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Sesamin ameliorates lipopolysaccharide/D-galactosamine-induced fulminant hepatic failure by suppression of Toll-like receptor 4 signaling in mice

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ABSTRACT

Sesamin has been described to exert anti-oxidant and anti-inflammatory properties. In present study, we investigated the potential effects and mechanisms of sesamin on lipopolysaccharide (LPS)-induced fulminant hepatic failure (FHF) in D-galactosamine (D-GalN)-sensitized mice. Our results showed that pretreatment with sesamin dose-dependently improved LPS/D-GalN-induced mortality and liver injury as indicated by reduced serum levels of aminotransferases and alleviated pathological damage as well as hepatocyte apoptosis in mice. Additionally, sesamin markedly attenuated LPS/D-GalN-induced adhesion molecules expression, and decreased neutrophils recruitment. Furthermore, sesamin inhibited LPS-induced tumor necrosis factor-alpha (TNF-α) production, p38 mitogen-activated protein kinases (MAPK) and NF-κB activation, and Toll like receptor (TLR) 4 expression in mice and in RAW264.7 macrophage cells. In summary, these results demonstrate that sesamin protects mice from LPS-induced FHF and the molecular mechanisms may down-regulate the expression of TLR4, block MAPK and NF-κB activation, decrease the production of TNF-α.

1. Introduction

Fulminant hepatic failure (FHF) is a life threatening and devastating clinical syndrome characterized by overwhelming hepatocyte necrosis and a sharp decline of liver dysfunction, resulting in hepatic encephalopathy and multiple organ dysfunction syndromes (MODS) associated with high mortality [1,2]. Up to now, liver transplantation is the only proven way to treat FHF [1,3]. But due to the costliness of surgery and shortage of the liver donor, only a small number of patients can receive it. Therefore, to find another effective and available therapy is particularly important.

Lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced liver injury is a well-established experimental murine model of FHF to find effective therapeutic agents [4,5]. In this model, D-GalN is a kind of amino sugar that consumes hepatic uridine triphosphate (UTP) and abrogates the biosynthesis of macro-molecules in liver. Consequently, a combination of LPS and D-GalN can induce specific liver injury with no effect on the other organs [6]. Injection of mice with LPS, the major component of gram-negative bacteria, triggers the signal pathway of Toll-like receptor (TLR) 4 mainly located on the cell surface of macrophages, which activates NF-κB and mitogen-activated protein kinases (MAPKs) to produce pro-inflammatory cytokines. These uncontrolled pro-inflammatory cytokines will lead to excessive inflammatory responses, and play central to the process of liver injury and determine the severity of liver injury and outcome of FHF [3,7–9]. Among these cytokines, tumor necrosis factor-alpha (TNF-α) is a critical effector in hepatic inflammatory responses and could be associated with hepatocyte apoptosis and necrosis [10,11].

Sesamin (Fig. 1A), a sesame lignan exacted from sesame seeds, has been described to possess anti-oxidant [12], anti-inflammatory [13,14], anti-cancer [15], and anti-hypertensive [16] effects. Previous studies have indicated that sesamin suppresses leukocyte infiltration, MAPKs and NF-κB activation, expression of intercellular cell adhesion molecule-1 (ICAM-1), and TNF-α production *in vivo* and *in vitro* models [17–20]. Besides, it also has been found that sesamin exhibits hepatoprotective effects against different models

