Original Article

Stattic alleviates acute hepatic damage induced by LPS/D-galactosamine in mice

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Abstract

Increasing evidence indicates that signal transducer and activator of transcription 3 (STAT3), a vital transcription factor, plays crucial roles in the regulation of inflammation. STAT3 has become a novel therapeutic target for intervention in inflammation-related disorders. However, it remains unclear whether STAT3 plays a part in acute hepatic damage. To investigate the effects of STAT3 here, LPS/D-GalN-induced hepatic damage was induced in mice, the STAT3 inhibitor Stattic was administered, and the degree of liver injury, inflammation, and hepatocyte apoptosis were investigated. The results showed that Stattic mitigated the hepatic morphologic abnormalities and decreased the level of aminotransferase in LPS/D-GalN-insulted mice. The results also indicated that Stattic decreased the levels of TNF-α and IL-6, prevented the activation of the caspase cascade, suppressed cleavage of PARP, and decreased the quantity of TUNEL-positive cells. These results suggest that Stattic provided protective benefits in LPS/D-GalN-induced hepatic damage, and the protective effects might be associated with its anti-inflammatory and anti-apoptotic effects. Therefore, STAT3 might become a novel target for intervening in inflammation-based and apoptosis-based hepatic disorders.

Keywords

Stattic, STAT3, acute hepatic damage, inflammation, apoptosis

Introduction

Acute hepatic damage is a life-threatening syndrome accompanied by a high morbidity around the world, and is induced by viruses, bacteria, drugs, toxins, and alcohol.[1] The pathogenesis of acute hepatic damage is complicated, and uncontrolled inflammation has been widely considered as the primary underlying mechanism.[2] LPS, a major component in the cell envelope of Gram-negative bacteria, induces overwhelming inflammation and acute hepatic damage in D-galactosamine (D-GalN)-sensitized mice.[3,4] The LPS/D-GalN-induced hepatic damage is a well-established model to explore the potential pathogenesis and therapeutic drugs of hepatic disorders.[5–7]

Signal transducer and activator of transcription 3 (STAT3) is a vital transcription factor that plays important roles in multiple cellular processes, such as cell survival, proliferation, inflammation, and apoptosis.[8,9] A growing body of evidence shows that inhibition of STAT3 plays an anti-inflammatory role in

inflammation-related experimental models.[10,11] For instance, STAT3 inhibitor dramatically reduced the level of TNF-α and IL-6 in LPS-stimulated mouse

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microglia BV-2 cells and mouse RAW264.7 macrophages.[10,11] However, it is still unclear whether STAT3 also plays a crucial role in LPS/D-GalN-induced hepatic damage.

Stattic is a selective STAT3 inhibitor, which prevents STAT3 activation and nuclear translocation.[12] Stattic has been widely employed to investigate the potential roles of STAT3 in regulation of inflammation, apoptosis, and tumor.[10,13,14] In the present study, the STAT3 inhibitor Stattic was administered in mice with LPS/D-GalN-induced hepatic damage, and the degree of liver injury, inflammation, and hepatocyte apoptosis were investigated.

Materials and methods

Materials and regents

Regents were obtained as follows: LPS (*Escherichia coli* 055: B5, L-2880), D-GalN (G0500), STAT3 inhibitor Stattic (S7947) were from Sigma-Aldrich (St. Louis, MO, USA). Abs against cleaved caspase-3, cleaved poly (ADP-ribose) polymerase (PARP), the HRP-conjugated anti-rabbit IgG, and the HRP-conjugated anti-mouse IgG were from Cell Signaling Technology (Ann Arbor, MI, USA). Ab against β-actin was purchased from 4A BIOTECH (Beijing, China). Mouse TNF-α ELISA Kit (EMC 102a) and mouse IL-6 ELISA Kit (EMC004) were from NeoBioscience Technology Company (Shenzhen, China). The test kits for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The Radio Immunoprecipitation Assay (RIPA) Lysis Buffer (P0013B), PMSF (ST506), the caspase-3 Activity Assay Kit (C1116), the caspase-8 Activity Assay Kit (C1152), and the caspase-9 Activity Assay Kit (C1158) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). The enhanced chemiluminescence (ECL) reagent was obtained from Advansta (Menlo Park, CA, USA).

Animals

All experimental protocols were approved by the Ethics Committee of Chongqing Medical University. Male BALB/c mice (6–8 wk old, 20 ± 2 g) were provided by the animal center of Chongqing Medical University and housed under the controlled temperature and light conditions (22–25°C and 12 h:12 h light/dark cycle), and were also allowed *ad libitum* access to water and diet.

