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Atractylodin ameliorates lipopolysaccharide and D-galactosamine-induced acute liver failure via the suppression of inflammation and oxidative stress

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ABSTRACT

Atractylodin (ACD) possesses versatile biological and pharmacological activities, including antibacterial, anti-inflammatory and hepatoprotective properties. However, the protective effects of ACD on lipopolysaccharide (LPS) and D-galactosamine (GalN)-induced acute liver failure (ALF) as well as the underlying molecular mechanisms remain unclear. In this study, our findings showed that ACD treatment could reduce the high lethality rate; decrease the serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), monocyte chemoattractant protein (MCP)-1, interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α), and ameliorate the pathological hepatic damage of ALF. Furthermore, ACD pretreatment inhibited toll like receptor 4 (TLR4), nuclear factor kappa B (NF-κB), the mitogen-activated protein kinase (MAPK) and NOD-like receptor protein-3 (NLRP3) activation pathway. Moreover, our research showed that ACD could dramatically increase superoxide dismutase (SOD) and glutathione (GSH) production, and reduce COX-2, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS) and malondialdehyde (MDA) production through upregulating the expression of the anti-oxidative enzymes heme oxygenase-1 (HO-1) and quinone (NQO1), which were related to the induction of nuclear transcription factor 2 (Nrf2) nuclear translocation. These results indicated that ACD exhibited anti-inflammatory activity, which was associated with the inhibition of inflammatory mediator production via the downregulation of the NLRP3 inflammasome and TLR4-NF-κB/-MAPK signaling pathways, and the antioxidative effects of ACD were connected with GSH and SOD activation through upregulation of the Nrf2-mediated signaling pathways.

1. Introduction

Acute liver failure (ALF) is a malignant disease resulting from an outbreak of liver-based septicemia and rapid hepatocellular death [1]. There are many factors that cause liver damage, such as viral infections, drug and food additive abuse and alcohol consumption [2]. ALF has a poor prognosis and high mortality in the clinic, and remains a worldwide health problem [3]. Moreover, there are no effective treatment methods to completely cure ALF, except for liver transplantation, and only a few patients can obtain a useable liver because of the lack of donors [4]. Thus, finding an efficient drug to overcome ALF is essential. Combinations of lipopolysaccharide (LPS) and D-galactosamine (GalN) produce liver failure, which is often associated with clinical symptoms in viral hepatitis [5]. This model has been recognized as a potential research method to study infectious hepatitis caused by viruses. GalN is a kind of hepatotoxic agent that leads to depletion of the uridine phosphate pool as a result of UDP-GalN derivative formation. This depletion can inhibit mRNA and protein synthesis [6]. Moreover, macrophages and Kupffer cells play important roles in GalN-induced hepatotoxicity mediated by reactive oxygen species (ROS) production. LPS found in the outer membrane of gram-negative bacteria can induce the release of cytokines, resulting in a cellular stress response [7]. Currently, it is clear that LPS/GalN causes hepatic inflammatory responses and oxidative stress, which can bring about ALF.

Many previous studies have revealed that the inflammatory response and oxidative stress play crucial roles in the LPS/GalN-induced pathological process [8,9]. Nuclear erythroid 2-related factor 2 (Nrf2), an important transcription factor in the cap'n'collar basic leucine zipper family, can protect against the oxidative damage triggered by injury and inflammation through antioxidant gene regulation [10,11]. Many research found that the lack of an active Nrf2 signaling pathway results in severe inflammation-induced oxidative stress [12]. Under oxidative

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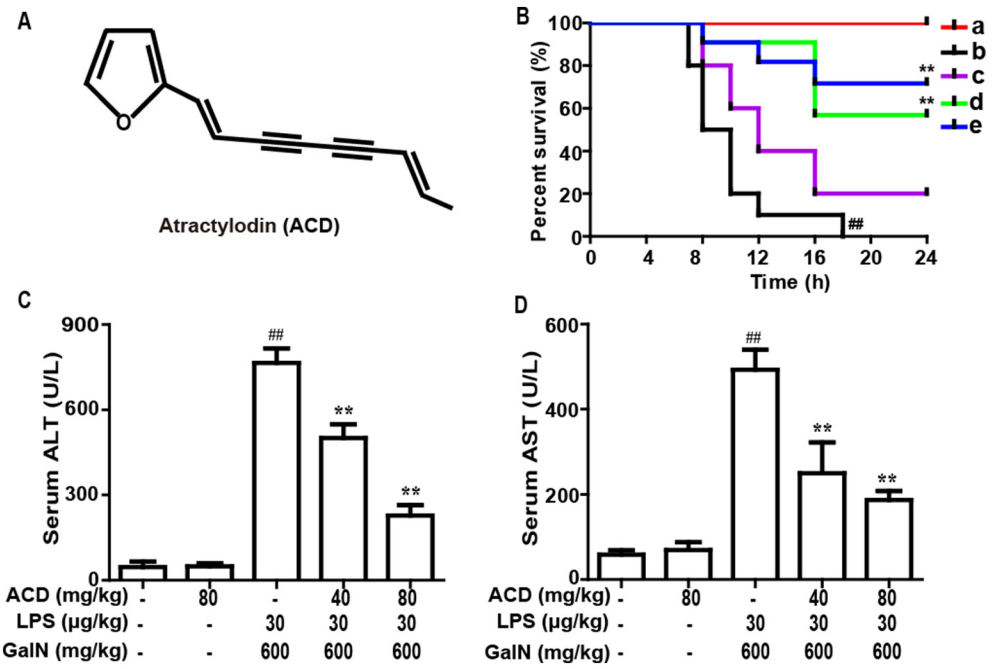

Fig. 1. Effect of ACD treatment on LPS/GalN-induced mortality as well as serum ALT and AST levels in mice. Mice were treated with different concentrations of ACD in presence or absence of LPS (30 μg/kg) and GalN (600 mg/kg). (A) The chemical structure of ACD. (B) Mice were pretreated with ACD (40 or 80 mg/kg) at twice every 12 h, and then LPS/GalN stimulation and determined the survival rate. (a) Control and ACD (80 mg/kg) group; (b) LPS/GalN treatment group; (c) LPS/GalN with ACD (20 mg/kg) group; (d) LPS/GalN with ACD (40 mg/kg) group; (e) LPS/GalN with ACD (80 mg/kg) group. The data is expressed as the percentage of surviving mice at each time point (n = 10/group). (C–D) After the ACD finally administered with 1 h, LPS (30 μg/kg) and GalN (600 mg/kg) were intraperitoneally injected for 6 h. Then, the serum levels of ALT and AST were measured. Values presented are expressed as the mean ± SEM (n = 5/group). $^{\#\#}p < 0.01$ vs. the control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the LPS/GalN group.

