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ORIGINAL ARTICLE

The protective mechanism of schisandrin A in D-galactosamine-induced acute liver injury through activation of autophagy

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Abstract

Context: The principal bioactive lignan of *Schisandra chinensis fructus,* commonly used for traditional Chinese medicine, is schisandrin A. Schisandrin A has been widely reported as being very effective for the treatment of liver disease. However, the mechanisms of its protective effects in liver remain unclear.

Objective: To explore the hepatoprotective mechanisms of schisandrin A.

Materials and methods: D-Galactosamine (D-GalN)-induced liver injury in mice was used as a model. Schisandrin A was examined for its protective mechanisms using hematoxylin–eosin (HE) staining, enzyme-linked immunosorbent assay (ELISA), western blotting and real-time PCR (RT-PCR).

Results: Aspartate amino-transferase (AST) and alanine transaminase (ALT) levels in the schisandrin A group were significantly decreased ($p < 0.01$) compared with those in the D-GalN-treated group. HE results showed that the pathological changes in hepatic tissue seen in the D-GalN-treated were reduced in the schisandrin A/D-GalN-treated group, with the morphological characteristics being close to those of the control (untreated) group. Western blotting results showed that schisandrin A can activate autophagy flux and inhibit progression of apoptosis. The immune function of the schisandrin A-pretreated group was assayed by flow cytometry. It was found that the mechanism may involve activated autophagy flux, inhibited apoptosis, and improved immunity in response to liver damage.

Conclusion: Our results show that the hepatoprotective mechanisms of schisandrin A may include activation of autophagy flux and inhibition of apoptosis. These results provide pharmacological evidence supporting its future clinical application.

Keywords

Apoptosis, autophagy flux, lignans, liver damage

History

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Introduction

D-Galactosamine (D-GalN)-induced acute liver injury is an established experimental model for the study of liver disease. D-GalN may induce the depletion of uracil nucleotides, and inhibits RNA and protein synthesis, thus leading to deterioration of cell membranes (El-Mofty et al., 1975; Yoshikawa et al., 1979). This model has been commonly used to screen hepatoprotective drugs (Nakagiri et al., 2003).

Schisandra chinensis fructus, from the fruit of *Schisandra chinensis* (Turcz) Baill., has been commonly used in traditional Chinese medicine for the treatment of liver diseases for centuries (Du et al., 2012). *Schisandra chinensis fructus* has been shown to contain many active lignans, including schisandrin A and Schisantherin A. Schisandrin A is the major bioactive lignan (Xu et al., 2005), and is an antioxidant which possesses hepatoprotective (Cheng et al., 2013; Yan et al., 2009; Yuan et al., 2010) and antitumor activities (Tang et al., 2011). It has been reported that the hepatoprotective mechanism of *Schisandra chinensis fructus* pollen extract may be related to its free radical scavenging effect, increasing antioxidant activity and inhibiting lipid peroxidation (Cheng et al., 2013). However, further possible mechanisms underlying hepatoprotective effects by schisandrin A are not known.

Autophagy is a metabolic process that degrades and recycles intracellular organelles and proteins, and has many connections to human physiology and to cancer (Guo et al., 2012; Lozy & Karantza, 2012). A recent study demonstrated that autophagy has a major impact on liver disease (Hidvegi et al., 2010; Rautou et al., 2010; Werling, 2011; Yin et al., 2008). However, whether the liver-protective effects of schisandrin A are associated with autophagy is not clear. In the present study, we investigated the effect of schisandrin A on autophagy.

There is a close relationship between autophagy and apoptosis (Andromachi et al., 2012). Hence, we also focused on the interaction between autophagy and apoptosis in liver injury. *Schisandra chinensis fructus* has often been used as a tonic medicine (Du et al., 2012), and a water-soluble

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low molecular weight polysaccharide from *Schisandra chinensis* has been shown to improve the immune response (Zhao et al., 2013). We, therefore, also examined the effects of schisandrin A on the immune function.

The present study investigated the hepatoprotective mechanisms of schisandrin A on D-GalN-induced liver injury through autophagy and apoptosis. Our results may help to develop future novel therapeutic strategies using schisandrin A for the treatment of liver disease.