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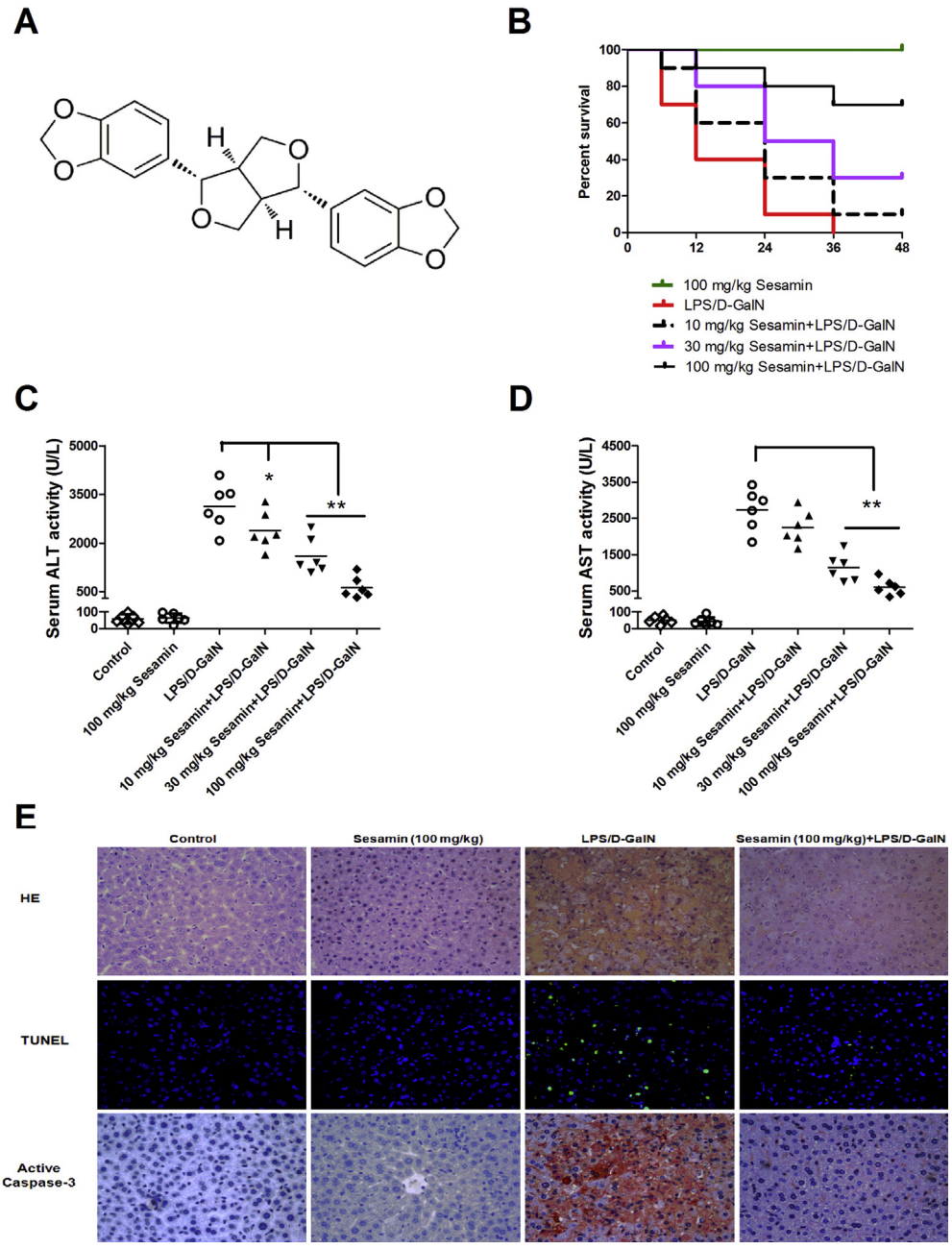

Fig. 1. Effects of sesamin on survival and liver injury of mice challenged by LPS/D-GalN. Mice were given oral gavage of sesamin (10, 30, 100 mg/kg, respectively) every 8 h one time for three times within 24 h before LPS (50 ug/kg)/D-GalN (800 mg/kg) injection. (A) Chemical structure of sesamin. (B) The survival rates were observed every 6 h within 48 h after LPS/D-GalN. The serum ALT (C) and AST (D) activities were measured at 6 h after LPS/D-GalN. (E) Liver tissues were obtained at 6 h after LPS/D-GalN for determining hepatic pathological changes and hepatocyte apoptosis by H&E, TUNEL, and IHC of the active caspase-3 staining (400 × magnifications). Each value is mean ± S.D. *P < 0.05, **P < 0.01 versus LPS/D-GalN group.

of liver injury such as CCL4-induced liver injury [21], nickel-induced liver injury [22] and hepatic ischemia-reperfusion injury [12]. However, whether sesamin exerts the protective effect on LPS/D-GalN-induced FHF and its underlying mechanisms remain unclear.