stress conditions, Nrf2 migrates to the nucleus from the cytoplasm, binds to the antioxidant response element (ARE) gene and enhances the transcription of ARE-responsive genes such as hemeoxygenase-1 (HO-1), NAD (P) H:quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase modifier subunit (GCLM), glutathione-S-transferases (GST), and glutamate-cysteine ligase catalytic subunit (GCLC) to mount strong antioxidant and cytoprotective responses [13,14]. In addition, in an APAP treatment experiment, compared to wild-type (WT) mice, Nrf2-null mice exhibited obviously increased liver damage [15]. These results demonstrate the importance of Nrf2-mediated regulation of cytokine-dependent hepatocyte apoptosis. In another study, researchers found that Nrf2 activation inhibited nuclear factor kappa B (NF-κB) expression which has direct relevance to inflammatory responses [16]. Numerous studies have revealed that Nrf2 is a promising drug target to protect against LPS/GalN-induced ALF [17,18]. However, whether atractylodin (ACD, Fig. 1A) attenuates LPS/GalN-induced liver failure by an Nrf2-mediated mechanism remains unclear. In other words, whether ACD can activate Nrf2 is a mystery. Therefore, exploring whether ACD exhibits positive effects on LPS/GalN-induced ALF has certain innovative and practical significance.

ACD is a product of an Asteraceae family member, extracted from the rhizome of *Atractylodes chinensis*, which is used for the treatment of rheumatic diseases, digestive disorders, night blindness, and influenza [19]. Recent studies have shown that ACD has anti-inflammatory activity. Chae and his colleagues found that ACD could inhibit interleukin-6 (IL-6) release by blocking NPM-ALK activation and MAPKs in HMC-1 cells [20]. In another experiment, investigators revealed that ACD ameliorated constipation and diarrhea by inhibiting NF-κB expression and the release of pro-inflammatory cytokines such as interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) [21]. Furthermore, ACD attenuates LPS-induced acute lung injury by inhibiting NOD-like receptor protein-3 (NLRP3) inflammasome and Toll like receptor 4 (TLR4) pathways [22]. In this study, ACD exhibited strong anti-inflammatory effects. Thus, our team aimed to evaluate whether ACD may protect against LPS/GalN-induced ALF and determine the underlying molecular mechanism.

2. Materials and methods

2.1. Materials

LPS (Escherichia colilipopolysaccharide, 055:B5), dimethylsulfoxide (DMSO) and GalN were obtained from Sigma-Aldrich Inc. (St. Louis, MO). ACD (purity > 98%) was purchased from Chengdu Pufei De Biotech Co., Ltd. The ROS, SOD, GSH, AST and ALT detection kits were purchased from Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). Kits of nuclear protein and cytoplasmic protein extraction as well as tissue lysis buffer were provided from Beyotime Biotechnology Corp., Ltd. (Nanjing, China). Pierce™ BCA protein assay kit was got from Thermo Scientific (NY, USA). Rabbit antibodies against iNOS, COX-2, NLRP3, TLR4, ASC, caspase-1, P-NF-κB, NF-κB, IκB, P-IκB, P-JNK, JNK, P-ERK, ERK, P-p38, p38 and mouse anti-IL-1β were offered by Cell signaling technology (MA, USA). Rabbit antibodies against Nrf2, NQO1, GCLC, GCLM, HO-1 and Keap1 were provided by Abcam. Mouse antibodies against Lamin B and β-actin were purchased from Proteintech Biotechnology (Wuhan China). All other chemicals were of analytical grade.

2.2. Animals and treatment

Male C57/BL6 mice aged 6 to 8 weeks and weighing approximately 18–22 g were purchased from Liaoning Changsheng Biotechnology (Liaoning, China). They were housed in sterile cages under normal temperature (24 ± 1 °C) and relative humidity (40%–70%) conditions with a 12-h light/dark cycle. Animal welfare and experimental procedures were carried out in accordance with the guide for the Care and Use of Laboratory Animals, which was published by the US National Institute of Health. This study was reviewed and approved by the Animal Welfare and Research Ethics Committee at Jilin University.

2.3. Experimental protocol

To evaluate the protective effects of pretreatment with ACD on LPS/GalN-induced ALF, mice were randomized into the following six groups: control group, LPS/GalN group, ACD (80 mg/kg) group, LPS/GalN with ACD (20 mg/kg), LPS/GalN with ACD (40 mg/kg) and LPS/GalN with ACD (80 mg/kg) group. The mice were intraperitoneally

(i.p.) injected with ACD twice (interval of 12 h) before the administration of LPS/GalN. After 1 h, mice were i.p. injected with LPS (30 μg/kg) and GalN (600 mg/kg) to induce ALF. The mice in the control group were given the same volume of PBS. Subsequently, the mice were observed the survival rates (n = 10/group) for 24 h or sacrificed (n = 5/group) at 5 h after LPS/GalN treatment to collect liver tissues and serum samples for biochemical assays.

2.4. Analysis of liver enzymes

All mice were anesthetized with ether, and then the serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by collecting blood retro-orbitally. The blood samples were kept stationary in a thermostatic water bath at 37 °C for 1 h. The serum was separated by centrifugation at 3000 rpm for 10 min. Subsequently, the ALT and AST levels of the mice were measured by test kits purchased from the Jiancheng Bioengineering Institute of Nanjing according to the instructions.

2.5. Enzyme-linked immunosorbent assays (ELISAs)

According to the previous method to extract serum, the serum was collected for measurement of TNF-α, IL-1β, IL-6, and MCP-1 secretion using mouse ELISA kits based on the manufacturer's instructions (BioLegend, Inc., CA, USA). The optical density of each well was measured at 450 nm.

2.6. Histopathological evaluation

After mice were sacrificed, fresh liver tissues samples were washed with saline, subsequently fixed in 10% neutral buffered formalin over 24 h, dehydrated, embedded in paraffin, and then cut into 5-μm-thick sections. The liver tissue sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) for hepatic pathological evaluation. All sections were evaluated for hepatic damage severity by a point-counting method using an ordinal scale. The score was graded according to a four-point scale from 0 to 3 as follows: 0, l, 2, and 3 represent no evidence of damage, moderate to severe injury with extensive nuclear pyknosis, loss of intercellular borders and severe necrosis with hemorrhage and neutrophil infiltration, respectively.

