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Water-soluble andrographolide sulfonate exerts anti-sepsis action in mice through down-regulating p38 MAPK, STAT3 and NF-κB pathways

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ABSTRACT

Andrographolide is a prescribed drug used for preventing and treating the common cold, influenza, viral infections or allergies. However, its poor water solubility enormously limits its bioavailability. In the present study, we aimed at examining and comparing the effect of andrographolide sulfonate (trade name: Xi-Yan-Ping Injection), a water-soluble form made from andrographolide through sulfonating reaction, on the treatment of murine sepsis model induced by lipopolysaccharide (LPS). Pretreatment with andrographolide sulfonate significantly decreased the levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and transaminase activities in serum, attenuated liver and lung damage, and improved the survival of mice with experimental sepsis. Andrographolide sulfonate also remarkably reduced the expression levels of TNF-α, IL-1β, IL-6 and inducible nitric oxide synthase in the injured liver from septic mice. Moreover, andrographolide sulfonate time-dependently suppressed the activation of p38 mitogen-activated protein kinase (MAPK) but not extracellular signal-regulated kinase (ERK1/2) or c-Jun NH_2-terminal kinase (JNK). Furthermore, pretreatment with andrographolide sulfonate markedly inhibited the activation of p65 subunit of nuclear factor-κB (NF-κB) as well as signal transducers and activators of transcription 3 (STAT3) in the injured liver from mice with endotoxic shock. Notably, andrographolide sulfonate showed a much stronger alleviation of LPS-induced sepsis in mice compared with andrographolide. Taken together, these results reveal that andrographolide sulfonate ameliorates sepsis in mice through suppressing p38 MAPK, STAT3 and NF-κB pathways and suggest that andrographolide sulfonate has an advantage of andrographolide for the treatment of endotoxin shock.

1. Introduction

Endotoxic shock is a systemic response to serious infection which was caused by lipopolysaccharide (LPS)-producing gram-negative bacteria [1,2]. It has a poor prognosis when it is associated with organ dysfunction, hypoperfusion, or hypotension. LPS initiates a cascade of events, including release of inflammatory mediators such as TNF-α and IL-1β [3]. The large amounts of cytokines and nitric oxide produced are thought to contribute to LPS-induced multiple organ system failure and mortality [4,5].

Andrographolide, a natural diterpenoid, is the major constituent of *Andrographis paniculata*, which is a plant indigenous to Southeast Asian countries that has been used as an official herbal medicine in China for many years. It has been reported that andrographolide possesses antibacterial, anti-inflammatory, antiviral, hepatoprotective, neuronprotective, antiangiogenic and anti-tumor activities [6–15]. Now it is an approved drug mainly used for treatment of various ailments including respiratory infection, bacterial dysentery and fever in China. In spite of its great therapeutic interest, the low oral bioavailability of andrographolide continues to be a major challenge in developing formulations for clinical efficacy. Pharmacokinetic study revealed poor bioavailability of andrographolide after oral administration of *Andrographis paniculata* extract [16]. The T_{max} of andrographolide was 1.5-2 h and C_{max} obtained after a single oral dose of 20 mg/kg of the dry extract in rats and multiple dose of tablets containing the dry extract (20 mg/kg) in human volunteers were 393 and 660 ng/ml, respectively [16]. The absolute bioavailability of andrographolide was only 2.67% for its poor water solubility and effluxed by P-glycoprotein [17]. For its poor water solubility, tablet is the main dosage form of andrographolide. As we know, drugs for infectious disease need to work as soon as possible to eliminate the etiopathogenisis and symptoms. Many efforts have been made to improve the bioavailability for andrographolide, such as andrographolide pH-sensitive nanoparticles [18], herbosome of andrographolide with a naturally occurring phospholipids [19]. Andrographolide sulfonate is a water-soluble form made from andrographolide through sulfonating reaction, which is an andrographolide sulfate mixture and has been made into an injection for intramuscular injection and intravenous

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drip. In the present study, we examined the anti-sepsis effect of andrographolide sulfonate and compared it with oral administration of andrographolide. We found that andrographolide sulfonate showed a much stronger and quicker alleviation of LPS-induced sepsis in mice than andrographolide.