Materials and methods

Materials used

D-GalN was obtained from Sigma (Saint Louis, MO). An extract of schisandrin A from *Schisandra chinensis* was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (HPLC $\geq$ 98%, Shanghai, China). Antibodies for western blotting were purchased from the following companies: p53, Cell Signaling (Shanghai, China); Bcl-2, Millipore (Billerica, MA); LC3, p62, Bax, caspase-3, and Beclin 1, Abcam; β-actin, Multi-sciences (Hangzhou, China); FITC-labeled second antibody, KPL (Columbia, MD). FITC anti-mouse CD4 and CD8α antibodies were purchased from Biolegend Company (San Diego, CA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), interleukin-1β (IL-1β), and IL-8 detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). UNIQ-10 TriZol reagent kit was purchased from Sangon Biotech Company (Shanghai, China). qRT-PCR M-MLV system kit was purchased from Invitrogen (Carlsbad, CA).

Hepatoprotective study

Male ICR mice aged 6–8 weeks (body weight 20 ± 2 g) were purchased from the Biomedical Technology Company Limited (Suzhou Belda, China). The mice were housed in a specific pathogen-free environment. A diet consisting of commercial pellets and sterile water was provided during the experiment. The use and care of experimental animals complied with the provisions of Soochow University (University Laboratory Animal Center permit number SYXK 2007-0035).

The mice were randomly divided into three groups, with 10 animals in each group. The three groups were a normal group (control group, i.g. saline, 10 ml/kg/d), a model group (in which, following i.g. saline, 10 ml/kg/d intra-peritoneal (i.p.) injection of D-GalN 800 mg/kg/d (dissolved in saline) was administered three times during the final stage of the experiment), and a schisandrin A group (dissolved in 0.5% hydroxypropyl-β-cyclodextrin; after 2 weeks of i.g. schisandrin A at 150 mg/kg/d, i.p. administration of D-GalN 800 mg/kg/d was performed three times during the final stage of the experimental period). Two weeks after the treatment, mice received an i.p. injection of 4% chloral hydrate (10 ml/kg) for anesthesia, blood was collected by enucleation of the eye and the mice were then euthanized. The liver was removed; part of it was stored at $-80\,^{\circ}$C for RNA extraction and protein assay, and another part was fixed in formalin. The spleen was removed for the preparation of a spleen lymphocyte suspension.

Enzyme activity and factors assay

Mouse blood was placed at room temperature for 2 h, and serum was then prepared by centrifugation for 15 min at 1500 rpm/min. AST and ALT activity of the serum was assayed using AST and ALT detection kit, respectively. Serum interleukin-1β (IL-1β) and IL-8 were assayed using ELISA kits (antibodies were obtained from Santa Cruz, CA).

Tissue morphology

A sub-sample of the liver tissue was placed in a 10% formalin solution, fixed for 48 h, and progressively dehydrated before embedding in paraffin. Slices were cut (4 μm thickness) from the paraffin blocks. The sections were subjected to hematoxylin and eosin (HE) staining, and morphological changes and cellular responses in the liver tissue were examined.

Western blot

Samples of the left liver lobe were washed with ice-cold PBS and proteins were collected in buffer containing 10 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mmol/L edetic acid, 1 mmol/L PMSF, 0.28 U/L aprotinin, 50 mg/L leupeptin, 1 mmol/L benzamidine, and 7 mg/L pepstatin A. Protein concentration was determined using the BCA kit. Equal amounts of protein (50 μg) were separated using SDS-PAGE (10%; Bio-Rad, Richmond, CA) and transferred to a nitrocellulose membrane. The membranes were treated with antibodies overnight at 4 °C and subjected to western blotting using chemiluminescence autoradiography (ECL kit, Amersham, Arlington Heights, IL). The results were normalized to loading controls (β-actin).

Flow cytometry analysis and factors associated with immunity detection

The percentage of CD4 and CD8α cells in spleen lymphocyte suspensions was measured using the FITC anti-mouse CD4, CD8α antibody with flow cytometry. Staining was performed according to the manufacturer's protocol. Five thousand cells per sample were analyzed using a flow cytometer (Epics-XL, Beckman, CA). All experiments were performed in triplicate.