2.7. Biochemical analyses of liver tissue samples

A fresh liver tissue sample from each mouse was ground and immediately dissolved in extraction buffer to analyze the biochemical content according to the relevant manufacturer's instructions. ROS are reactive chemical species containing oxygen and include peroxides, superoxide, hydroxyl radicals, singlet oxygen and alpha-oxygen. During exposure to oxidative stress, the ROS content can rapidly increase, possibly leading to obvious damage to cell structures and even death. Dichlorofuorescin diacetate (DCFH-DA) is the most sensitive and widespread intracellular reactive oxygen detection probe. In the presence of ROS, DCFH is DCF, a material with strong green fluorescence, which cannot cross cell membranes. The amount of chemiluminescence was determined with an excitation wavelength of 500 ± 15 nm and an emission wavelength of 530 ± 20 nm. Superoxide dismutase (SOD) plays a vital role in the balance of oxidation and antioxidation. SOD can combine with WST-1 and produce a yellow water soluble formazan dye. The liver tissue samples were homogenized, and the SOD levels were tested by using colorimetry. GSH is a low-molecular-weight free radical scavenger that can clear free radicals, which are associated with oxidative damage. GSH content was determined at 405 nm by quantitative colorimetric analysis. Malondialdehyde (MDA) levels reflect the degree of lipid peroxidation in the body. MDA can indirectly reflect the severity of free radical attacks on cells. MDA content was determined at 532 nm by quantitative colorimetric analysis.

2.8. Separation of nuclear and cytosolic protein

The protein of cytoplasmic and the nuclear were extracted by using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Beijing, China), according to the manufacturer's instructions. All experimental procedures were carried out on the ice.

2.9. Western blot analysis

The liver tissue samples from mice were lysed in Radio Immunoprecipitation Assay (RIPA) buffer with 1 mM phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitors (Thermo Fisher scientific, USA) for 30 min at 4 °C. The protein concentrations were detected by using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), and 20 mg of protein was transferred onto a polyvinylidene fluoride (PVDF) membrane following separation on a 10% SDS-polyacrylamide gel. The membrane was blocked with 5% non-fat milk for 1 h and incubated overnight at 4 °C with a 1:1000 dilution of specific primary antibodies. On the second day, the membrane was washed with TBST three times, usually for 5 min, and incubated in a 1:5000 dilution of secondary antibodies conjugated to horseradish peroxidase at room temperature for 1 h. The membrane was washed 3 times, and visualized by a chemiluminescence (ECL) western blotting detection system. Band intensities were quantified using ImageJ gel analysis software.

2.10. Statistical analyses

The survival curve result was analyzed by Log-rank (Mantel-Cox) Test, and other statistical analysis was performed by the one-way ANOVA. All statistical calculations were carried out using the GraphPad Prism 6.0 software. All data were expressed as mean ± SEM. Statistical significance was accepted at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Effects of ACD on cell viability in HepG2 and on mortality in LPS/GalN-induced ALF in mice

In the present study, the effect of ACD on cell viability was tested by MTT assay. Our result showed that ACD at many concentrations (10–40 μM) was not toxic to HepG2 cells, whereas 80 μM ACD exhibited a mild cytotoxicity (Fig. S1). Next, to explore the effects of pretreatment with ACD on LPS/GalN-induced ALF, mice were i.p. injected with LPS (30 μg/kg) and GalN (600 mg/kg) mixed with different doses of ACD (40 or 80 mg/kg). As shown in Fig. 1B, LPS/GalN caused the mice to die successively within 24 h, and the survival rate was 100% (10/10). However, when the mice were pretreatment with ACD (40 or 80 mg/kg), the trend was obviously changed. The survival rate about 50% (5/10) and 70% (7/10) were detected, respectively. Meanwhile, post-treatment with ACD (80 mg/kg) also efficiently increased the survival rate up to 40% when compared with LPS/GalN group (Fig. S2). These results suggested that ACD effectively protected against the high fatality rate induced by LPS/GalN.

3.2. Effects of ACD on LPS/GalN-induced changes in the hepatic function index in mice

To further evaluate liver damage severity, serum AST and ALT levels were considered first. Thus, the mouse serum was collected for ALT and AST measurements. As illustrated in Fig. 1C–D, compared with the control group, ACD (80 mg/kg) had no obvious effect on the serum levels of ALT and AST. Only treatment of LPS/GalN dramatically augmented the serum AST and ALT levels, and it was interesting that ACD (40 or 80 mg/kg) suppressed these increases in a dose-dependent manner. The decreased enzyme levels indicate that ACD could improve