2. Materials and methods

2.1. Preparing and HPLC analysis of andrographolide sulfonate

Andrographolide sulfonate (trade name: Xi-Yan-Ping Injection) was commercially available and has been used clinically for many years. The active agent that was eventually synthesized is a mixture of several different compounds. The preparation process was described previously [20]. Briefly, 50 g andrographolide was vacuum dehydrated in 60 °C for 3 h, crushed and passed through 100-mesh screen. 25 ml sulfuric acid was added slowly after 50 ml of ethanol was added and mixed. The reaction continued with stirring for 72 h. 40 ml 95% ethanol were then added and pH was adjusted to 7.0 with 50% NaOH. The mixture was left still standing for 12 h after ethanol content adjusted to 85%. Lastly, the reaction mixture was filtrated and vacuum dehydrated. HPLC analysis was applied on a Waters 600 series HPLC system consisting of a Waters 600 pump, a 2487 UV detector, an online degasser and an LC Work Station equipped with Empower™ software. Andrographolide sulfonate was applied to Diamonsil C18 column (5 μm, 250 mm × 4.6 mm) and the gradient eluted programme (acetonitrile: potassium dihydrogen phosphate) was 0 min, 8:92; 10 min, 35:65; 60 min, 70:30; respectively. The potassium dihydrogen phosphate consisted of 1.361 g potassium dihydrogen phosphate and 1.0 g sodium 1-heptanesulfonate in 1000 ml water. Column temperature was set up at 25 °C and the flow rate was 1 ml/min. The mobile phase was degassed by ultrasonic and filtered though a 0.22 μm membrane filter. Before sample analysis, the column was stabilized with mobile phase for at least 30 min. The structures of andrographolide sulfonate were confirmed by comparison of MS, ^{1}H NMR and ^{12}C NMR. Fig. 1 shows the HPLC spectrum of andrographolide sulfonate. The total sulfonated forms of andrographolide are up to 95%.

2.2. Animals

BALB/c mice, 6–8 weeks of age, were purchased from Model Animal Center of Nanjing University (Nanjing, China). They were maintained with free access to pellet food and water in plastic cages at 21 ± 2 °C and kept on 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 (Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All efforts were made to reduce the number of animals used and to minimize animals' suffering.

2.3. Agents

LPS from *Escherichia coli* (0111:B4), and andrographolide were purchased from Sigma-Aldrich (St. Louis, MO). Andrographolide sulfonate (trade name: Xi-Yan-Ping Injection) was provided by Jiangxi Qingfeng Pharmaceutical Co., Ltd (Ganzhou, China). Kits for determining serum alanine transaminase (ALT), aspartate transaminase (AST) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ELISA kits for TNF-α and IL-1β were purchased from Dakewe Biotech Company (Shenzhen, China). Anti-phosphorylation of JNK (Thr183/Tyr185), anti-phosphorylation of ERK1/2 (Thr202/Tyr204), anti-phosphorylation of p38 (Thr180/Tyr182), anti-phosphorylation of p65, anti-phosphorylation of STAT3 were purchased from Cell Signaling Technology (Beverly, MA). Anti-GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

2.4. LPS-induced septic shock in mice

BALB/c mice were administered LPS at 5 mg/kg i.p. and survival was monitored continuously for 100 h ($n = 8$ per group). Andrographolide sulfonate (1, 3, or 10 mg/kg) was administered i.v. and andrographolide suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na) were given via i.g. administration immediately after LPS injection. Serum was collected at the indicated time points after LPS administration to measure the serum levels of ALT, AST and cytokines.

2.5. RT-PCR and real-time PCR

RT-PCR and real-time PCR were performed as described previously [21]. Briefly, RNA samples were treated by DNase and subjected to quantitative PCR, which was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green I dye (Biotium, Inc.), and threshold cycle numbers were obtained using ABI Prism 7000 SDS software version 1.0. Conditions for amplification were 1 cycle of 94 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. The primer sequences used in this study were as follows: *tnf-α* forward 5'-CGAGTGACAAGCCTGTAGCCC-3'; *tnf-α* reverse 5'-GTCTTTGAGATCCATGCCGTTG-3'; *il-1β* forward 5′-CTTCAGGCAGGCAGTATCACTC-3′; *il-1β* reverse 5′-TGCAGTTGTCTAATGGGAACGT-3′; *il-6* forward 5′-ACAACCACGGCCTTCCCTAC-3′; *il-6* reverse 5′-TCTCATTTCCACGATTTCCCAG-3′; *iNOS* forward