Quantitative real-time PCR analysis

Total RNA from the left liver lobe was isolated using the UNIQ-10 TriZol reagent kit. Reverse transcription was performed using the qRT-PCR M-MLV system kit according to the manufacturer's protocol. The forward and reverse primers used were as follows: P53-F, 5′-GCCATCTACAAGA AGTCACAACAC-3′ and P53-R, 5′-CTGTCGTCCAGATAC TCAGCATAC-3′. Quantitative real-time PCR analysis was performed using the SYBR Green real-time PCR Master Mix Kit (Invitrogen, Carlsbad, CA). Briefly, each 50 μl PCR reaction mixture contained 25 μl SYBR Premix Ex Taq™, 1 μl PCR forward primer and 1 μl PCR reverse primer, 1–2 μl total cDNA and dH_2O (up to 50 μl). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in mRNA expression from the real-time PCR experiments (Livak & Schmittgen, 2001).

Statistical analysis

The data are presented as mean ± SD from at least three samples per data point. Statistical analysis was performed using ANOVA. A p value <0.05 was considered statistically significant.

Results

D-GalN induces liver injury

To confirm whether the model was successful, we determined the activity of serum AST and ALT. Twelve hours after the third D-GalN injection, ICR mice in the model group showed higher serum ALT (697.38 IU/L) and AST (379.42 IU/L) than mice in the control group (37.87 IU/L and 27.36 IU/L, respectively; both $p<0.01$). The schisandrin A/D-GalN-treated mice had lower serum ALT (73.55 IU/L) and AST (47.53 IU/L) than the model group mice (both $p<0.01$). These results show that D-GalN treatment successfully induced liver injury (Figure 1B). Consistent with these results, the HE staining showed morphological changes including serious balloon-like lesions, liver cell edema, and piecemeal necrosis in the liver tissue of animals in the model group. The morphology of liver tissue of animals pretreated with schisandrin A was similar to the control group (Figure 1A).

Schisandrin A activates autophagy

The role of autophagy in liver acute injury is not well established. LC3-II level, or the ratio of LC3-II and LC3-I, have been shown to be related to the formation of autophagic vesicles. P62 is a crucial substrate in autophagic flux. If LC3-II is increased and P62 is decreased autophagic flux is inhibited, whereas autophagy is activated (Ma et al., 2013). Expression of LC3-II was increased by 65% and p62 was decreased by 29.8% after intragastric administration of schisandrin A for 2 weeks compared with the D-GalN treated group, whereas D-GalN inhibited autophagic flux (Figure 2A and B). However, the expression of another autophagic protein, beclin 1, decreased by 56.8% in mice pretreated with schisandrin A compared with those treated with D-GalN (Figure 3A).

Schisandrin A inhibits apoptosis

Many apoptotic and anti-apoptotic signals depend on interactions between Bcl-2 and other members of the Bcl-2 family. The expression of anti-apoptotic (Bcl-2) and pro-apoptotic (caspase-3) factors in liver samples from ICR mice were evaluated. The expression of Bcl-2 and caspase-3 was down-regulated 38.8% and 17.45% respectively in mice in the schisandrin A/D-GalN-treated group compared with those in the model group (Figure 3A and B).

