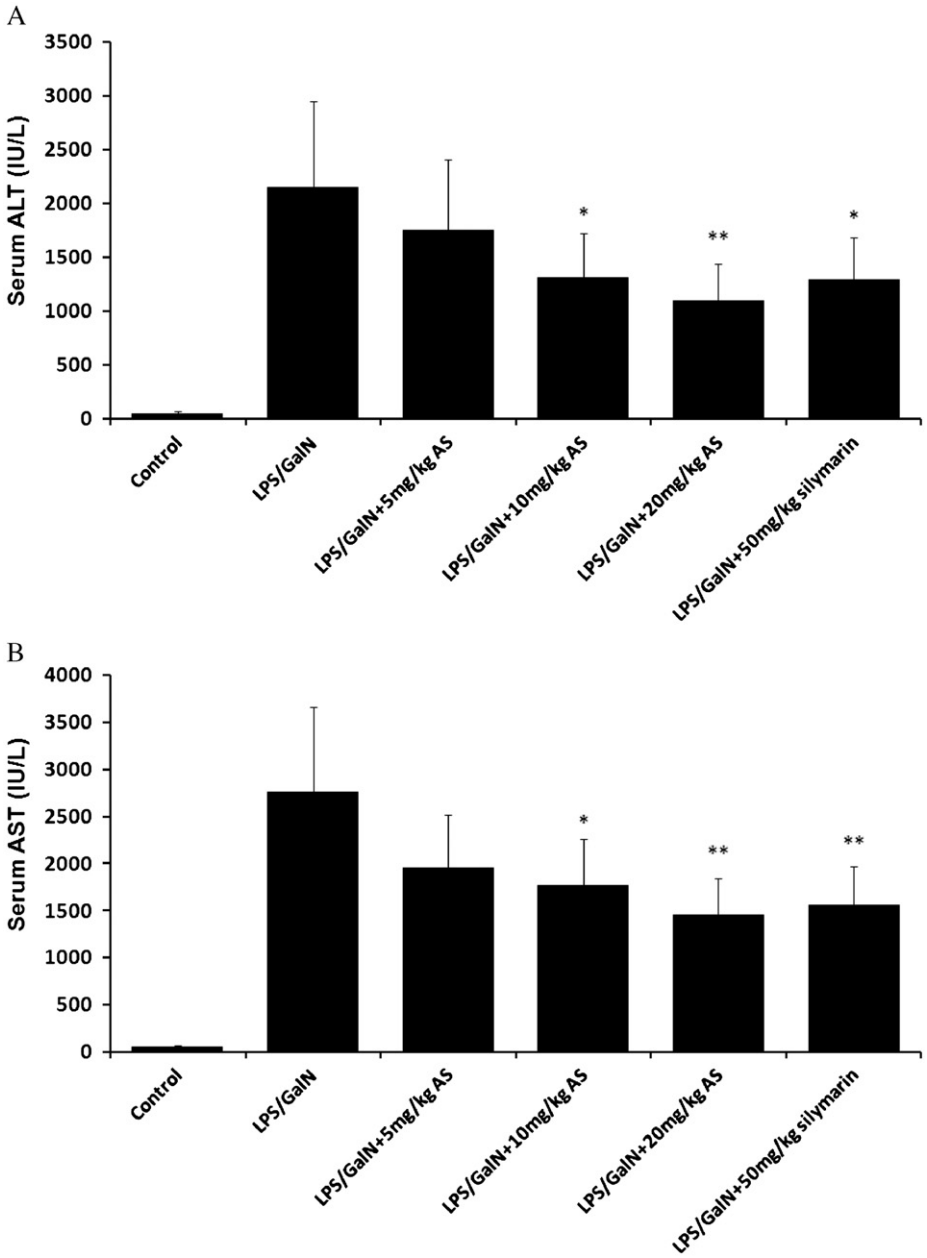

Fig. 3. Effects of AS on the liver injury induced by LPS/D-GalN in mice (n=7). Mice were administered orally with AS (5, 10 and 20 mg/kg) or silymarin (50 mg/kg) or PBS once daily for 3 days before LPS/D-GalN administration. Serum samples were collected 6 h after challenge, and serum transaminase activity was determined. (A): Serum ALT concentration; (B): Serum AST concentration. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with LPS/ D-GalN administration group.

antibody at 4 °C overnight. Then the membrane was treated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000). Antibody binding was visualized with an ECL chemiluminescence system and short exposure of the membrane to X-ray films (Kodak, Japan).