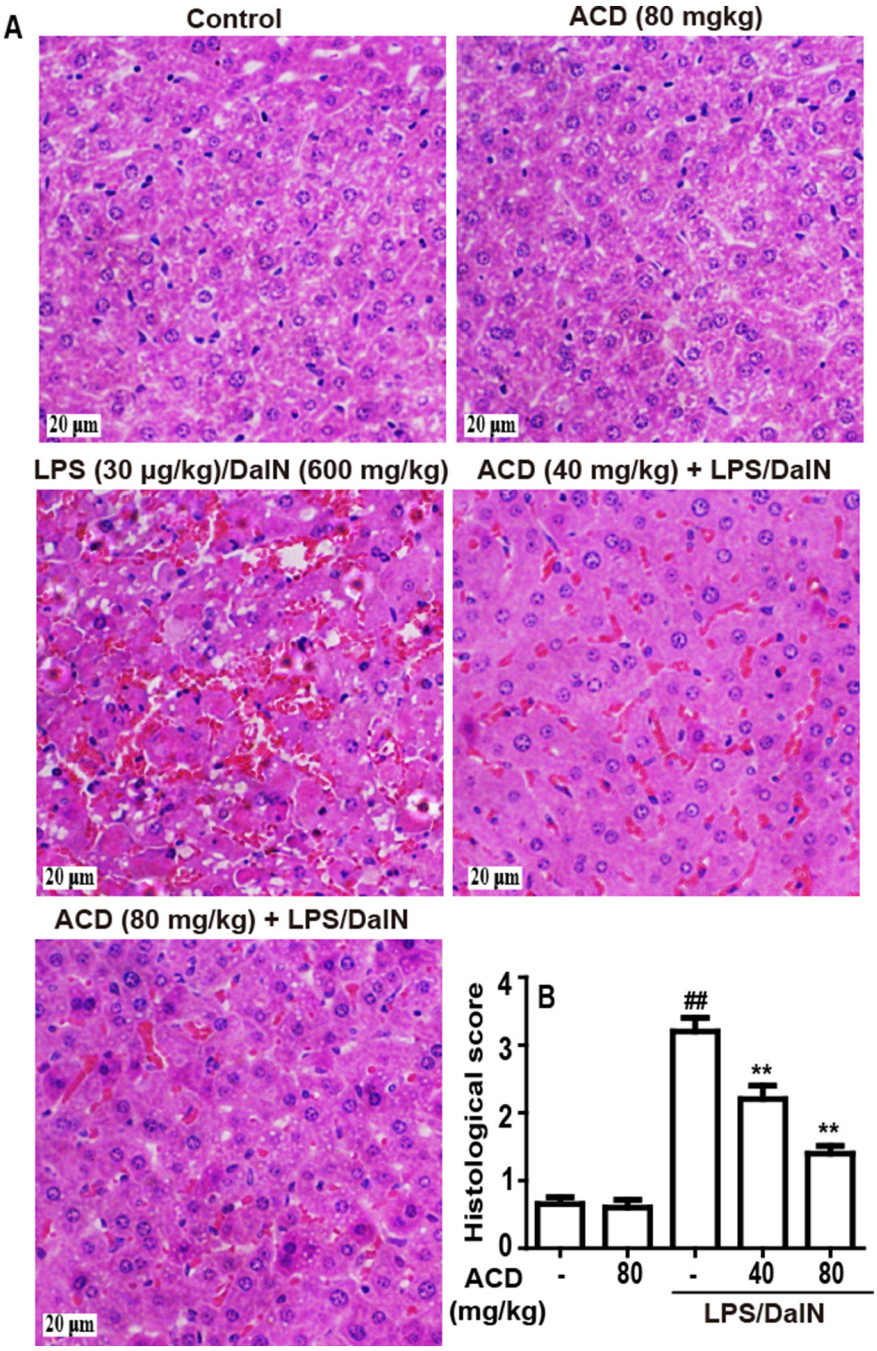

Fig. 2. Effects of ACD administration on hepatic histological evaluation. (A) Mice were given intraperitoneal injection of ACD (40 or 80 mg/kg) and LPS/GalN, and then 6 h later, hepatic change was evaluated (B) The severity of liver positively graded by using a four-point scale from 0 to 4, representing no injury, mild injury, moderate injury, severe injury and urgent injury respectively. Values presented are expressed as the mean ± SEM (n = 5/group). $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. LPS/GalN group; $^{\#\#}p < 0.01$ vs. control group.

LPS/GalN-induced liver damage. In contrast, LPS/GalN-induced liver damage was stable and successful in vivo experiments. Next, histological changes were examined to further assess the degree of liver tissue damage. As illustrated in Fig. 2A, LPS/GalN induced evident pathologic hepatic changes, as indicated by extensive hemorrhage, necrosis, neutrophil infiltration and liver cell cord derangement in the LPS/GalN group compared with the control group or the ACD only treatment group. However, treatment of ACD (40 or 80 mg/kg) markedly weakened the liver damage induced by LPS/GalN. As shown in Fig. 2B, the liver damage score was evaluated.

3.3. Effects of ACD treatment on LPS/GalN-induced oxidative stress in mice and HepG2 cells

To investigate whether ACD protected against hepatic damage by ameliorating oxidative stress, an index related to oxidation products was determined. The results showed that ACD (40 or 80 mg/kg) treatment reduced ROS and MDA production (Fig. 3A–B) and distinctly enhanced the levels of GSH and SOD (Fig. 3C–D), which play critical roles in protecting against LPS/GalN-induced oxidative stress. Meanwhile, we discovered that ACD evidently inhibited H_2O_2-stimulated ROS generation in HepG2 cells (Fig. S3). These evidences suggested that ACD possessed antioxidative stress capacity.

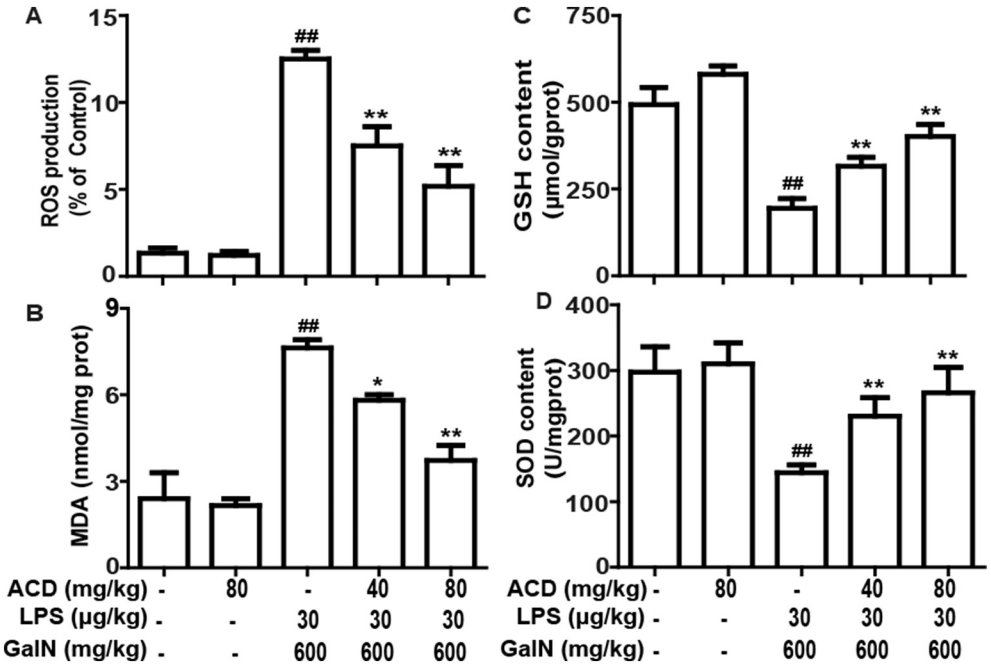

Fig. 3. Effects of ACD administration on LPS/GalN-induced ROS, MDA, SOD and GSH production in liver tissues. Mice were pretreated with ACD (40 or 80 mg/kg) in existence of LPS (30 μg/kg) and GalN (600 mg/kg). (A) ACD inhibited ROS production induced by LPS/GalN. ROS levels were measured by DCFDA fluorescence intensity. (B) ACD obviously inhibited MDA production of liver induced by LPS/GalN. (C) ACD significantly activated GSH production in liver. (D) ACD improved the release of SOD in liver. All biochemistry levels were assayed by using the Griess reaction. Values presented are expressed as the mean ± SEM (n = 5/group). $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ vs. the control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the LPS/GalN group.