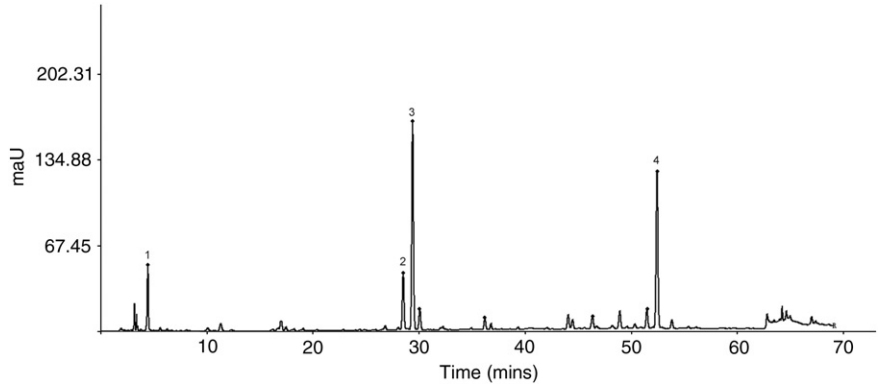

Fig. 1. HPLC chromatogram of andrographolide sulfonate. Peak 1: 17-hydro-9-dehydroandrographolide-3, 19-disodium sulfonate; Peak 2: 17-hydro-9-dehydroandrographolide-3-sodium sulfonate; Peak 3:17-hydro-9-dehydroandrographolide-19-sodium sulfonate; Peak 4: 17- hydro-9-dehydroandrographolide.

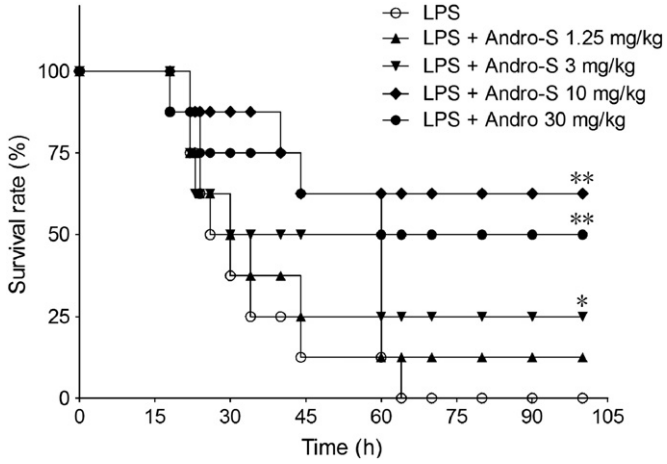

Fig. 2. Andrographolide sulfonate prevented septic shock and improved the survival rate in mice. Mice were i.v. injected with saline (LPS alone), i.v. with andrographolide sulfonate and i.g. with andrographolide after LPS challenge (5 mg/kg, i.p.). Then the survival rates were monitored continuously. *$P<0.05$ vs. LPS group. Andro: Andrographolide; Andro-S: Andrographolide sulfonate. There were eight mice in each group.

5'-ACATCGACCCGTCCACAGTAT-3'; *iNOS* reverse 5'-CAGAGGGGTAGGCTTGTCTC-3'; *β-actin* forward 5′-TGCTGTCCCTGTATGCCTCT-3′; *β-actin* reverse 5′-TTTGATGTCACGCACGATTT-3′.

2.6. Western blot

Protein from mouse liver was extracted in lysis buffer (30 mmol/L Tris, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The protein content of the supernatant was determined by BCA protein assay Kit (Pierce, Richford, IL). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membrane was blocked with 5% nonfat milk for 1–2 h at room temperature. The blocked membrane was incubated with the indicated primary antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase (HRP)-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

2.7. Statistical analysis

Data are expressed as mean ± SEM. ANOVA with post hoc two comparisons is used to evaluate the differences between various experimental and control groups. Mortality differences between groups were evaluated by the Kaplan–Meier method. *P* values less than 0.05 were considered significant.