Hoechst 33342 Staining

To analyze the typical morphological signs of apoptosis, paraffin-embedded sections were deparaffinized and stained with Hoechst 33342, a fluorescent dye that has been widely used for analysis of nuclear morphology, in an aqueous dilution of 1:10,000 for 5 to 10 minutes. Hoechst 33342-stained tissues were examined with a fluorescence microscope and photographed. For quantification of apoptosis, 100 nuclei were randomly viewed, and apoptosis was counted.

Caspase-3 protease activity

Caspase-3 protease activity in the liver tissue was measured using a caspase-3 colorimetric assay kit according to the manufacturer's instructions. Briefly, after homogenization of whole liver tissue in cell lysis buffer, homogenates were centrifuged for 1 min at 10,000 g, and the supernatant (100 g protein) was incubated with Asp-Glu-Val-Asp-p-nitroanilide (pNA) and reaction buffer for 90 min at 37 °C. Absorbance was measured at 405 nm as caspase-3 activity.

Measurement of MPO activity

Frozen liver tissues were thawed and homogenized in phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide. The enzyme activity was determined spectrophotometrically using a MPO detection kit according to the manufacturer's instructions. MPO activity was assessed according to the absorbance measured at 450 nm and normalized by the total protein concentration of the same sample.

Statistical Analysis

Results were analyzed using Student's t test or by ANOVA where appropriate. All data in this study were expressed as mean