3.4. Effects of ACD decrease inflammatory responses in LPS/GalN-induced ALF

ALF is closely associated with the release of pro-inflammatory cytokines, such as MCP-1, TNF-α, IL-1β and IL-6. Thus, the serum TNF-α, IL-1β, IL-6 and MCP-1 levels were determined by ELISA. As shown in Fig. 4A–D, compared with the control vehicle, ACD (80 mg/kg) had no effect on the serum levels of MCP-1, TNF-α, IL-1β and IL-6. However, compared with the control group, the LPS/GalN-treated group exhibited significantly increased pro-inflammatory cytokines levels. Compared with LPS/GalN alone, ACD (40 or 80 mg/kg) pretreatment distinctly reduced the release of pro-inflammatory cytokines compared to the LPS/GalN group in a dose-dependent manner. Furthermore, we examined the protein expression of iNOS and COX-2, which are closely connected with the development of the inflammatory response. As shown in Fig. 4E–G, ACD (40 or 80 mg/kg) ameliorated LPS/GalN-induced COX-2 and iNOS activity.

3.5. Effects of ACD treatment on LPS/GalN-induced TLR4-NF-κB and -MAPK activation in mice

TLR4, as the upstream of NF-κB and MAPK signaling pathway, is a typical inflammatory pathway. Thus, we next investigated the effects of ACD treatment on TLR4-NF-κB and -MAPK signaling pathways. As is shown in Fig. 5A and C–F, compared to the control group, LPS/GalN induced the expression of TLR4 protein and the phosphorylation of NF-κB and IκBα. ACD pretreatment reduced IκBα phosphorylation and subsequently activated the phosphorylation of NF-κB (p65), which allowed p65 translocate to the nucleus. To confirm this result, NF-κB expression was detected in the nucleus and cytoplasm. The results suggested that LPS/GalN improved the nuclear translocation rate of NF-κB. Nevertheless, ACD inhibited NF-κB translocation into the nucleus (Fig. 5B and G). As shown in Fig. 6A–D, treatment with LPS/GalN obviously activated MAPK phosphorylation. Moreover, ACD (40 or 80 mg/kg) inhibited the phosphorylation of MAPKs induced by LPS/GalN.

3.6. Effects of ACD treatment on LPS/GalN-induced NLRP3 inflammasome activation in mice

The NLRP3 inflammasome plays an important role in inflammatory responses. In the present study, the experimental results indicated that NLRP3 could be activated by LPS/GalN stimulation in the liver of the mice. Moreover, downstream cell signaling pathway molecules, including ASC, caspase-1 and IL-1β, also exhibited the same change. In other words, LPS/GalN elevated the protein expression of ASC, cleaved caspase-1 and mature IL-1β. However, ACD (40 or 80 mg/kg) inhibited the LPS/GalN-induced NLRP3, ASC, cleaved caspase-1 and mature IL-1β expression increases. In addition, the western blotting results revealed that the ACD-only (80 mg/kg) treatment could not lead to NLRP3 inflammasome activation (Fig. 7A–E).

3.7. Effect of ACD on the Nrf2-mediated signaling pathway in LPS/GalN-induced ALF mice

Previous studies have discovered that a large amount of ROS is released in LPS/GalN-induced ALF. ALF may be connected with oxidative stress. Nrf2, an antioxidant protein, usually protects against oxidative damage. Therefore, we investigated whether ACD could induce changes in Nrf2 and downstream molecule expression. Western blotting results revealed that ACD (40 and 80 mg/kg) elevated the expression of Nrf2, GCLC, GCLM, NQO1 and HO-1 (Fig. 8A and C–F), but did not exhibit an obvious change of Keap1 in LPS/GalN-induced ALF (Fig. S4A–B). In addition, ACD (80 mg/kg) obviously promoted Nrf2 translocation to the nucleus (Fig. 8B and G). Furthermore, we measured the effect ACD on the expression of Keap1 and Nrf2 protein using western blot analysis in HepG2 cells. As presented in Fig. S4C-D, ACD treatment could effectively reduce the expression of Keap1 protein and enhance the Nrf2 nuclear translocation in HepG2 cells.

4. Discussion

Liver failure is a life-threatening condition that demands urgent medical care [2]. Most often, liver failure develops gradually over many years. However, ALF is a rarer condition that occurs rapidly (in as little as 48 h) and this rapid onset means that patients may miss the chance to receive the right treatment. ALF, also known as fulminant hepatic