Here, we explore the protective effects and molecular mechanisms of sesamin on LPS/D-GalN-induced FHF. Our results indicated that sesamin improved mortality, alleviated liver damage, reduced hepatic inflammation in mice by LPS/D-GalN injection. Notably, sesamin dampened LPS-activated TLR4 signal by down-regulation of TLR4 expression, blocking MAPK and NF-κB activation, and inhibiting TNF-α production.

2. Material and methods

2.1. Reagents and antibodies

Sesamin (purity > 95%) was purchased from Aladdin Industrial Corporation (Shanghai, China). LPS (Escherichia coli, 0111:B4) and D-GalN were purchased from Sigma (St. Louis, USA).

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) detection kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The enzyme-linked immunosorbent assay (ELISA) kit for TNF-α was purchased from Bender MedSystems (Vienna, Austria). In Situ Cell Death Detection Kit was from Roche Applied Science (Basel, Switzerland). Rabbit anti-mouse TLR4, Myeloperoxidase (MPO) and β-action antibodies were purchased from Abcam (Cambridge, UK). Intercellular adhesion molecule-1 (ICAM-1), endothelial cell adhesion molecule-1 (ECAM-1), Active caspase 3, phospho-p38 MAPK, and phospho-IκB were purchased from Cell Signaling Technology (Boston, USA). Anti Ly6G-PE mouse antibody was purchased from Miltenyi Biotec (Cologne, Germany).

2.2. Experimental design and protocol

Male Balb/c-mice (6–8 weeks, 18–22 g) were purchased from the Laboratory Animal Center of Chongqing Medical University (Chongqing, PR China), then fed under specific pathogen-free conditions. All the experiments were in accordance with the guidelines from Institutional Animal Care and Use Committee of Chongqing Medical University. Mice were given oral gavage of sesamin (10, 30, 100 mg/kg, dissolved in 0.5% carboxylmethylcellulose sodium salt in 0.9% normal saline, respectively) every 8 h one time for three times within 24 h before LPS (50 μg/kg)/D-GalN (800 mg/kg) administration. The mortality and liver injury were assessed subsequently.

For *in vitro* experience, the mouse macrophage cell line RAW 264.7 cells (ATCC, Rockville, MD) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, and maintained at 37 °C with 5% CO_2. The different dose of sesamin (1, 3, 10 μM, respectively) was added 30 min before LPS (100 ng/ml) stimulation.

2.3. Analysis of liver enzymes

Blood samples were collected by cardiac puncture to analyze ALT and AST activities as index of hepatocellular injury according to the manufacturer's directions.

2.4. Analysis of histopathology

Liver tissues maintained in 4% paraformaldehyde were dehydrated and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (HE) and assessed using light microscopy.

2.5. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining

Hepatocytes apoptosis in paraffin-embedded tissues were undertaken with an in situ cell death detection kit. Liver sections were incubated with proteinase K solution for 30 min at 37 °C. Then TUNEL reaction mixture was added on samples to incubate for 1 h at 37 °C in a humidified box. The TUNEL-labeled cells were imaged using a fluorescence microscopy (Nikon, Tokyo, Japan).

2.6. Immunohistochemistry (IHC) staining

The active caspase 3 was detected using IHC method according to the manufacturer's instructions. In brief, liver sections were incubated with the primary active caspase 3 antibody for overnight, followed by incubation with horseradish peroxidased-labeled second antibody for 30 min. Sections were then stained with AEC (DAKO, Cambridge shire, UK).

2.7. ELISA assay

The TNF-α levels in sera and supernatants were detected by using a mouse TNF-α ELISA kit according to the manufacturer's directions. In Brief, samples and standards were added into microwells coated with anti-mouse TNF-α antibody. And then, a biotin conjugated secondary antibody and horseradish peroxidase (HRP)-conjugated streptavidin was sequentially added into microwells. Finally, a color substrate solution reactive with HRP was detected. The actual concentration of samples TNF-α was calculated according to the standard curve.

2.8. Flow cytometric analysis

The liver was perfused through the portal vein with calcium-free buffer followed by collagenase-containing buffer. The liver was removed, minced and pressed through a 200-gauge stainless steel mesh, and then centrifuged at 50 *g* for 5 min. The supernatants containing hepatic leukocytes were separated and collected. Leukocytes were stained with the Ly6G-PE antibody and analyzed by flow cytometry.