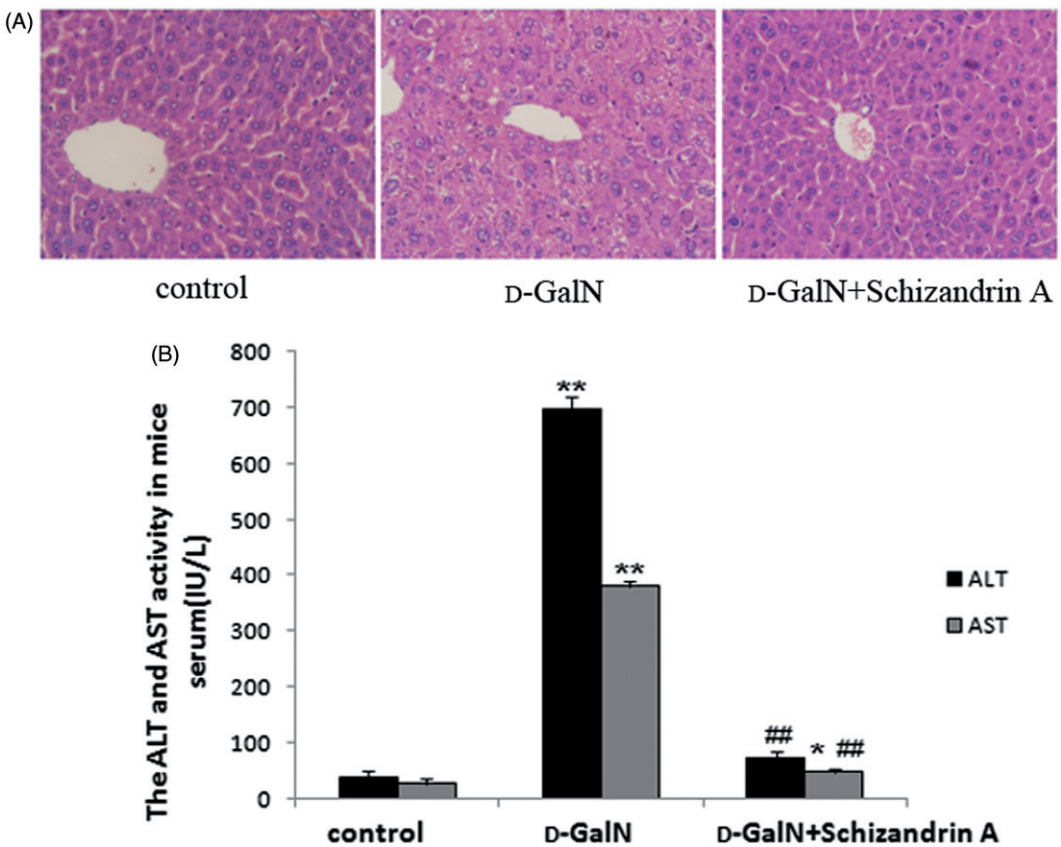

Figure 1. Effects of schisandrin A on D-GalN-induced liver injury. D-GalN was administered at 800 mg/kg/d, i.p. once every 24 h, a total of three times during the final stages of the experiment. ICR mice received i.g. treatment with schisandrin A (150 mg/kg/d); 2 weeks after i.g. schisandrin A, i.p. D-GalN 800 mg/kg/d was administered three times during the final stages of the experiment. A control group received i.g. physiological saline (10 ml/kg/d) for 2 weeks. (A) Representative HE-stained paraffin-embedded liver sections (400 × magnification). (B) ALT and AST activity levels in serum. Data represent mean ± SD ($n=10$/group/time point; **$p<0.01$, compared with the control group; ## $p<0.01$, compared with the D-GalN-treated group).

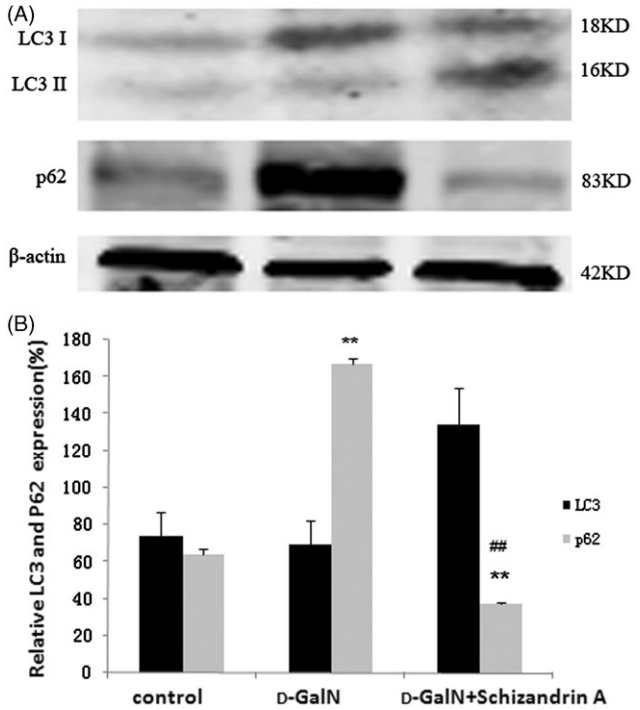

Figure 2. Schisandrin A activates autophagy. (A) The expression of LC3 and p62 proteins was analyzed by western blot. The protein levels of LC3I 18KD, LC3II 16KD and p62 83KD are shown, and β-actin was used as an internal control. (B) Relative expression of LC3 and p62. Optical densities of respective protein bands were analyzed using Sigma Scan Pro5. Statistical comparisons between treated and control groups were performed using ANOVA (**$p<0.01$ versus control, ## $p<0.01$, compared with the D-GalN-treated group).