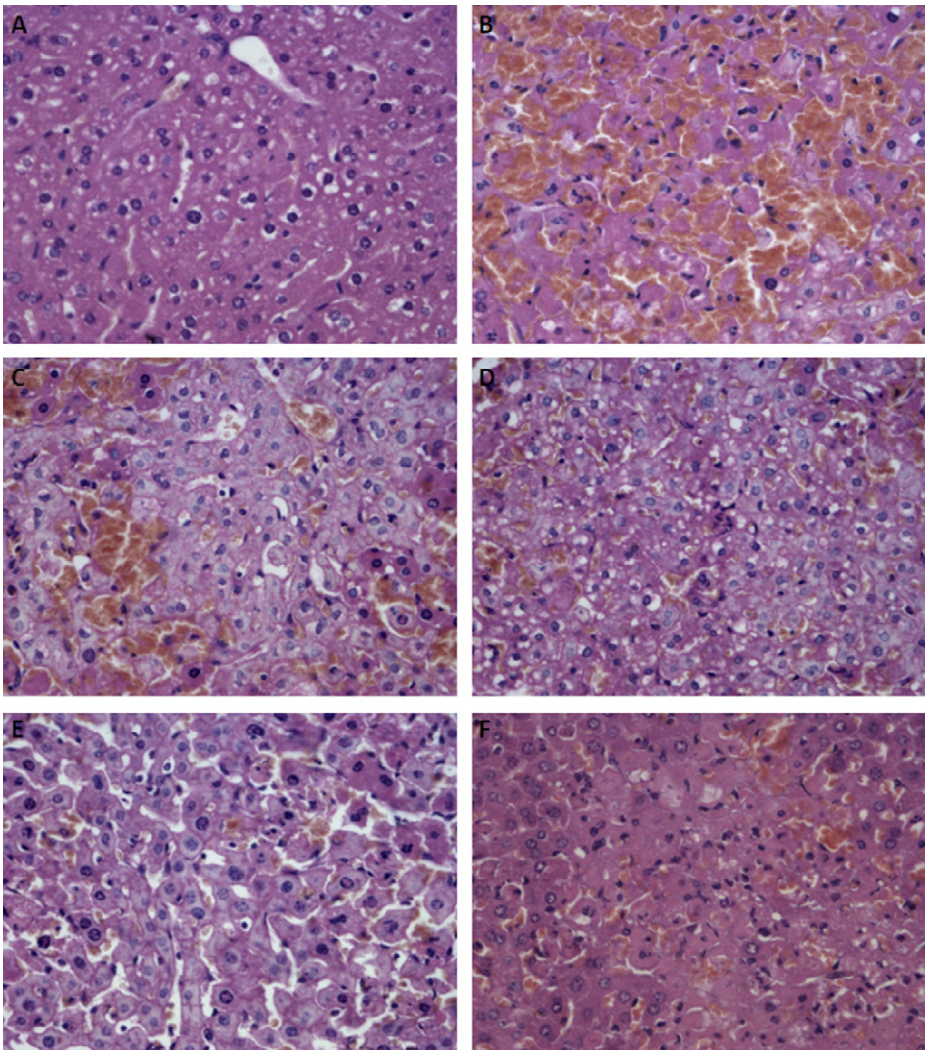

Fig. 4. Histology of liver sections (n=7, H&E stain, Original magnification × 400). (A): Control group treated with PBS. (B): Group treated with LPS/D-GalN. (C): Group pretreated with 5 mg/kg AS once daily for 3 days before LPS/D-GalN administration. (D): Group pretreated with 10 mg/kg AS once daily for 3 days before LPS/D-GalN administration. (E): Group pretreated with 20 mg/kg AS once daily for 3 days before LPS/D-GalN administration. (F): Group pretreated with 50 mg/kg silymarin once daily for 3 days before LPS/D-GalN administration.

± standard (S.D.). Survival statistics were compared with a Kaplan-Meier curve and log-rank test. *P* values less than or equal to 0.05 was considered significant.

Results

Effect of AS on mortality of LPS/D-GalN-treated mice

To evaluate whether AS affected the outcome of sepsis induced by LPS/D-GalN, the survival rate of mice was compared. Kaplan-Meier survival curves showed that LPS/D-GalN administration caused almost all animals death within 48 h; however, pretreatment with AS dose-dependently improved the survival of LPS/D-GalN-treated mice (Fig. 2). In positive drug-treated group, silymarin also increased the survival of mice compared with the model group.

Effect of AS on LPS/D-GalN-induced hepatotoxicity

Serum aminotransferases were assayed to evaluate the hepatotoxicity of mice. Results indicated that there existed significantly increasing plasma levels of ALT and AST 6 h after LPS/D-GalN administration, which were reduced by pretreatment with AS in a dose-dependent manner and silymarin (50 mg/kg) (Figs. 3A and B).

Next, Histological examination of liver tissue section showed that severe hepatocytes swelling and necrosis and leukocyte infiltration were observed in mice at 6 h after LPS/D-GalN administration. The magnitude of hepatocytes necrosis and leukocyte infiltration was decreased in AS-pretreated or silymarin-pretreated animals (Fig. 4).

To further investigate neutrophil infiltration in liver tissue, MPO activities were assayed. MPO can be used as a marker of tissue neutrophil infiltration. As shown in Fig. 5, LPS/D-GalN administration induced a marked increase in hepatic MPO activity at 6 h and pretreatment with AS (5, 10, 20 mg/kg) or silymarin (50 mg/kg) reduced the elevation of hepatic MPO activity induced by LPS/D-GalN, which was consistent with the reduced leukocyte infiltration observed in histological examination.