Acute liver injury mouse model

Mice were randomly divided into four groups ($n = 8$): the control group, the Stattic group, the LPS/D-GalN group, and the Stattic + LPS/D-GalN group. The mice in the Stattic group were only injected intraperitoneally with Stattic (5 mg/kg, dissolved in DMSO:olive oil = 1:19). The mice in the LPS/D-GalN group were injected intraperitoneally with LPS (10 μg/kg, dissolved in Normal Saline) combined with D-GalN (700 mg/kg, dissolved in Normal Saline) to induce acute liver injury. The mice in the Stattic + LPS/D-GalN group received Stattic (5mg/kg) 0.5 h prior to LPS (10 μg/kg)/D-GalN (700 mg/kg) injection. The mice in the control group were only treated with the same dosage of vehicle. The mice were anesthetized and sacrificed at 1.5 h after LPS/D-GalN challenge to collect plasma samples for measuring the level of TNF-α. Meanwhile, the mice underwent the same procedure were sacrificed at 6 h after LPS/D-GalN injection to collect blood samples and liver tissues for evaluating the other indexes.

Measurement of aminotransferase

To measure the plasma levels of ALT and AST, blood collected at 6 h after LPS/D-GalN administration was centrifuged at 5000 rpm at 4°C for 15 min to separate the plasma sample. The plasma samples were added into the 96-well plate. The ALT or AST matrix fluid was added into the plate after 5 min of incubation. Then the plate was incubated at 37°C for 30 min. After that, 2-4-dinitrophenylhydrazine was added into each well, and the plate was incubated at 37°C for 20 min. Finally, the NaOH solution was added, and the levels of ALT and AST were evaluated according to the absorbance measured at 490 nm.

Evaluation of liver histology

The right liver lobe was fixed in 4% paraformaldehyde. Then the sample was embedded in paraffin. Next, sections 4 μm thick were cut and stained with hematoxylin and eosin (HE) to assess histological damages using light microscopy (Olympus, Tokyo, Japan).

Analysis of inflammatory cytokines

The blood for the test of TNF-α was harvested at 1.5 h after LPS/D-GalN administration. Plasma level of TNF-α was measured by mouse TNF-α ELISA Kit. The blood for plasma IL-6 test was harvested at 6 h after LPS/D-GalN administration. Plasma level of IL-6 was measured by mouse IL-6 ELISA Kit. The analysis was performed according to the instructions of the manufacturer.

Determination of caspase activity

To evaluate the activity of caspase-3, caspase-8, and caspase-9, lysis buffer was added to the liver tissue, which was then homogenized and centrifuged at 8000 g at 4°C for 15 min. The supernatants were collected to detect the activities of caspase-3, caspase-8, and caspase-9 by using specific assay kits according to the instructions of the manufacturer. The activity of caspase was evaluated according to the absorbance measured at 405 nm. Then the results were normalized to the protein concentration and shown as percentage of the specific value.

Western blot analysis

The total protein was extracted from the liver sample, and the total protein concentration was determined using the BCA protein assay kit. Equal amount of total protein was separated using SDS-PAGE and then transferred to nitrocellulose membrane. After blocking with 5% nonfat milk in TBST (TBS + 0.1% Tween-20) for 2 h, the blots were incubated overnight with primary Abs to cleaved caspase-3 and cleaved PARP, followed by three washes in TBST for 10 min each. HRP-Conjugated anti-rabbit or anti-mouse IgG was applied for 2 h followed by three washes in TBST for 10 min each. Ab binding was visualized with ECL reagents and the ChemiDoc Touch Imaging System (Bio-Rad). The intensity of bands was quantified using software ImageJ.

TUNEL assay

The In Situ Cell Death Detection Kit (Roche) was used to detect the level of apoptosis in the liver tissue according to the manufacturer's instructions. The terminal transferase reactions produced a dark-brown precipitate in the nuclei of the apoptotic cells. The images were observed under a light microscope (Olympus, Tokyo, Japan).

Statistical analysis

All data are shown as mean ± SD. The statistical significance of the difference between the groups was analyzed by one-way ANOVA with the Tukey's post hoc test. Experimental results were statistically significant when the P value was <0.05.