RAW 264.7 cells were stained with PE-labeled anti-TLR4 antibody. After washed 3 times with PBS, TLR4 levels of cell surface were subsequently analyzed by flow cytometry.

2.9. Quantitative reverse transcription polymerase chain reaction (QRT-PCR)

Total RNA was extracted from liver samples using a Total RNA extraction reagent (Takara, Japan) according to the manufacturer's protocol. After synthesizing of the complementary DNA (cDNA), cDNA samples were used for Real-Time PCR reaction which initiated at 95 °C for 30 s, followed 39 cycles of amplification of denaturation at 95 °C for 5 s, annealing at 60 °C for 45 s. The sequences of TNF-α 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), and the β-actin 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) which is used as an internal standard.

2.10. Western blotting

Total proteins were isolated from hepatic tissues using the protein extract kit (Piece Biotechnology, Rockford, USA) according to the manufacturer's protocol. Protein concentrations were detected by the BCA protein assay kit. 40 μg proteins were loaded on 12% polyacrylamide-sodium dodecyl sulfate (SDS) gel and transferred to nitrocellulose membrane. The membranes were incubated with primary antibody at 4 °C overnight and then incubated with a HRP-conjugated secondary antibody for 1 h at room temperature. Antibody binding was visualized with an ECL chemiluminescent system and developed using X-ray films exposure.

2.11. Luciferase reporter assay

RAW264.7 cells were transiently cotransfected with both pNF-κB-Luc or pAP-1-Luc plasmid and the pRL-TK *Renilla* plasmid by using Lipofectamine 2000 (Invitrogen, USA). After 24 h, the cells were stimulated with 100 ng/ml LPS with or without 10 μM sesamin; cell lysis was measured for luciferase activity according to the luciferase assay reagent kit (Promage,USA). The NF-κB or AP-1 reporter activity was divided by the activity of the *Renilla* control reporter to normalize the transfection efficiency.

2.12. Statistical analysis

All results were analyzed using Student's test or by one-way analysis of variance (ANOVA) appropriately. All data in this study were expressed as mean ± standard (S.D.). The level of statistical significance was p values less than or equal to 0.05. Kaplan–Meier curve and log-rank test was applied in survival statistics.

3. Results

3.1. Effect of sesamin on LPS/D-GalN-induced lethality and liver injury in mice

Injecting LPS/D-GalN to mice caused 100% mortality rate within 48 h (Fig. 1B). Pretreatment with sesamin improved mortality of LPS/D-GalN-challenged mice in a dose-dependent manner, especially at a dose of 100 mg/kg. Accordingly, LPS/D-GalN challenge significantly elevated serum aminotransferases activities at 6 h, which were reduced dose-dependently by sesamin (Fig. 1C and D). Microscopic examination of liver sections revealed that LPS/D-GalN induced severe morphaological damages, which are characteristic of architecture disruption, hemorrhage, and extensive hepatic necrosis and apoptosis. When sesamin was pretreated 30 min before LPS injection, only focal hepatocyte death was observed (Fig. 1E). In a line with the result of HE staining, TUNEL and IHC of active caspase 3 analysis indicated that LPS/D-GalN induced a large amount of apoptotic hepatocytes and active caspase 3 expression in the liver sections. In contrast, pretreatment with sesamin showed less apoptotic hepatocytes as well as reduced caspase 3 activation (Fig. 1E).

3.2. Effect of sesamin on LPS/D-GalN-induced neutrophil recruitment and adhesion molecules expression

To confirm whether sesamin affects neutrophil recruitment into the liver after LPS challenge, we analyzed intrahepatic neutrophil accumulation by flow cytometry with Ly6G, a marker of neutrophil. As shown in Fig. 2A, $Ly6G^+$ cells (neutrophils) in the liver dramatically increased after LPS challenge as compared with control, but this increase was significantly inhibited by sesamin. These changes of $Ly6G^+$ neutrophils were consistent with expression of MPO, which is the other marker of neutrophil (Fig. 2B). We then detected the expression of some adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and endothelial cell adhesion molecule-1 (ECAM-1) that are responsible for interaction between neutrophils and endothelial cells. As expected, ICAM-1 and ECAM-1 expression elevated 6 h after LPS/D-GalN challenge. In contrast, sesamin effectively suppressed ICAM-1 and ECAM-1 expression (Fig. 2B).