Effect of AS on LPS/D-GalN-induced hepatocellular apoptosis and caspase-3 activity

Hoechst 33342 staining was used to detect apoptotic hepatocytes. Administration of LPS /D-GalN induced high numbers of apoptotic cells, whereas pretreatment with AS showed dose-dependently lessened hepatocellular apoptosis (Fig. 6A). It was also observed that the hepatic caspase-3 activity was markedly elevated in mice challenged with LPS and D-GalN. It is notable that this LPS/D-GalN-induced increase in caspase-3 activity was dose-dependently reduced in AS-treated mice (Fig. 6B). Positive drug silymarin also showed that lessened LPS/D-GalN-induced hepatocellular apoptosis and reduced the hepatic caspased-3 activity induced by LPS/D-GalN (Fig. 6A and B).

Effect of AS on LPS/D-GalN-induced TNF-α production

Because TNF-α is a critical mediator of liver injury induced by LPS/D-GalN, we postulated that the protective effect of AS against LPS/D-GalN-induced liver injury was mediated by inhibiting elevation of TNF-α levels. Therefore, we measured serum and hepatic TNF-α levels at 1.5 h after LPS/D-GalN administration. As shown in Figs. 7A and B, administration of LPS/D-GalN markedly induced elevation of serum and hepatic TNF-α levels; pretreatment with AS (5, 10, 20 mg/kg) or silymarin (50 mg/kg) attenuated LPS/D-GalN-mediated promotion of TNF-α levels. A similar observation was made regarding the TNF-α mRNA levels in the hepatic tissues (Fig. 7C).

Effect of AS on LPS/D-GalN-activated MAPK signal pathway

Activation of MAPK signaling pathway plays a key role in the regulation of TNF-α transcription. As expected, in the liver tissues of LPS/D-GalN-treated mice, phosphorylation of ERK, JNK and p38 was increased at 30 minutes, pretreatment with AS or silymarin

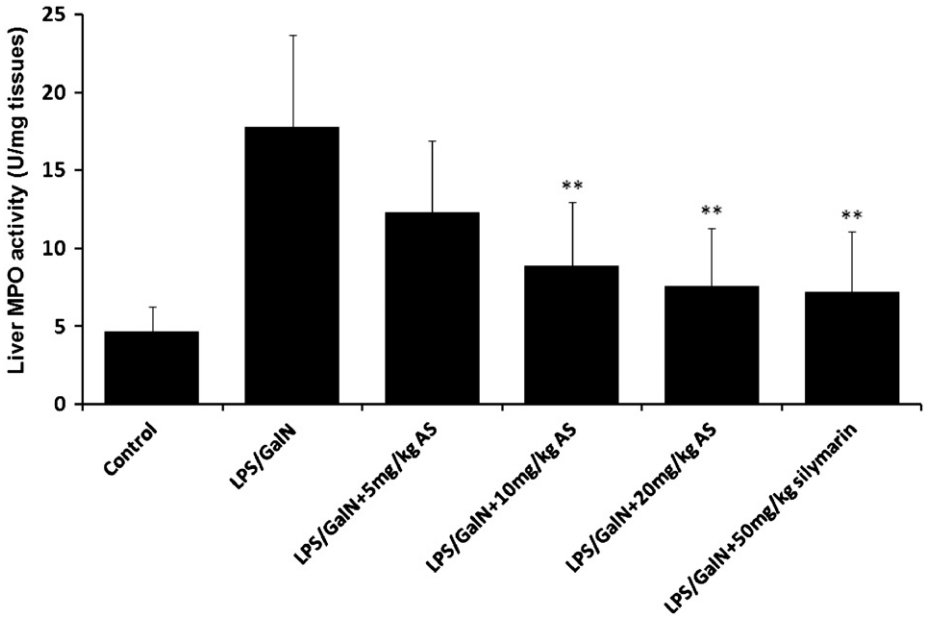

Fig. 5. Effects of AS on LPS/D-GalN-induced changes of MPO activity (n=7). Mice were administered orally with AS (5, 10 and 20 mg/kg) or silymarin (50 mg/kg) or PBS once daily for 3 days before LPS/D-GalN administration. Liver samples were collected 6 h after challenge, and MPO activity was determined. $^{**}P < 0.01$ compared with LPS/ D-GalN administration group.