Results

Stattic decreased LPS/D-GalN-induced liver injury

The levels of ALT and AST were the indicators to estimate the degree of liver injury.[15] In this study, the levels of plasma ALT and AST obviously increased at 6 h after the injection of LPS/D-GalN, as compared with the control group (Figure 1). However, the LPS/D-GalN-induced up-regulation of ALT and AST was significantly suppressed in the Stattic + LPS/D-GalN group (Figure 1).

Stattic attenuated LPS/D-GalN-induced histological abnormalities

HE staining showed that the liver with LPS/D-GalN exposure presented marked histological abnormalities, including hepatocellular necrosis, disordered arrangement of hepatic lobule, and infiltration of inflammatory cells. But these histological alterations induced by LPS/D-GalN were dramatically ameliorated in mice receiving Stattic treatment (Figure 2).

Stattic decreased LPS/D-GalN-induced production of inflammatory cytokines

TNF-α and IL-6 are major pro-inflammatory cytokines in LPS/D-GalN-induced liver injury model.[16,17] In this study, compared with the control group, there was significantly increasing in TNF-α and IL-6 level in LPS/D-GalN group (Figure 3). However, compared with LPS/D-GalN group, TNF-α and IL-6 level obviously decreased in the Stattic + LPS/D-GalN group (Figure 3).

Stattic suppressed LPS/D-GalN-induced up-regulation of caspase activity