3.3. Effect of sesamin on LPS/D-GalN-induced TNF-α production and TLR4 signal activation in mice

Serum level of TNF-α was measured by ELISA in LPS/D-GalN-challanged mice with or without sesamin (Fig. 3A). At 6 h after LPS administration, serum TNF-α levels were remarkably increased as compared with vehicle control, whereas, pretreatment with sesamin significantly inhibited LPS/D-GalN-induced TNF-α production. Because serum level might not specifically reflect TNF-α expression in the liver, protein and mRNA expressions of TNF-α in the liver were determined by Western blotting and QRT-PCR. As shown in Fig. 3B and C, pretreatment with sesamin (100 mg/kg) markedly suppressed LPS/D-GalN-induced hepatic TNF-α protein and mRNA expressions.

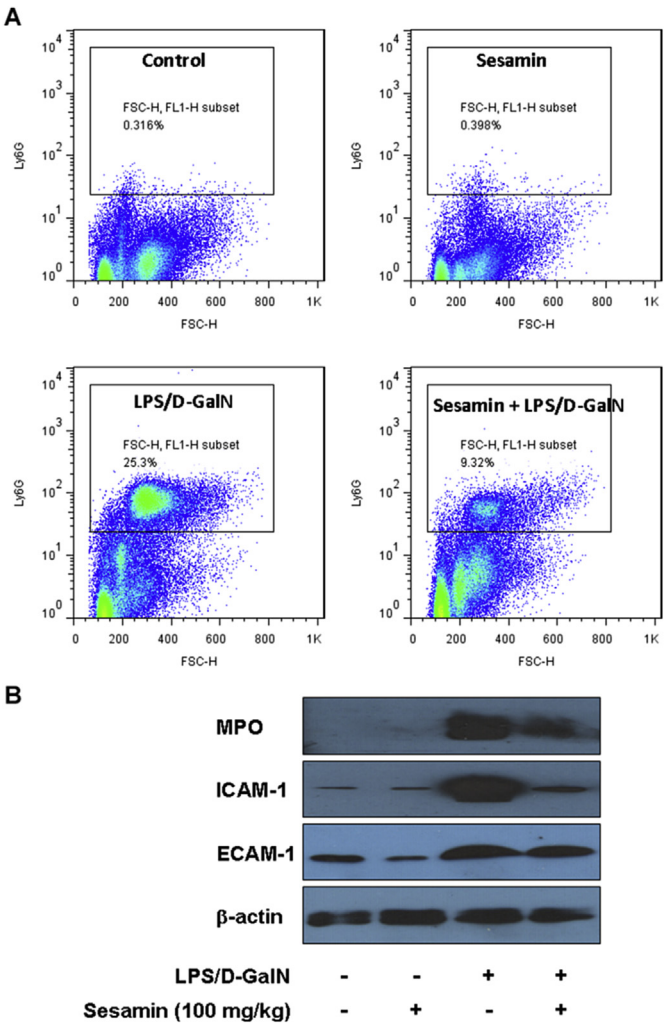

Fig. 2. Effects of sesamin on LPS-induced hepatic neutrophil accumulation and adhesion molecules expression in D-GalN-sensitized mice. Mice were given oral gavage of sesamin (100 mg/kg) every 8 h one time for three times within 24 h before LPS (50 μg/kg)/D-GalN (800 mg/kg) administration. (A) Hepatic tissues leukocytes were isolated at 6 h after LPS/D-GalN for evaluating hepatic neutrophils accumulation. (B) Liver tissues were collected at 6 h after LPS/D-GalN for determining the expression of MPO, ICAM-1, and ECAM-1 by western blotting.

Since TLR4 signal pathway mediates LPS-induced TNF-α production, we asked whether sesamin could affect TLR4 signal pathway *in vivo*. Western blotting analysis showed that phosphorylated p38 MAPK and IκB, as well as TLR4 were up-regulated in hepatic tissues of mice challenged by LPS/D-GalN. However, pretreatment with sesamin inhibited these up-regulated signal molecules (Fig. 3D).