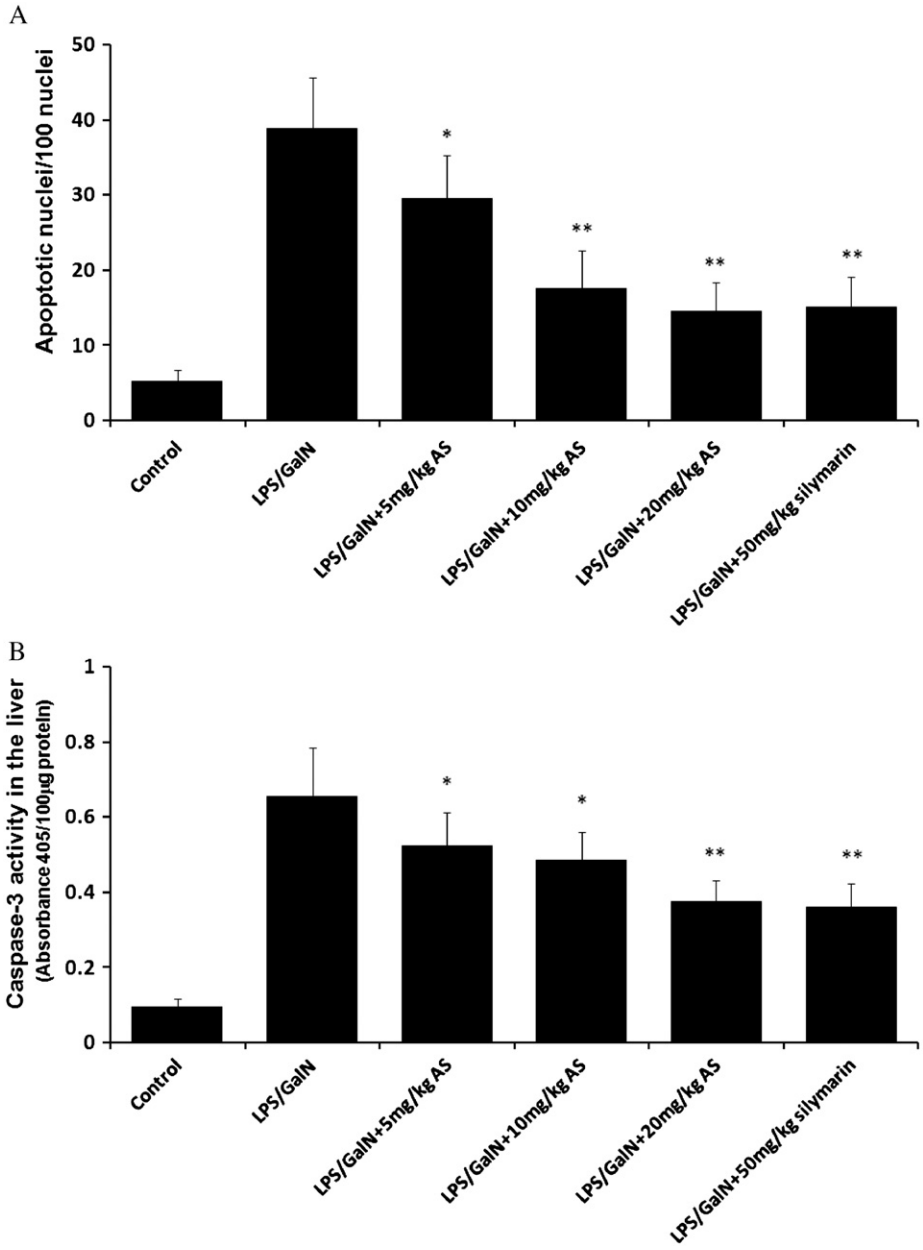

Fig. 6. Effects of AS on LPS/D-GalN-induced hepatocellular apoptosis and caspase-3 activity (n=7). Mice were administered orally with AS (5, 10 and 20 mg/kg) or silymarin (50 mg/kg) or PBS once daily for 3 days before LPS/D-GalN administration. Liver samples were collected 6 h after challenge, and hepatocellular apoptosis and caspase-3 activity was assayed. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with LPS/ D-GalN administration group.

dose-dependently inhibited LPS-activated phosphorylation of these three molecules in the liver tissues of mice (Fig. 8).