Apoptosis is a programmed cell death accompanied by activation of a series of caspases.[18] To confirm whether Stattic plays a part in LPS/D-GalN-induced hepatocyte apoptosis, the activities of caspase-3, caspase-8, and caspase-9 were measured. As shown in Figure 4, the activities of caspase-3, caspase-8, and caspase-9 significantly increased in LPS/D-GalN group, whereas the tendency was dramatically reversed by Stattic. Meanwhile, the Western blot analysis showed that the level of cleaved caspase-3 dramatically increased in LPS/D-GalN group, and Stattic significantly suppressed the level of cleaved caspase-3 induced by LPS/D-GalN-exposure (Figure 5).

Stattic alleviated LPS/D-GalN-induced hepatocyte apoptosis

Cleavage of PARP was regarded as a marker of cells undergoing apoptosis.[19] The Western blot analysis showed that the level of cleaved PARP markedly increased in the LPS/D-GalN group, and the up-regulation of cleaved PARP induced by LPS/D-GalN exposure was obviously inhibited by Stattic (Figure 6). In the meantime, the TUNEL assay showed that the
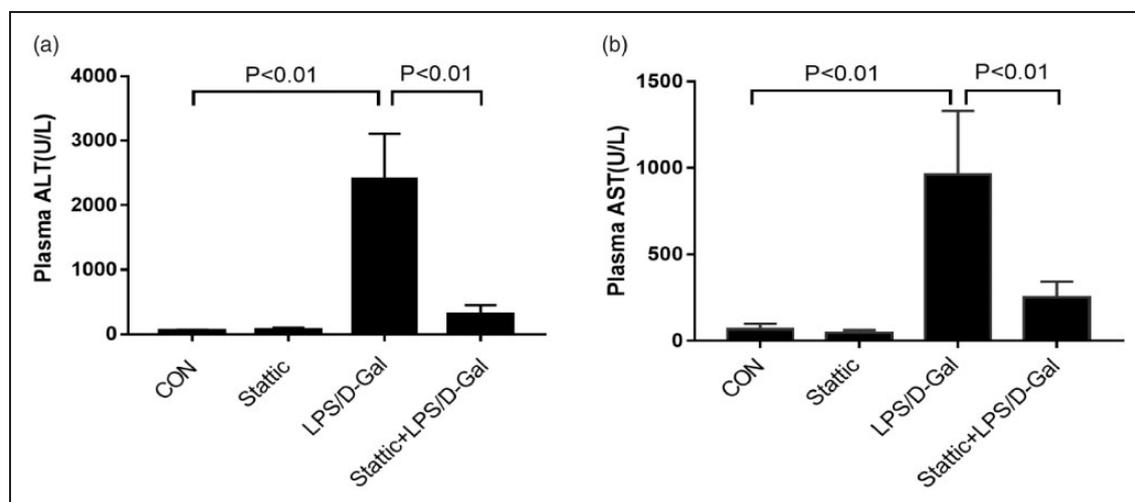
Figure 1. Stattic restrained LPS/D-GalN-induced elevation of plasma ALT and AST. Acute liver injury was induced by LPS/D-GalN, and Stattic was injected 0.5 h prior to LPS/D-GalN exposure. Plasma samples were collected at 6 h after LPS/D-GalN exposure. The levels of ALT (a) and AST (b) in plasma were measured. Data were expressed as the mean $\pm$ SD, $n = 8$.
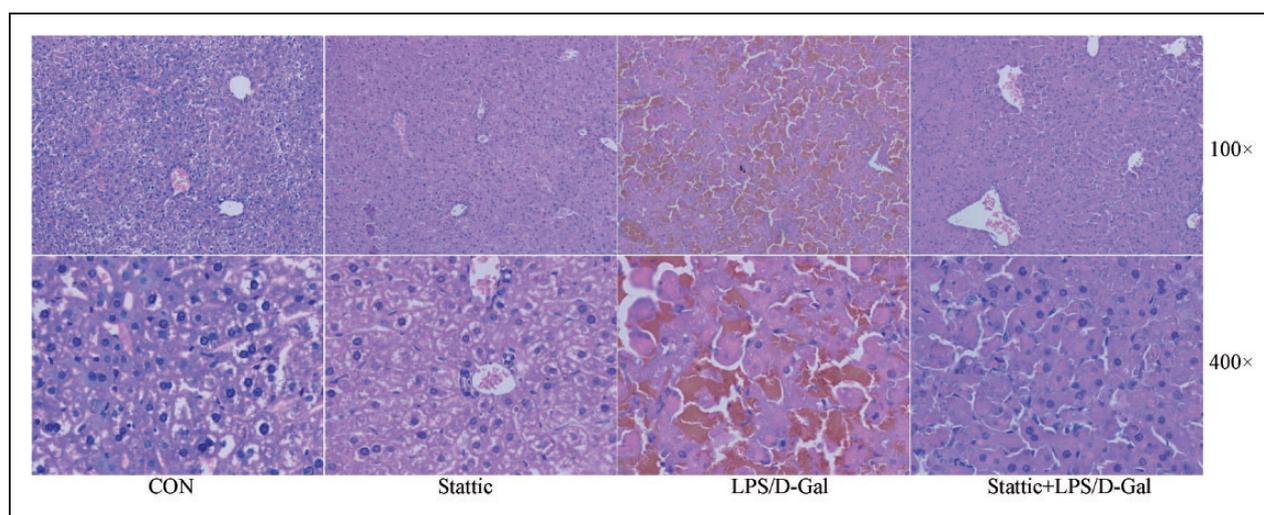