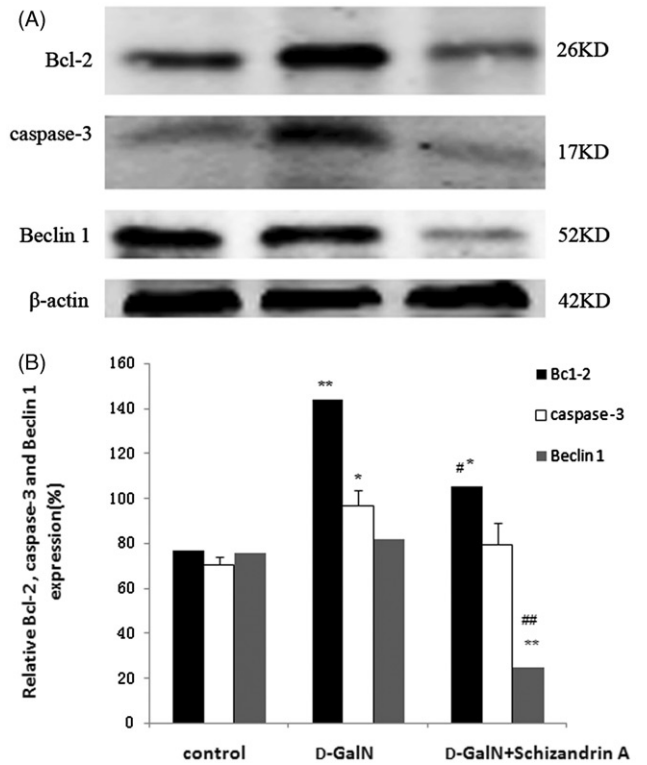

Figure 3. Schisandrin A inhibits apoptosis in liver injury. (A) Bcl-2, caspase-3, and beclin 1 protein expression. (B) The relative expression of Bcl-2, caspase-3, and beclin 1. Optical densities of respective protein bands were analyzed using Sigma Scan Pro5. Statistical comparisons between treated and control groups were performed using ANOVA (*$p<0.05$, **$p<0.01$ versus control; #$p<0.05$, ##$p<0.01$, compared with the D-GalN-treated group).

Effects of schisandrin A on p53 genes

To investigate whether schisandrin A influences the expression of the p53 gene, we studied the change of p53 at the protein and mRNA level. The expression of p53 protein in the schisandrin A/D-GalN-treated group was 52% lower than in the model group ($p<0.05$), whereas the expression of p53 protein increased by 68.3% in the model group compared with the control group ($p<0.05$). At the mRNA level, the relative expression of p53 in the schisandrin A group was 0.345 times that of the control group, while there was no change in the relative expression of p53 in the model compared with control. These findings demonstrate that schisandrin A affects p53 expression at both the protein and RNA level (Figure 4).

Schisandrin A enhances immune function

We hypothesized that schisandrin A affects the body's immune function. Hence, the activity of T lymphocytes and macrophages was analyzed. The CD4/CD8α ratio may reflect the activity of T lymphocytes. Our results showed that the CD4/CD8α ratio of spleen cells in the model group was 1.22, significantly lower than that of the control group (2.22). The CD4/CD8α ratio of spleen cells in the schisandrin A/D-GalN treated group was 1.72, close to that the control group (Figure 5A). The changes of the content of IL-8 and IL-1β may reflect macrophage activity. In this study, the IL-8 and IL-1β content of the schisandrin A group was significantly lower than the model group ($p<0.01$ Figure 5B). These results indicate that schisandrin A enhanced the body's immune function.

Discussion

In this study, the protective effect of schisandrin A against D-GalN-induced liver damage in mice was investigated, and a new possible mechanistic framework was provided.