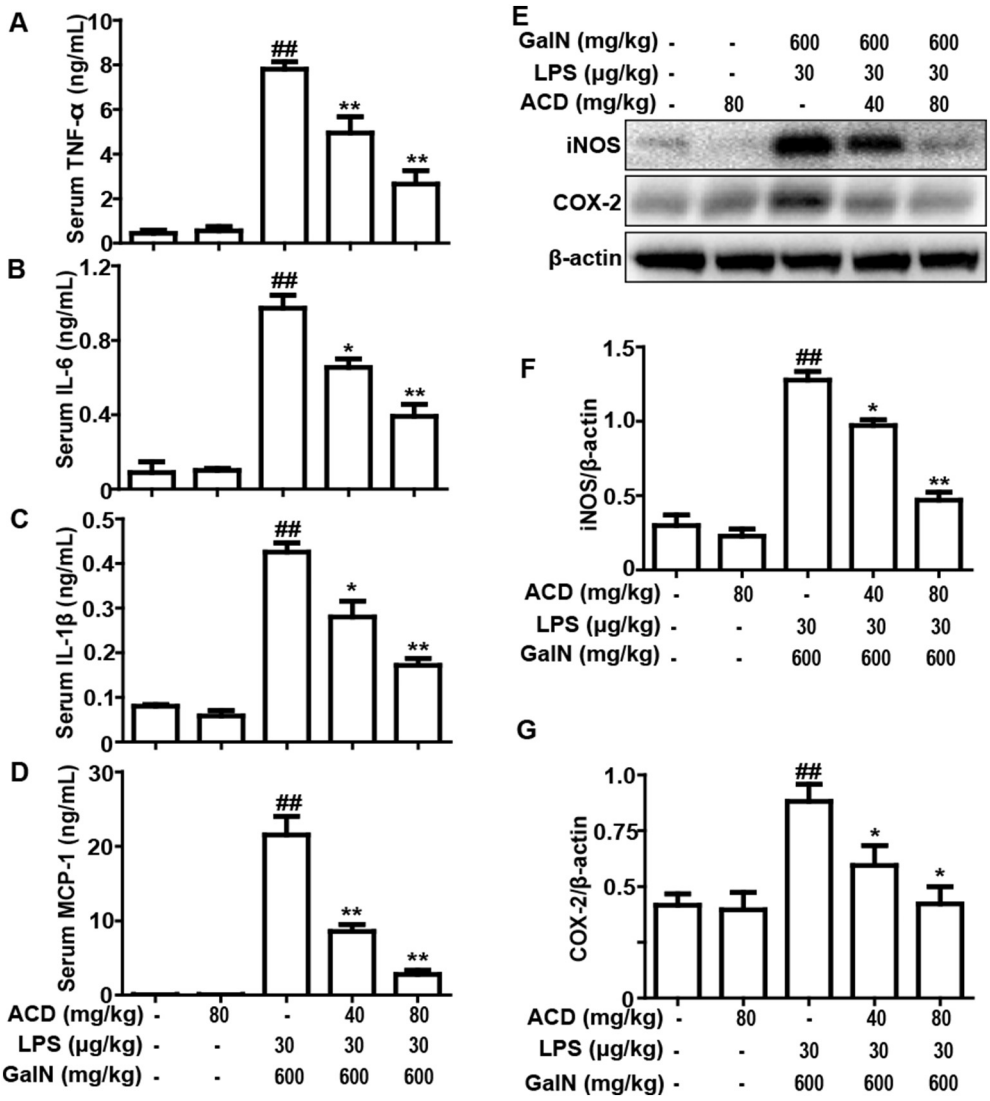

Fig. 4. Effects of ACD treatment on serum inflammatory cytokines and oxidative stress-related biochemistry. (A–D) ACD (40 or 80 mg/kg) treatment inhibits TNF-α, IL-1β, IL-6 and MCP-1 release in LPS/GalN-induced ALF. (E) Effects of ACD on iNOS and COX-2 expression were measured by western blot. (F–G) Quantification of relative protein expressions were performed by densitometric analysis. Values presented are expressed as the mean ± SEM (n = 5/group). $^{\#\#}p < 0.01$ vs. the control vehicle; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the LPS/GalN group.

failure, can cause serious bleeding and increases in intracranial pressure [4]. In many situations, it is very difficult to handle, and a liver transplant may be the only cure. Therefore, it is necessary to screen effective drugs for resolving liver damage. In our study, the protective effects of ACD on LPS/GalN-induced ALF in mice were examined. The results showed that ACD improved LPS/GalN-induced liver damage by upregulating Nrf2 signaling pathway and inhibiting TLR4 and NLRP3 inflammasome activation.

In a previous study, LPS/GalN caused high mortality in mice, which is a feature of ALF [23]. Thus, we investigated whether ACD protects against the high fatality rate of LPS/GalN-induced ALF. The results of the survival experiment indicated that ACD obviously reduced the number of dead mice. Next, ALT and AST were investigated as biochemical indicators of liver damage [24]. Our results showed that ACD significantly inhibited LPS/GalN-induced ALT and AST production. In addition, histological analysis of the liver found that ACD ameliorated LPS/GalN-induced acute hemorrhage and necrosis and restored the hepatic structure.

Oxidative stress plays a vital role in LPS/GalN-induced ALF [25]. To verify this hypothesis, we detected ROS, SOD, MDA and GSH production, which is associated with oxidative stress. ROS, an important inducer of oxidative stress, can lead to cell apoptosis and necrosis at medium and high concentrations [26]. ROS react with the cell membrane, and then produce MDA [27]. Thus, MDA was used to assess oxidative stress in this study. The results suggested that ACD significantly inhibited the elevation of ROS and MDA production and improved the production of antioxidative genes GSH and SOD. Inflammation is often accompanied by the development of oxidative stress [28–31]. Our results was consistent with previous research, LPS/GalN results in the release of inflammatory cytokines, including TNF-α, IL-1β, IL-6 and MCP-1, which leads to acute liver failure [32,33].

The evidence indicates that LPS/GalN-induced acute liver injury is closely associated with inflammatory responses. The ELISA results showed that ACD inhibited LPS/GalN-induced pro-inflammatory cytokine production. Moreover, COX-2 expression in normal tissue cells is extremely low; however, when inflammation is induced under stimulation conditions, the level of COX-2 will increase several fold [34,35]. iNOS, as a key inflammatory enzyme, plays an important role in involving in the pathogenesis of liver injury and in turn LPS/GalN could result in hepatic iNOS protein overexpression in mice [36,37]. In summary, LPS/GalN stimulated inflammatory responses and oxidative stress in liver tissues. However, ACD protected against LPS/GalN-