Figure 2. Stattic alleviated LPS/D-GalN-induced histological abnormalities in the liver. Acute liver injury was induced by LPS/D-GalN, and Stattic was injected 0.5 h before LPS/D-GalN exposure. Liver samples were harvested at 6 h after LPS/D-GalN exposure. Liver sections were stained with hematoxylin and eosin for morphological evaluation. The morphological abnormalities of liver sections were assessed under a light microscope (original magnification: 100$\times$ and 400$\times$) ($n = 4$).

TUNEL-positive staining cells were markedly increased in LPS/D-GalN-treated mouse livers, but the increasing positive staining cells induced by LPS/D-GalN were strongly inhibited by Sattic treatment (Figure 7).

Discussion

LPS/D-GalN-induced hepatic injury is a well-established model. In the model, D-GalN needs to be administrated with LPS at the same time to induce acute liver injury.[5–7] Administration of D-GalN initiates the depletion of hepatic UTP, which leads to an impaired macromolecular biosynthesis, such as RNA, proteins, glycogen, etc. These biochemical changes in the liver increase the sensitivity of mice to LPS more than several thousand-fold by treatment of D-GalN.[20] Hence, in D-GalN-sensitized mice, LPS, even at a very small dose, can induce acute liver injury, which is characterized by overwhelming inflammation and hepatocyte apoptosis.[20–22] Because the LPS/D-GalN-induced hepatic injury shows similar changes as acute hepatic
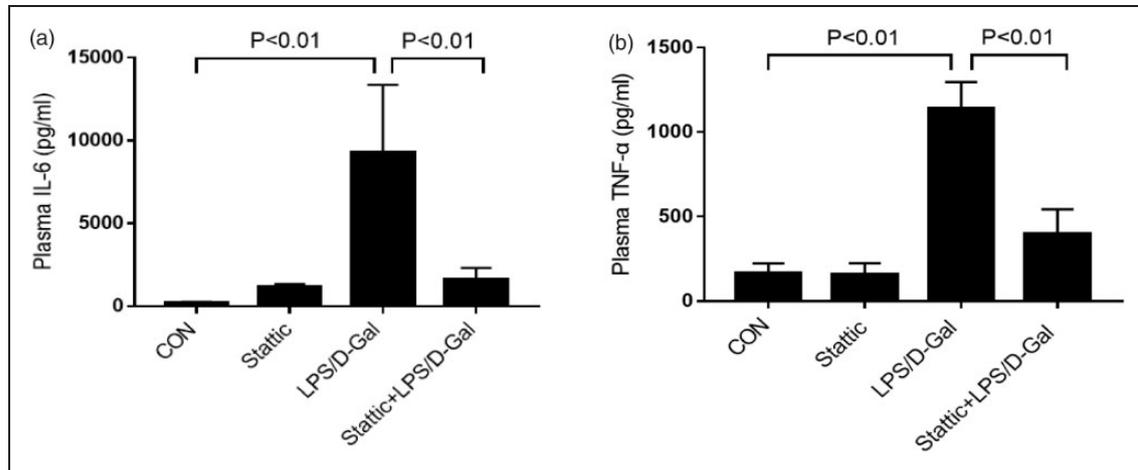
Figure 3. Stattic suppressed LPS/D-GalN-induced production of IL-6 and TNF-α. Acute liver injury was induced by LPS/D-GalN, and Stattic was injected 0.5 h before LPS/D-GalN exposure. Plasma samples for the test of IL-6 were collected at 6 h after LPS/D-GalN exposure. Plasma samples for TNF-α measurement were collected at 1.5 h after LPS/D-GalN exposure. The levels of IL-6 and TNF-α in the plasma were determined by ELISA. Data were expressed as the mean ± SD, n = 8.
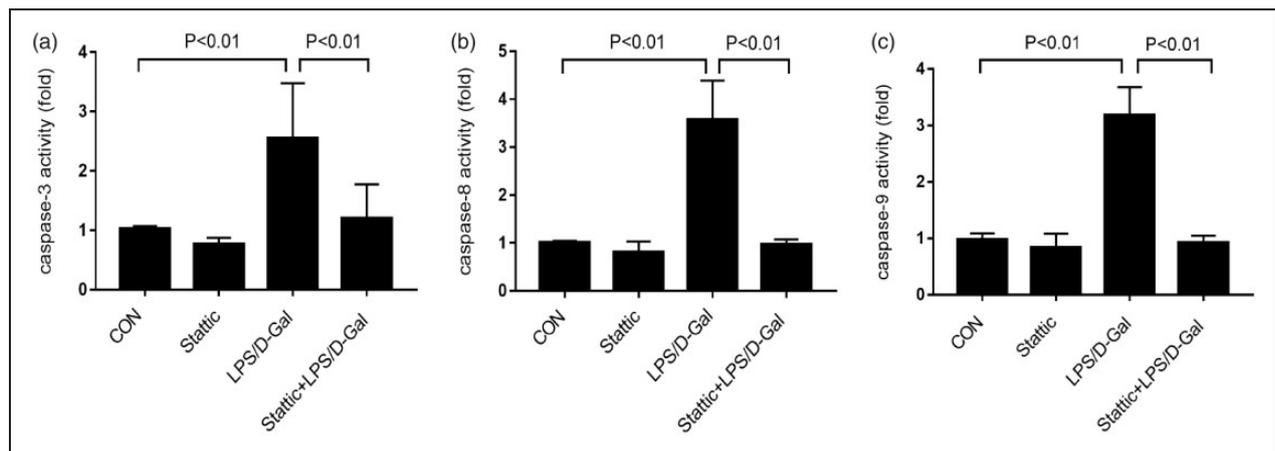

Figure 4. Stattic decreased LPS/D-GalN-induced up-regulation of the activity of caspase-3, caspase-8, and caspase-9. Acute liver injury was induced by LPS/D-GalN, and Stattic was injected 0.5 h before LPS/D-GalN exposure. The liver tissue was collected at 6 h after LPS/D-GalN exposure. The activities of caspase-3 (a), caspase-8 (b), and caspase-9 (c) in the liver tissues of mice were detected by the caspase Activity Assay Kit. Data were expressed as the mean ± SD, n = 8.

injury in clinical patients, the model is widely used to study the pathogenesis and therapeutic intervention of acute hepatic injury.[5–7]

Recently, increasing evidence indicates that STAT3 plays crucial roles in the regulation of inflammation and apoptosis.[10,13] STAT3 has become a novel therapeutic target for intervention in inflammation-related disorders,[10,11] but it remains unclear whether STAT3 plays a part in acute hepatic damage. In the present study, the results showed that Stattic mitigated the hepatic morphologic abnormalities, such as hepatocellular necrosis and destruction of hepatic lobule, and decreased the level of aminotransferase in LPS/D-GalN-insulted mice. These results suggested that

Stattic plays a protective role in LPS/D-GalN-induced hepatic damage.