Discussion

D-GalN is a specific hepatotoxic agent metabolized exclusively in hepatocytes, which reduces intracellular pool of uracil nucleotides, thus inhibiting the synthesis of RNA and proteins. When given together with a low dose of LPS, D-GalN highly sensitizes animals to develop lethal liver injury showing biochemical and metabolic changes akin to fulminant hepatic failure (Galanos et al., 1979; Keppler et al., 1968). In our study, an intraperitoneal injection of LPS/D-GalN in mice resulted in a more serious liver injury that was associated with a significantly higher mortality in model mice than in AS -treated mice. Intake of AS also significantly reduced the elevation of activities of AST, ALT in serum induced by LPS/D-GalN. Additionally, It has been suggested that apoptosis is recognized as an important step in the development of liver injury (Patel and Gores, 1995). Caspase-3, which is a cystein-protease of the CED-3/ICE family, plays a major role in the execution of apoptosis (Nicholson and Thornberry, 1997). The present study showed AS might attenuate hepatocyte apoptosis by inhibiting caspase-3 activity in liver tissues.

TNF-α is known to be a pleiotropic cytokine that contributes to the triggering of an inflammatory cascade involving the induction of cytokines including IL-1, IL-6, IFN-γ, nitric oxide and cell adhesion molecules, etc. (Hishinuma et al., 1990; Tiegs et al., 1989). In respect of apoptosis, TNF-α combined with TNF-α receptor on the hepatocyte membrane activates caspase-3 and eventually induces apoptosis at an early stage through a series of signal transmission. It's deserved to be mentioned that