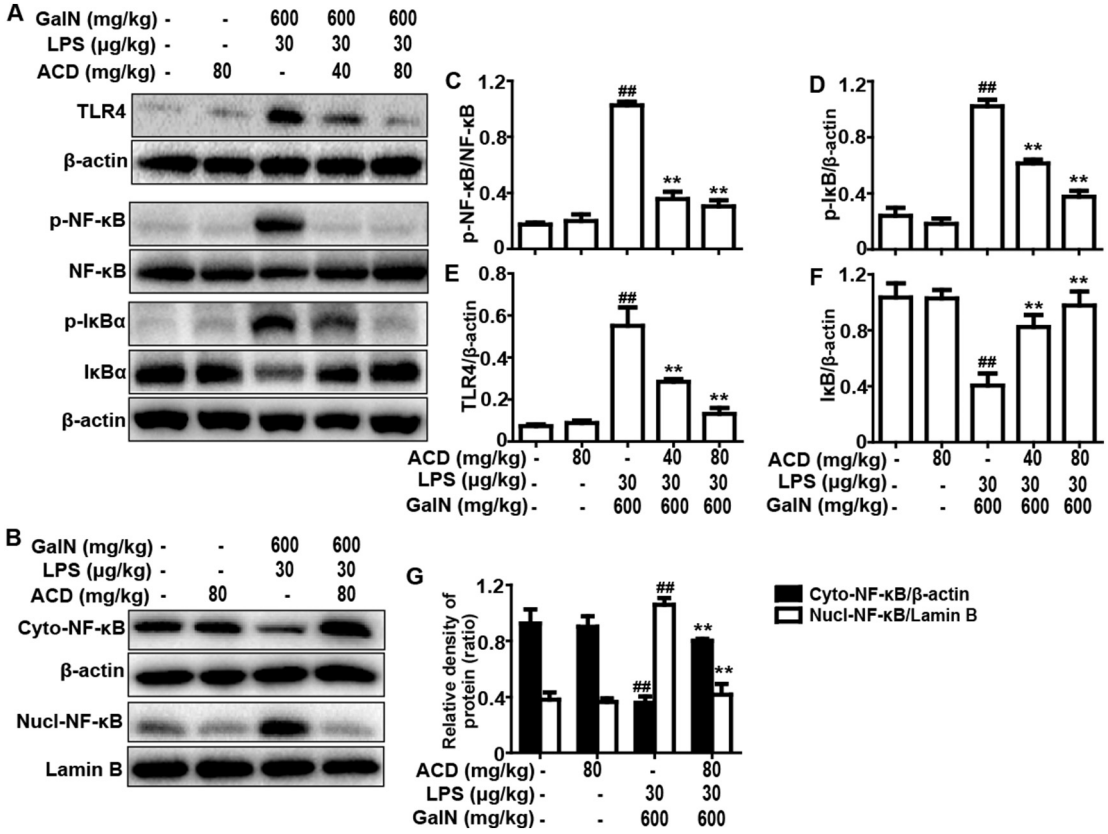

Fig. 5. Effects of ACD treatment on TLR4-NF-κB signaling pathway in LPS/GalN-induced ALF. All liver tissues of mice were harvested and the homogenate was analyzed by western blot. (A–B) Western blots of TLR4 and NF-κB are shown to study the role of ACD in ALF induced by LPS/GalN (C–G) Quantifications of TLR4, NF-κB, IκBα, P-IκBα, and P-NF-κB (p65) expression were normalized than that of b-actin. Typical data were obtained in three independent experiments, and one of the three representative experiments is shown. Values presented are expressed as the mean ± SEM (n = 5/group). $^{\#\#}p < 0.01$ vs. the control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the LPS/GalN group.

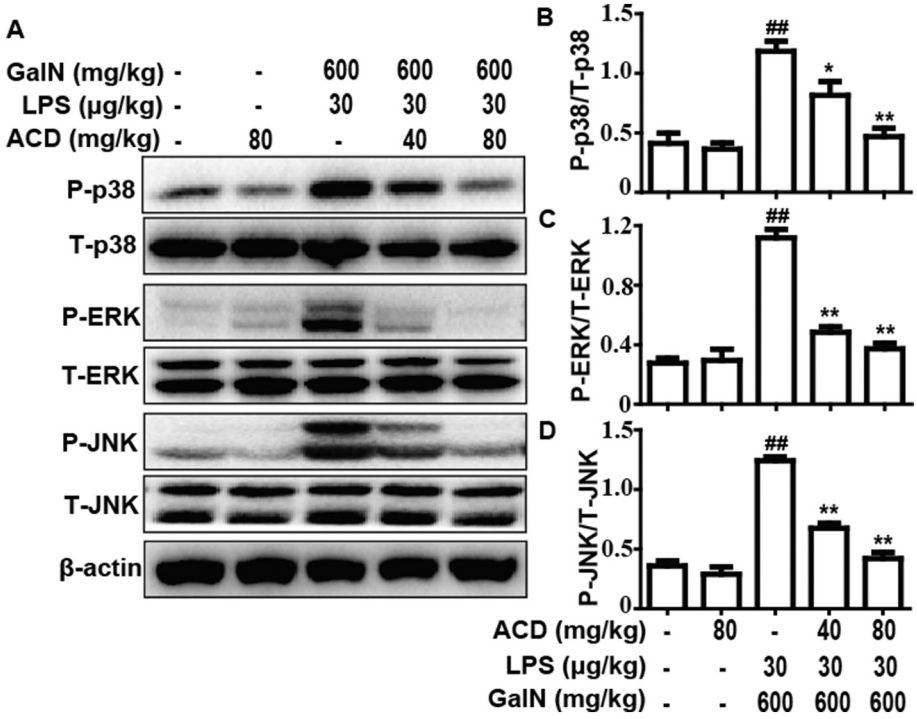

Fig. 6. Effects of ACD treatment on LPS/GalN-induced MAPK signaling pathways activation in mice. All liver tissues of mice were harvested and the homogenate was analyzed by western blot. (A) Western blots of MAPK are shown to study the role of ACD in ALF induced by LPS/GalN (B–D) Quantifications of the relative expression of P-JNK/T-JNK, P-ERK/T-ERK and P-p38/T-p38 was measured by densitometric analysis. Typical data were obtained in three independent experiments, and one of the three representative experiments is shown. Values presented are expressed as the mean ± SEM (n = 5/group). $^{\#\#}p < 0.01$ vs. the control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the LPS/GalN group.

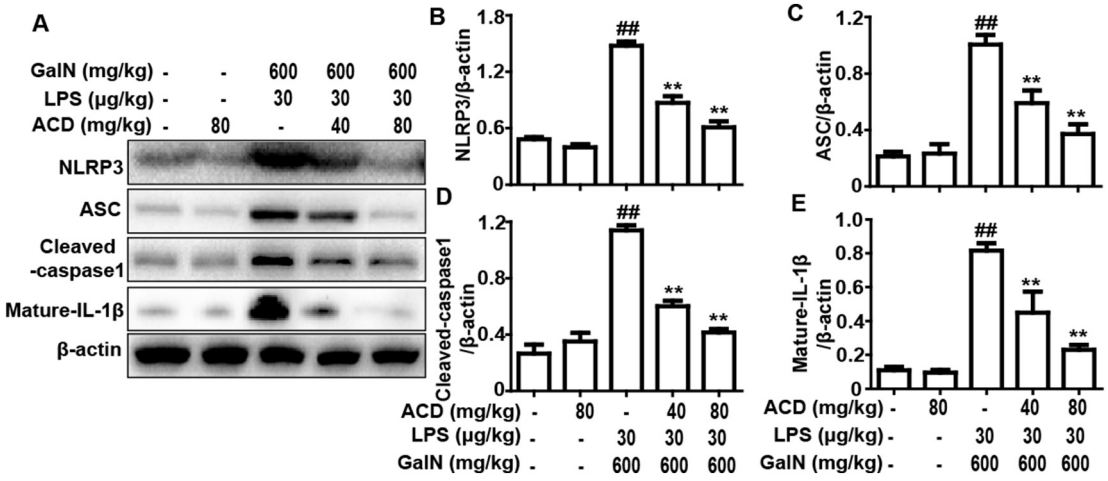

Fig. 7. Effects of ACD treatment on LPS/GalN-induced NLRP3 inflammasome activation in mice. Mice were injected with ACD (40 and 80 mg/kg) to mice, and then LPS (30 mg kg) and GalN (600 mg/kg) were administrated and liver tissue homogenates were analyzed by western blot. (A) Western blots of NLRP3 inflammasome are shown to study the role of ACD in ALF induced by LPS/GalN (B–E) Quantifications of the relative expression of NLRP3, ASC, caspase-1 and IL-1β were measured by densitometric analysis and β-actin was acted as an internal control. Typical data were obtained in three independent experiments, and one of the three representative experiments is shown. Values presented are expressed as the mean ± SEM (n = 5/group). $^{\#\#}p < 0.01$ vs. the control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the LPS/GalN group.