It is widely accepted that overwhelming inflammation is the crucial mechanism in the development of LPS/D-GalN-induced hepatic damage,[2] and TNF-α and IL-6 are the major pro-inflammatory cytokines in the model.[16,17] The present results showed that TNF-α and IL-6 were obviously up-regulated in the LPS/D-GalN-insulted mice, which was markedly reversed by Stattic. Therefore, the protective effect of Stattic in LPS/D-GalN-induced liver injury might be due to its anti-inflammatory property.

In agreement with the present results, accumulating studies have revealed that inhibition of STAT3 displays
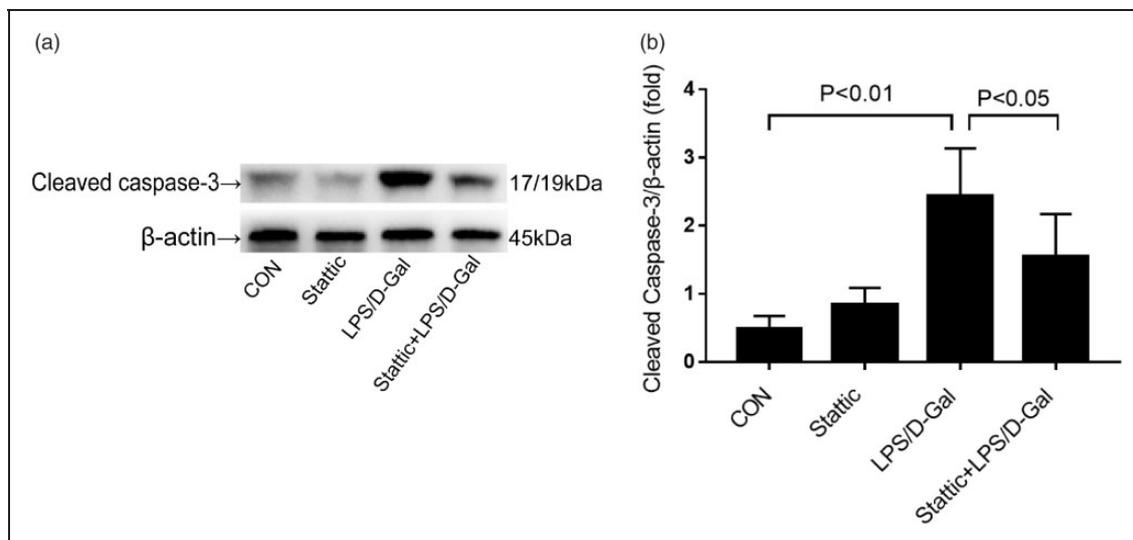
Figure 5. Stattic suppressed LPS/D-GalN-induced up-regulation of cleaved caspase-3. Acute liver injury was induced by LPS/D-GalN, and Stattic was injected 0.5 h prior to LPS/D-GalN exposure. Liver samples were harvested at 6 h after LPS/D-GalN exposure. (a) The levels of cleaved caspase-3 in the liver were determined by Western blot analysis. (b) The blots were semi-quantified. Data were expressed as the mean $\pm$ SD, $n = 4$.
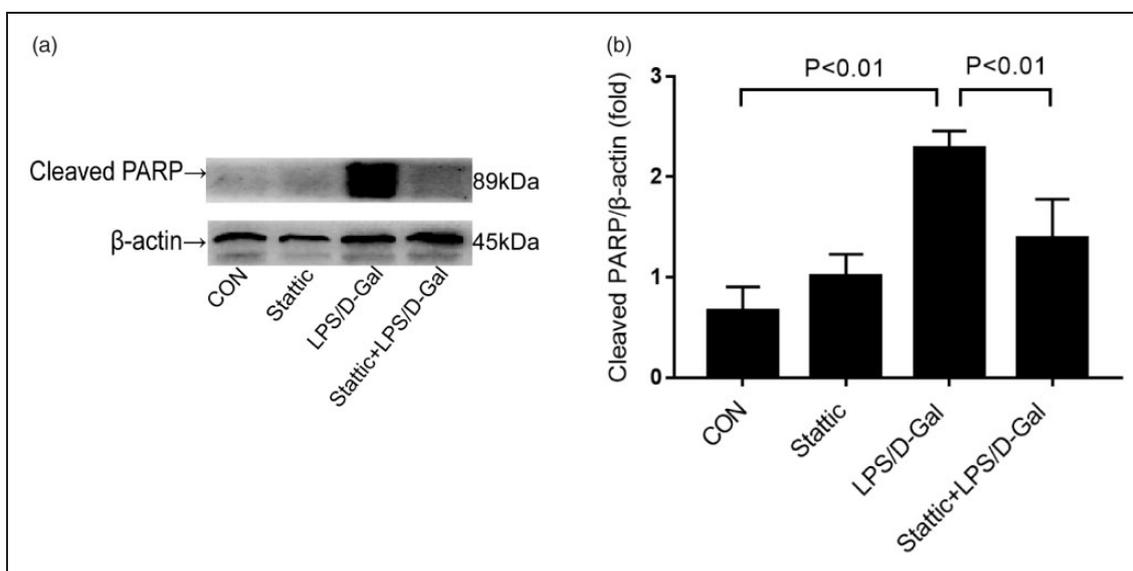