3.4. Effect of sesamin on LPS-induced TNF-a production and TLR4 signal activation in macrophage

Although many various cells are capable of producing TNF-α, macrophages are its principal source and play the crucial role on LPS/D-GalN-induced FHF [6,11]. Thereby, we used a murine macrophage line to evaluate the role of sesamin on LPS-activated signal pathway. RAW 264.7 macrophages were pretreated with sesamin 30 min before LPS (100 ng/ml), then TNF-α levels in the supernatants were assessed. Obviously, LPS induced a marked increase of TNF-α at 6 h, which was inhibited by sesamin in a dose-dependent manner (Fig. 4A).

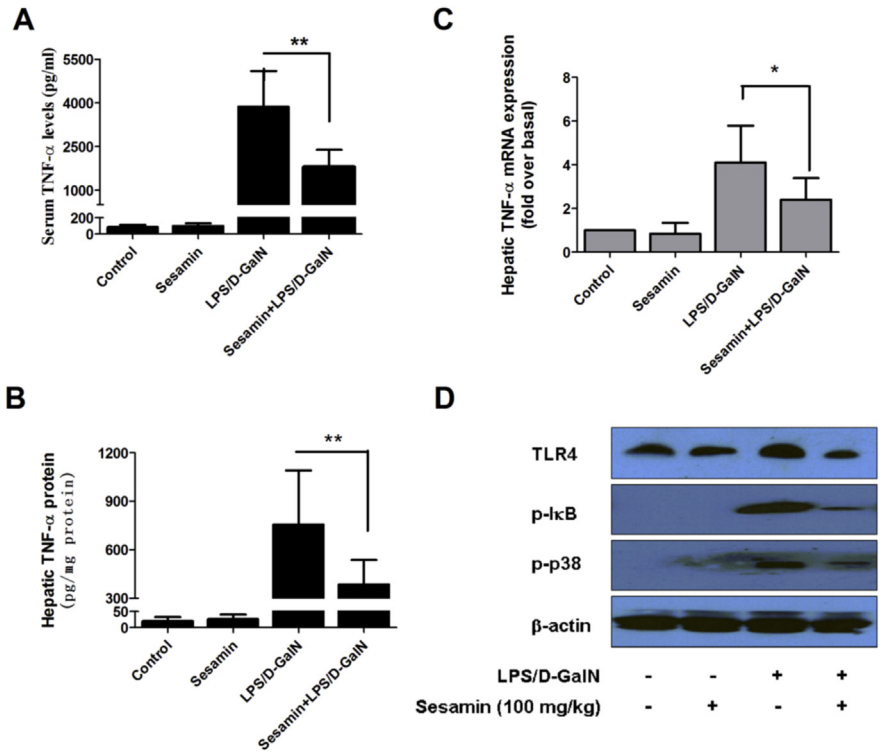

Fig. 3. Effects of sesamin on TNF-α production and TLR4 signal activation in mice induced by LPS/D-GalN. Mice were given oral gavage of sesamin (100 mg/kg) every 8 h one time for three times within 24 h before LPS (50 μg/kg)/D-GalN (800 mg/kg) injection. Serum samples and liver tissues were collected at 6 h after LPS/D-GalN. (A) Serum and (B) hepatic TNF-α protein levels were measured by ELISA, (C) Hepatic TNF-α mRNA expression levels were analyzed by qRT-PCR. (D) Expression of TLR4, p-IκB, and p-p38 in hepatic tissues were determined by western blotting. Each value is mean ± S.D. (n = 6), $^{*}P < 0.05$, $^{**}P < 0.01$ versus LPS/D-GalN group.

Further, RAW 264.7 cells were transfected with AP-1 or NF-κB promoter luciferase reporter gene to determine whether the protective effect of sesamin is associated with inhibiting AP-1 and NF-κB activities. Compared with control, LPS induced dramatically increased transcriptional activities of AP-1 and NF-κB, which were markedly inhibited by sesamin (Fig. 4B and C).