D-GalN/lipopolysaccharide (LPS)-induced acute hepatitis is well known (Duan et al., 2011), and we used D-GalN-induced acute liver injury in mice to exclude the impacts of LPS on macrophages. We hypothesized that schisandrin A may affect the body's immune function. Moreover, the changes associated with D-GalN-induced liver damage are similar to those associated with acute viral hepatitis (Nakagiri et al., 2003). Autophagy plays an important role in viral hepatitis (Tang et al., 2009), however, the role of autophagy in D-GalN-induced liver damage is not well understood. To our understanding, autophagy may be a ''double-edged sword'' in both tumorigenesis and the development of liver disease (Degenhardt et al., 2006; Levine & Klionsky, 2004), with potential positive or negative effects. Schisandrin A exhibits a liver protective effect through modulation of autophagy and apoptosis. Schisandrin A activated autophagic flux and inhibited apoptosis in liver injury, while D-GalN inhibited autophagic flux to some extent. Interestingly, the expression of another autophagic protein, beclin 1, decreased in the schisandrin A/D-GalN-treated group. At the same time,

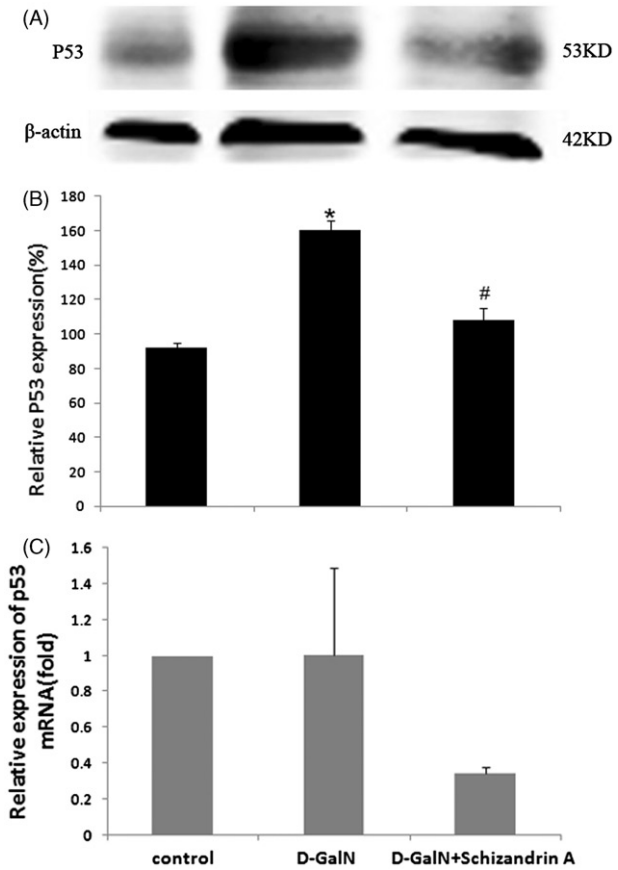

Figure 4. Effect of schisandrin A on the p53 gene. (A) p53 protein expression. (B) Relative expression of p53 (*$p < 0.05$ versus control; #$p < 0.05$ compared with the D-GalN-treated group). (C) The relative expression of mRNA p53.

the expression of the anti-apoptotic protein Bcl-2 was also markedly reduced in mice pretreated with schisandrin A, whereas its level was increased in the D-GalN group. Expression of beclin 1 and Bcl-2 in B lymphocytes has been reported to show parallel phenomena (Arsov et al., 2008). Autophagy and apoptosis in the liver are interconnected processes. However, the interaction between beclin 1 and Bcl-2 in liver injury has yet to be thoroughly explored. Some studies have indicated that Bcl-2 and beclin 1 are able to regulate each other (Kim et al., 2007) and that these two proteins can combine and lead to the inhibition of autophagy (Arsov et al., 2008). An imbalance between autophagy and apoptosis probably favors the apoptotic process (Andromachi et al., 2012).

Furthermore, expression of the pro-apoptotic protein caspase-3 was significantly decreased in the schisandrin A/D-GalN group compared with the D-GalN treated group. Down-regulation of caspase-3 indicates that schisandrin A inhibited apoptosis. Some studies have shown that the level of active caspase-3 was increased in a vehicle/CCL_4-treated group, and decreased in response to treatment with gomisin A/CCL_4, to a level close to that of the un-treated group (In et al., 2013).