Figure 6. Stattic suppressed LPS/D-GalN-induced elevation of cleaved PARP. Acute liver injury was induced by LPS/D-GalN, and Stattic was injected 0.5 h prior to LPS/D-GalN exposure. Liver samples were harvested at 6 h after LPS/D-GalN exposure. (a) The levels of cleaved PARP in the liver were determined by Western blot analysis. (b) The blots were semi-quantified. Data were expressed as the mean $\pm$ SD, $n = 4$.

an anti-inflammation effect. Previous studies have indicated that Stattic dramatically reduced the expression of TNF-α and IL-6 in LPS-induced murine microglia BV-2 cells.[10] WP1066, another STAT3 inhibitor, significantly reduced the level of TNF-α and IL-6 in LPS- or IFN-γ-stimulated mouse RAW264.7 macrophages.[11] *In vivo*, STAT3 inhibitor Nifuroxazide significantly decreased the mRNA and protein level of TNF-α and IL-18 and reduced infiltration of inflammatory cells in

diabetes-associated renal injury.[23] LLL12, an allosteric STAT3 inhibitor, also attenuated the infiltration of inflammatory cells and the level of TNF-α and IL-1β in LPS-induced acute lung injury.[24] Interestingly, in carbon tetrachloride-induced acute hepatic damage, compound 24, a STAT3 inhibitor, significantly downregulated the level of IL-6.[25] These above data indicate that inhibition of STAT3 plays a protective effect in the intervention of inflammation. In addition, it should be
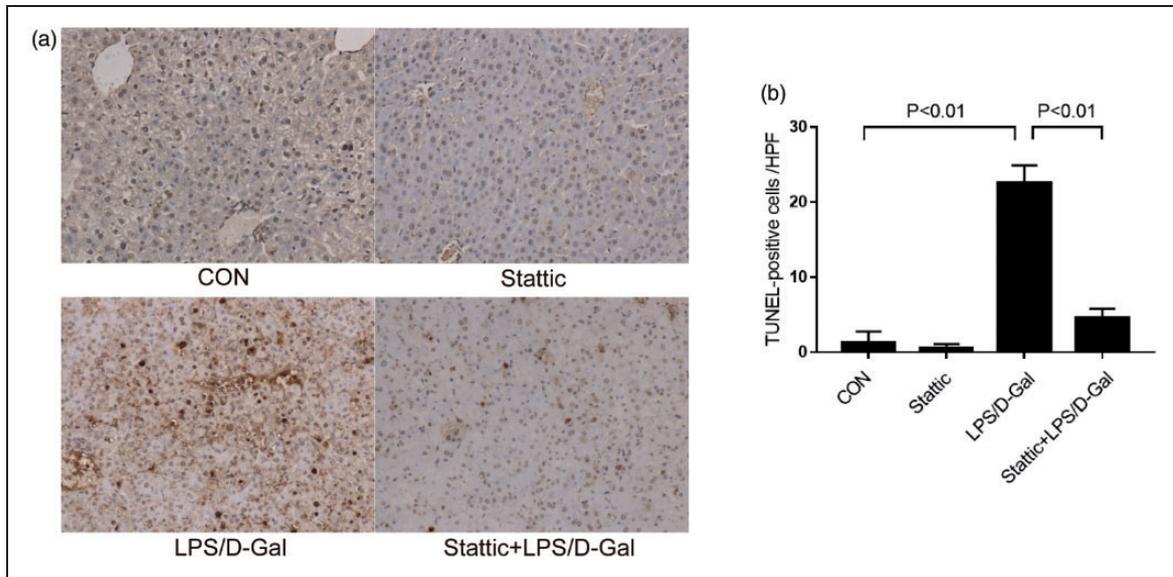
Figure 7. Stattic suppressed hepatocyte apoptosis in LPS/ᴅ-GalN-exposed mice. Acute liver injury was induced by LPS/D-Gal, and Stattic was injected 0.5 h before LPS/ᴅ-GalN exposure. Liver samples were harvested at 6 h after LPS/ᴅ-GalN exposure. The apoptotic cells were determined by TUNEL assay, and the TUNEL-positive cells showed a dark-brown nucleus. (a) The representative sections of each group are shown (original magnification 200×). (b) The numbers of TUNEL-positive cells in 10 randomly selected fields were counted under a microscope. Data were expressed as the mean ± SD, $n = 4$.