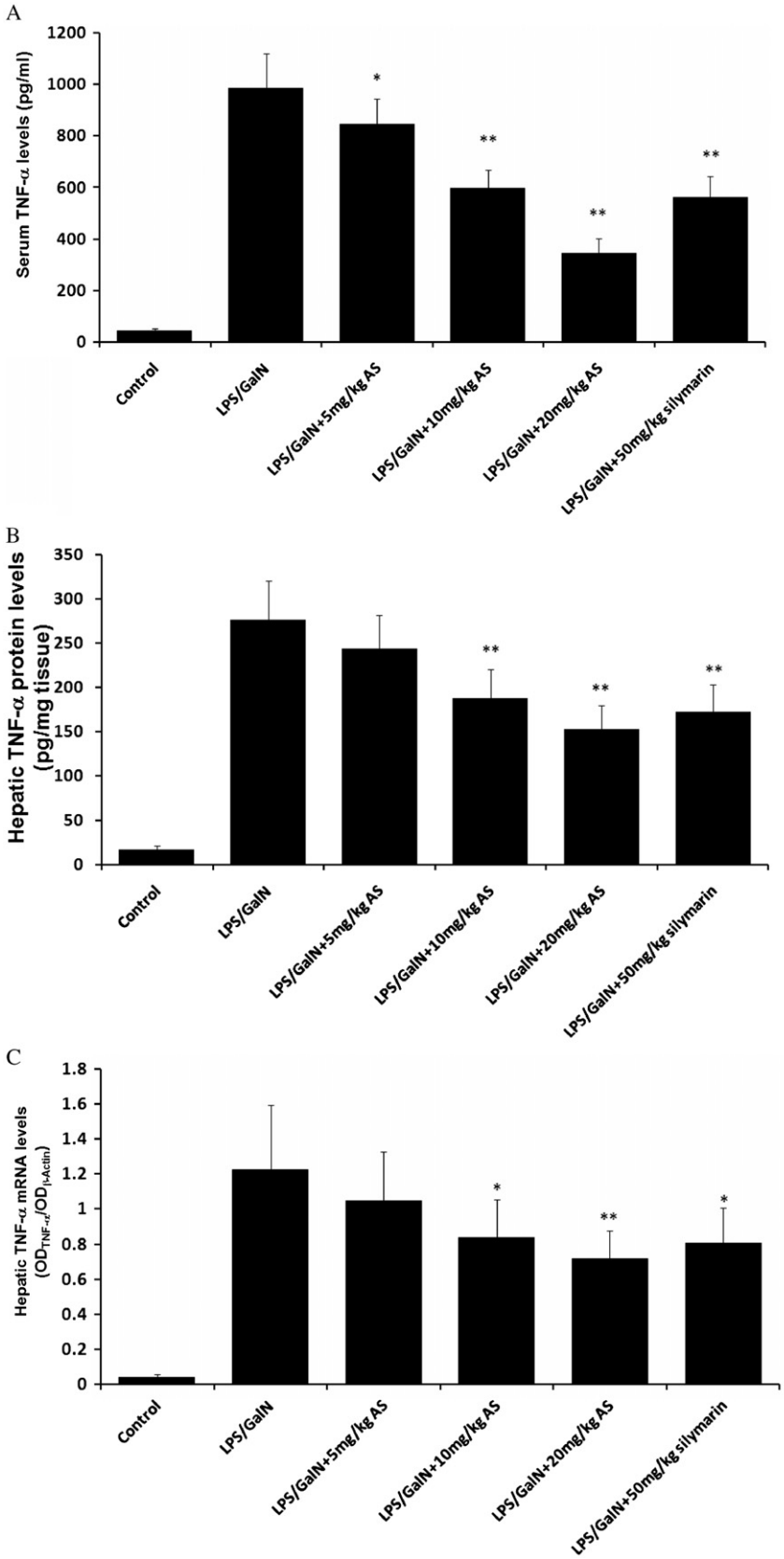

Fig. 7. Effects of AS on LPS/D-GalN-induced changes of TNF-α ($n=7$). (A): Serum TNF-α levels. (B): Hepatic TNF-α protein levels. (C): Hepatic TNF-α mRNA expression levels. Mice were administered orally with AS (5, 10 and 20 mg/kg) or silymarin (50 mg/kg) or PBS once daily for 3 days before LPS/D-GalN administration. Serum and hepatic samples were collected 1.5 h after challenge, serum and hepatic TNF-α was assayed by ELISA, hepatic TNF-α mRNA was assay by RT-PCR. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with LPS/D-GalN administration group.

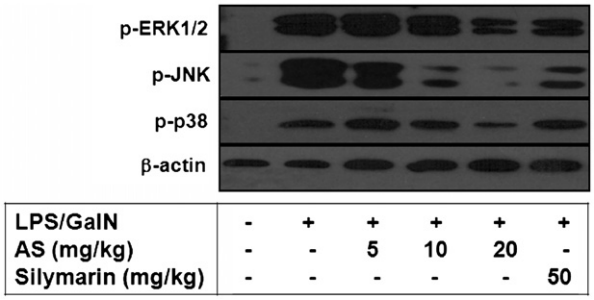

Fig. 8. Effects of AS on LPS/D-GalN-induced phosphorylation of ERK, JNK and p38 in the liver tissues. Mice were administered orally with AS (5, 10 and 20 mg/kg) or silymarin (50 mg/kg) or PBS once daily for 3 days before LPS/D-GalN administration. Liver tissue samples were collected 0.5 h after challenge, and phosphorylation of ERK, JNK and p38 was determined by western blotting.