Finally, we analyzed TLR4 expression of macrophages by flow cytometry. As indicated in Fig. 4D, pretreatment with sesamin suppressed LPS-induced the expression of TLR4 on the cell surface of macrophages.

4. Discussion

Administration of LPS and D-GalN induces acute liver injury of mice and resembles clinic fulminant hepatic failure [3]. As anticipated, in this study, administration of LPS/D-GalN led to a high mortality rate and severe liver injury. Further, we characterized the protective effect of sesamin on LPS/D-GalN-induced FHF as assessed improved mortality rate, reduced ALT and AST activities, ameliorated hepatic pathological damage, and decreased liver inflammation with neutrophil infiltration.

TNF-α is a multifunctional cytokine which plays pivotal roles in LPS-induced inflammatory pathological processes. Knockout of TNF-α gene or treatment with TNF-α antibody almost completely blocked LPS/D-GalN-induced liver injury [11,23]. There are two distinct stages in LPS-induced liver injury. The early stage occurs at the initial few hours after LPS challenge and is related to the production of TNF-α from Kupffer cells, leading to mild hepatocyte apoptosis [10,11]. The later stage is also initiated by TNF-α, which induces adhesion molecules to recruit neutrophils into liver. These neutrophils lead to massive hepatocyte necrosis by releasing oxidants and proteases [24–27]. Our present results found that sesamin effectively alleviated LPS-induced hepatocyte apoptosis and hepatic inflammation with adhesion molecules expression and neutrophils infiltration. To further investigate whether the beneficial effect of sesamin is associated with inhibiting TNF-α production, we measured TNF-α production in the serum and hepatic tissues. The data revealed that sesamin markedly suppressed LPS/D-GalN-induced TNF-α production as evaluated by down-regulated expression of hepatic TNF-α mRNA and protein. Moreover, *in vitro* experiment demonstrated that sesamin inhibit LPS-induced TNF-α production in macrophages. These results suggest that sesamin could inhibit LPS-induced TNF-α transcriptional expression, which might mediate its hepatoprotection.

It is widely accepted that AP-1 and NF-κB are the important transcriptional factors of TNF-α expression during LPS-activated signaling pathway [9,28,29]. The canonical AP-1 and NF-κB signal pathway is initiated when LPS binds to it receptor. Consequently, p38 MAPK and I-κB are phosphorylated and thus AP-1 and p65 NF-κB are activated to translocate from the cytoplasm into the nucleus, where they induce transcription of cytokines such as TNF-α [9]. Inhibition of the upstream kinase IKK or p38 MAPK with antagonists attenuated LPS-induced inflammatory response as well as TNF-α production *in vivo* and *in vitro* [29,30]. Previous studies have provided compelling evidence that sesamin inhibits the activation of NF-κB and p38 MAPK activation in various cell models [20,31]. Therefore, we deduced that the inhibition of sesamin on LPS-induced TNF-α production might be through blocking the activation of AP-1 and NF-κB. As expected, *in vivo* experiments with Western blotting showed that sesamin markedly alleviated LPS-induced activation of p38 MAPK and NF-κB in the liver of mice. Likewise, *in vitro* experiments revealed that sesamin potently