noted that inhibition of STAT3 plays pro-inflammatory roles.[26,27] Some reports indicated that inhibition of STAT3 reversed the anti-inflammatory effect of Dexmedetomidine on LPS-induced acute lung injury by increasing the level of IL-1β and TNF-α, and reversed the inhibitory effect of Panaxatriol saponins on inflammatory response of Oxygen and Glc Deprivation/Reoxygenation-treated Microglial cells by enhancing the expression of TNF-α and IL-6.[26,27]

Accumulating evidence indicated that hepatocyte apoptosis is the characteristic change in LPS/ᴅ-GalN-induced hepatic damage.[28] However, available results show that the effect of STAT3 in apoptosis is intricate and contradictory. Plenty of evidence has shown that inhibition of STAT3 possesses pro-apoptotic function, because STAT3 shows persistent activation in multiple tumors, and inhibition of STAT3 promotes the apoptosis of tumor cells.[29,30] However, some results have also shown that inhibition of STAT3 possesses anti-apoptotic function.[31,32] For instance, the STAT3 inhibitor S3I-201 suppressed the apoptosis of glomerular mesangial cells and reduced the apoptotic rate in folic acid-induced glomerular mesangial cell injury.[31] A previous study also showed that mammary gland involution accompanied extensive apoptosis of epithelial cells, while knockout of STAT3 decreased the apoptosis of mammary epithelial cells at the onset of involution.[32] Therefore, the apoptosis-regulatory effect of STAT3 appears to be tissue- or cell-dependent. In the current study, the results showed that treatment of

Stattic prevented the activation of the caspase cascade, suppressed cleavage of PARP, and decreased the quantity of TUNEL-positive cells. These results indicated that Stattic can inhibit the hepatic apoptosis induced by LPS/ᴅ-GalN treatment. Hence, the protective effect of Stattic in LPS/ᴅ-GalN-induced liver injury may also depend on its anti-apoptotic function.

It is well known that TNF-α is a major death receptor ligand, which triggers an extrinsic pathway of apoptosis.[33] Previous results have verified that TNF-α is a crucial pro-apoptotic factor in LPS/ᴅ-GalN-induced liver injury, which activates the caspase cascade, cleaves structural proteins, and induces hepatic apoptosis.[16] According to the current results, TNF-α evidently declined following the treatment with Stattic in LPS/ᴅ-GalN-induced liver injury. Hence, it is reasonable that anti-apoptotic function of Stattic largely depends on its effect in inhibiting TNF-α production.

It should be noted in this study that ᴅ-GalN sensitization varies with age.[34] Previous study showed that the basal level of Stat3 in liver does not significantly change at various ages of mice, but the activation of Stat3 is impaired in aged animals.[35] Therefore, the study needs to be conducted in mice with the same or similar age to avoid the impact of age.

Taken together, the current study showed that treatment with the STAT3 inhibitor Stattic mitigated hepatic damage, prevented inflammatory response, and suppressed hepatocyte apoptosis in LPS/ᴅ-GalN-induced hepatic damage in mice. The

beneficial effects of Stattic might be associated with its anti-inflammatory and anti-apoptotic effects, but the detailed mechanisms remain to be further studied. This study indicated that STAT3 inhibitors, including Stattic, seem to be possess potential value in intervention in inflammation-related hepatic disorders.

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