P53 plays a key role in the process of apoptosis (Yan et al., 1997). P53-mediated apoptosis involves multiple mechanisms including modulation of the expression of Bcl-2, Bax, and caspases (Mihara et al., 2003). In the present study, schisandrin A also affected the expression of the p53 gene. The relative mRNA and protein expression of P53 decreased in response to pretreatment with schisandrin A, whereas protein expression of P53 increased in mice which were

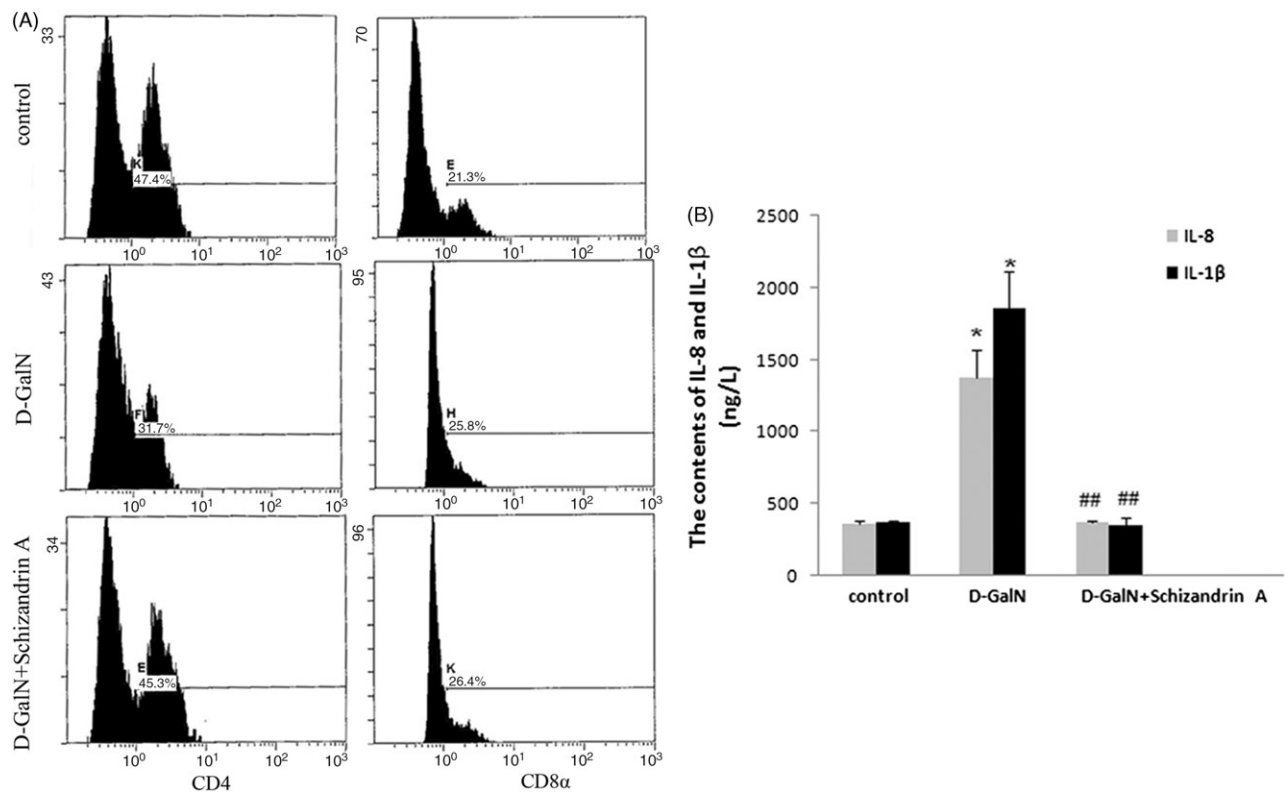

Figure 5. Schisandrin A affects the body's immune responses. (A) Effects of autophagy regulation on CD4 and CD8α as measured using an FITC assay. (B) IL-8 and IL-1β content (*$p < 0.05$ versus control group and ##$p < 0.01$ versus D-GalN-treated group).

treated with D-GalN. D-GalN is known to selectively block transcription and indirectly influence hepatic RNA and protein synthesis as a consequence of endotoxin toxicity (Tang et al., 2004).

In addition, our results show that schisandrin A participates in the regulation of immune function. Schisandrin A enhanced the activity of T lymphocytes and decreased the activity of macrophages.

Conclusion

Our results have revealed a new protective mechanism of schisandrin A in acute liver injury through autophagy and apoptosis. This further understanding of the mechanisms may provide the basis for potential clinical application of schisandrin A.

Declaration of interest

The authors report no conflicts of interest. This work was supported by grants from a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions in 2011.

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