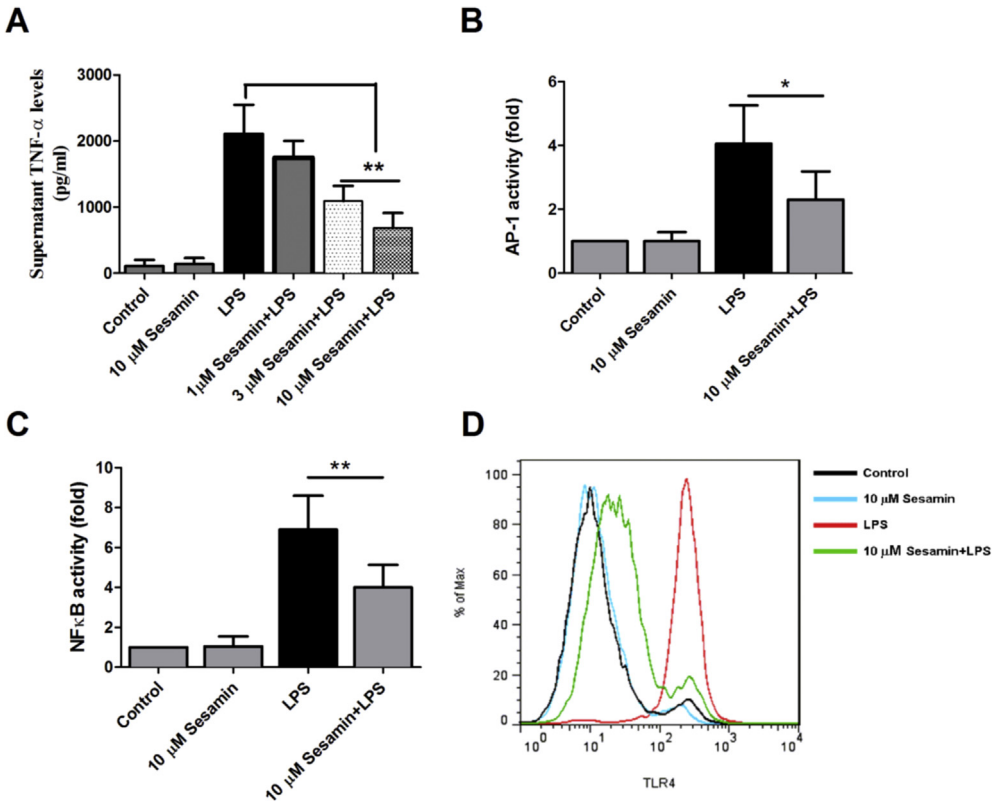

Fig. 4. Effects of sesamin on LPS-induced TNF-α production, AP-1 and NF-κB activation, and TLR4 expression in RAW264.7 macrophage cells. RAW264.7 cells were pretreated with various concentrations of sesamin (1, 3, 10 μM, respectively) 30 min before LPS stimulation or PBS. The supernants and cells were collected at 6 h after LPS stimulation for further analysis. (A) TNF-α production was measured by ELISA. (B) AP-1 and (C) NF-κB activation were determined by a luciferase reporter gene. (D) TLR4 expression levels were detected by flow cytometry. Each value is mean ± S.D. (n = 6), $^{*}P < 0.05$, $^{**}P < 0.01$ versus LPS/D-GalN group.

suppressed LPS-activated AP-1 and NF-κB activities in macrophages. All these results indicate that sesamin inhibits LPS-induced TNF-α production through blocking MAPK and NF-κB activation.

Generally, TLR4 recognizes LPS and plays the critical role in inflammatory process. LPS activates NF-κB mobilization and MAPK pathways through its direct binding to TLR4, ultimately causing the production of pro-inflammatory cytokines such as TNF-α [9]. Accumulating evidence reveal that TLR4 contributes to the pathogenesis of LPS/D-GalN-induced liver injury. Mice with *Tlr4* knockout or TLR4 antagonist treatment significantly protected mice from LPS/D-GalN-induced lethality [32,33]. To address whether sesamin affects TLR4 expression, we first determine TLR4 expression in liver tissues by Western blotting. Our result indicated that sesamin inhibited LPS-induced TLR4 expression in the liver of mice. Virtually, Kupffer cells or other macrophages are the best-characterized target of LPS in the liver, where they have a key role in hepatic inflammatory responses by triggering TLR4 signal pathway [34]. Therefore, we determine whether sesamin down-regulates TLR4 expression on the surface of macrophages. Using a macrophage cell line model of *in vitro*, we found that TLR4 expression on the surface of macrophages elevated 6 h after LPS stimulation and sesamin effectively attenuated this increasing tendency. Data above suggest that the inhibitive effect of sesamin on LPS-induced TNF-α production may be through down-regulation of the expression of TLR4 on surface of macrophages (Kupffer cells), resulting in blocking MAPK and NF-κB activation.

In conclusion, sesamin exhibits a protective effect on liver injury induced by LPS/D-GalN, improves the liver function, and thus decrease the lethality. Its potential mechanism might be related to the suppression of TLR4 expression of macrophages, blocking of MAPK and NF-κB signaling pathway, and the following inhibiting TNF-α production.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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