induced inflammatory responses and oxidative stress. To explore the molecular mechanism of ACD protection against LPS/GalN-induced inflammation and oxidative stress, our team screened some key molecular targets. Many studies have suggested that the NLRP3 inflammasome is closely associated with the pathogenesis of ALF. Once stimulated, NLRP3 recruits the ASC protein and recruits procaspase-1 to the inflammasome complex. Procaspase-1 is cleaved to generate caspase-1, which activates the inflammatory cytokine IL-1β [38,39]. To investigate the anti-inflammatory mechanism of ACD, the NLRP3 inflammasome was evaluated. The results of the western blot analysis revealed that ACD obviously inhibited LPS/GalN-induced NLRP3, ASC, caspase 1 and IL-1β activation. NF-κB is a nuclear transcription factor

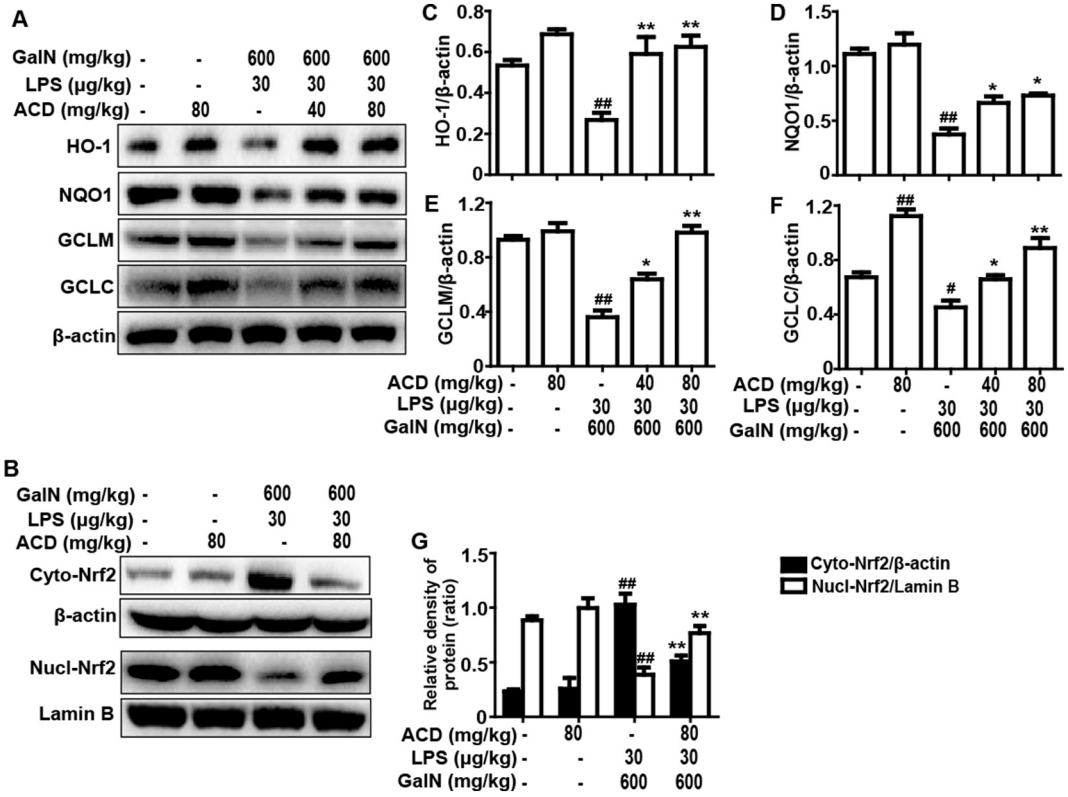

Fig. 8. Effect of ACD on Nrf2, NQO1 and HO-1 protein expression in LPS/GalN-induced ALF. All liver tissues of mice were harvested and the homogenate was analyzed by western blot. (A) The level of Nrf2, NQO1 and HO-1 were measured by western blot. (B) Nuclear and cytoplasmic levels of Nrf2 were checked by western blot. (C–G) Quantifications of the relative expression were performed by densitometric analysis. Typical data were obtained in three independent experiments, and one of the three representative experiments is shown. Values presented are expressed as the mean ± SEM (n = 5/group). $^{\#\#}p < 0.01$ vs. the control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the LPS/GalN group.

that plays a critical role in inflammation [40]. Activation of NF-κB is mediated by IκB phosphorylation and subsequent degradation by a kinase (IKK). NF-κB can then freely enter the nucleus and promote inflammatory cytokines production [41]. Thus, to explore the anti-inflammatory effect of ACD, the expression of IκBα and NF-κB was detected by western blotting. The results showed that ACD observably inhibited the phosphorylation of IκBα and NF-κB. Our results showed that LPS/GalN stimulated the phosphorylation of NF-κB and IκBα. In addition, numerous reports have suggested that MAPK family members, such as JNK, ERK and p38, play a critical role in the pro-inflammatory process [42]. The results revealed that the phosphorylation of JNK, ERK and p38 increased significantly after LPS/GalN administration, whereas ACD treatment obviously inhibited the MAPKs and NF-κB signaling pathways activation. Importantly, TLR4, as the upstream of NF-κB and MAPK signaling pathway, is a typical inflammatory pathway, which plays a vital role in LPS/GalN-induced ALF [43–45]. In our study, ACD treatment efficiently reduced the expression of TLR4 protein increased by LPS/GalN.

There is increasing evidences that Nrf2, a nuclear translation factor, is known for its excellent antioxidant capacity [46]. A previous study reported that Nobiletin protected against LPS/GalN-induced acute liver injury by activating Nrf2 signaling pathways [47]. In addition, Lv and his colleague found that corilagin exhibits antioxidant activities mediated by Nrf2 activation against acetaminophen-induced ALF [15]. Thus, Nrf2 may be an important target and play a vital role in ALF. In addition, the activation of Nrf2 leads to the induction of many cytoprotective proteins, including HO-1 and NQO1 [15]. Our experimental results suggested that ACD not only stimulated Nrf2 translocation into the nucleus but also activated the expression of the downstream genes HO-1, NQO1, GCLC, and GCLM in LPS/GalN-induced ALF.

5. Conclusion

In conclusion, our findings revealed that ACD treatment effectively prevented LPS/GalN-induced acute death in mice. Moreover, ACD inhibited the release of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and MCP-1, which are connected to blocking the NLRP3 inflammasome and TLR4-MAPK/-NF-κB signaling pathways. Furthermore, ACD effectively enhanced HO-1, NQO1, GCLC and GCLM expression, which was largely dependent on the upregulation of the Nrf2 signaling pathway. In the future, combination therapies utilizing the effects of antioxidative and anti-inflammatory agents may be good strategies to effectively alleviate ALF.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.04.005.

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