TNF-α-induced neutrophil transmigration at the later stages of liver injury has been shown to be a critical step in hepatocyte necrosis (Chosay et al., 1997; Kaplowitz, 2000). Therefore TNF-α is in association with a wide range of inflammatory or auto-immune diseases including various liver lesions. TNF-α is postulated to play a critical role in induction of liver injury associated with LPS exposure, as suggested by studies showing that neutralization of circulating TNF-α with a specific antiserum or antibody ameliorate the LPS-induced hepatic injury (Aggarwal et al., 2006; Matsumoto et al., 2002). Furthermore, experiments with TNF-α knockout or TNF-receptor p55 knockout mice confirmed the central role of TNF-α in LPS/D-GalN induced hepatotoxicity (Marino et al., 1997; Pfeffer et al., 1993). In this study, the administration of LPS/D-GalN significantly up-regulated the expression of hepatic TNF-α and elevated plasma TNF-α levels. In contrast, pretreatment with AS dose-dependently reduced LPS/D-GalN-induced hepatic TNF-α mRNA expression and serum TNF-α release, diminished the increase of caspase-3 activity induced by TNF-α. These findings are consistent with previous studies in other animal models, which demonstrated that AS decreased TNF-α production.

MAPKs pathway is assumed key signal pathway regulating TNF-α, although the detailed mechanism is still unkown (Guha and Mackman, 2001; Kawai and Akira, 2007). MAPKs are a family of serine/threonine protein kinases of highly conserved enzymes, which transmit environmental stimuli into the nucleus, and have been shown to participate in regulating cytokine production in response to a broad range of stimuli, including ischemia/reperfusion, ultraviolet light, heat shock, and microbial infection (Guan, 1994; Johnson et al., 2005). Once activated by dual phosphorylation on tyrosine and threonine, the MAPKs modulate the functional responses of cells through phosphorylation of transcription factors and activation of other kinases (Kaminska, 2005). The three major MAPK proteins, p38 MAPK, JNK and ERK, are thought to play different roles in inflammatory diseases in different capacities. JNK and p38 MAPK are involved in growth arrest and apoptosis, whereas the ERK cascade is normally associated with mitogenesis and differentiation (Hung et al., 1998). However, it's still demonstrated that MAPKs have overlapping substrate specificities and phosphorylation of regulatory sites is shared among multiple protein kinases (Lee et al., 1994). They can all be activated by LPS, leading to the production of inflammatory mediators such as TNF-α, IL-6 and NO. Many studies also have shown that the MAPKs are required for cytokines, especially TNF-α induced by LPS in vivo or vitro inflammatory models including LPS/D-GalN mice model (Bhat et al., 1998; Das et al., 2009; Mi Jeong et al., 2009). Furthermore, the experiments concerned with the inhibitors of MAPKs such as p38 MAPK inhibitor SB203580, JNK inhibitor SP600125, and ERK inhibiter PD98059 have suggested that the MAPKs play critical role in TNF-α expression (Bogoyevitch et al., 2004; Hsu et al., 2009; Schuh and Pahl, 2009). The present study showed activation of p38 MAPK, JNK and ERK all increased significantly in LPS/D-GalN mice, which was in concert with higher level of TNF-α later measured in the same group. While AS cut down the high phosphorytion of MAPKs protein in a dose-dependent manner, TNF-α mRNA and protein expression in liver tissues and serum TNF-α were also decreased by AS. Based on these findings, it's easy to deduce that AS might exert its hepatoprotective effect through suppressing MAPKs as well as TNF-α, and there must be a close and delicate relationship between them.

To conclude, a hepatoprotective effect of AS, namely improvement of liver function, alleviation of liver pathological injury, attenuation of cell apoptosis and reduction in lethality on murine liver injury induced by LPS/D-GalN, was described in this report. The potential mechanisms underlying hepatoprotection might be associated with inhibiting TNF-α expression and its important regulator MAPKs. It needs to be further studied as well as the precise mechanism whether AS is a promising agent for the treatment of hepatic failure in patients.

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