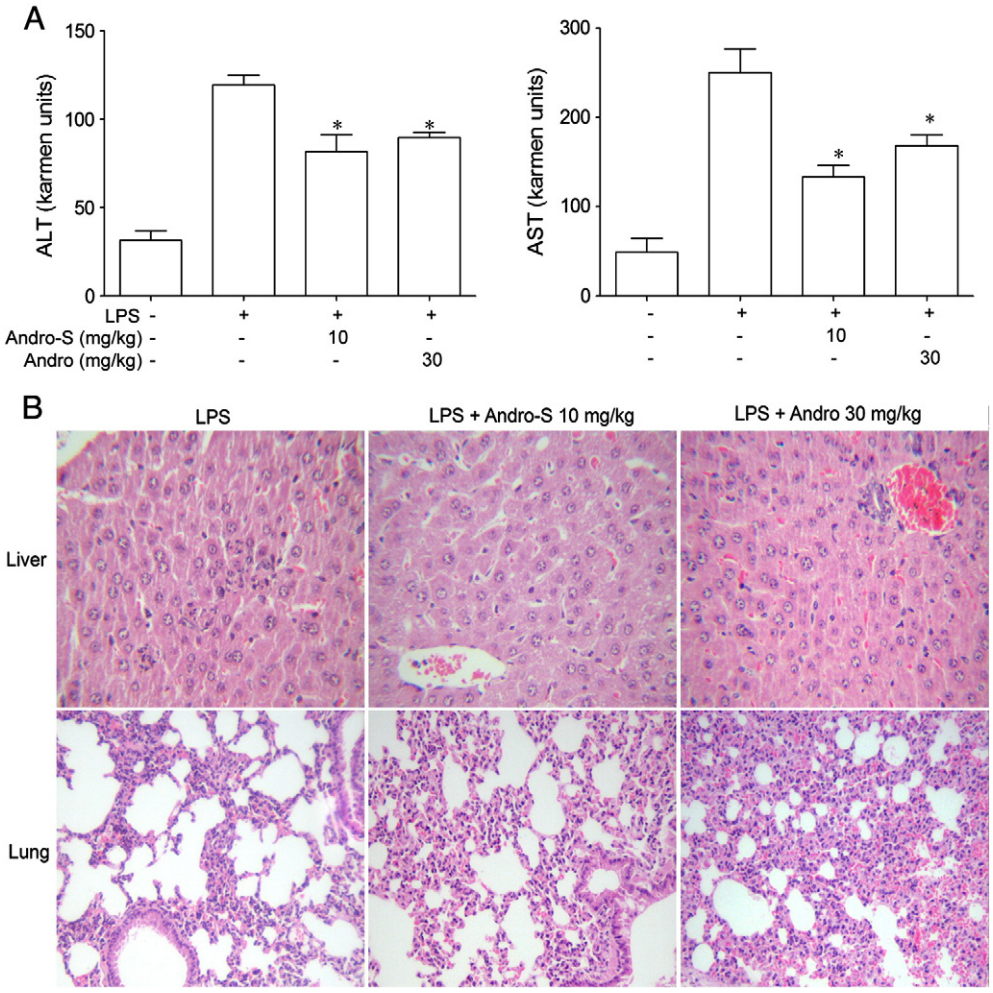

Fig. 3. Andrographolide sulfonate attenuated LPS-induced liver and lung inflammation. Serum levels of ALT and AST were measured 12 h after LPS injection (A) and photomicrographs of representative mouse lungs and livers with H&E staining were shown (B, original magnification ×100). Values were shown as the means ± SEM of eight mice. *$P<0.05$ vs. LPS group. Andro: Andrographolide; Andro-S: Andrographolide sulfonate.

3. Results

3.1. Andrographolide sulfonate ameliorated survival rate of mice with septic shock induced by LPS

Andrographolide sulfonate (trade name: Xi-Yan-Ping Injection) was commercial available and it contained more than 95% sulfonated forms of andrographolide (Fig. 1). In response to a lethal dose of LPS, mice in model group displayed decreased activity, piloerection, periocular discharge, and diarrhea. The survival rate of mice at 100 hours after LPS injection was only 12.5 % (Fig. 2). Notably, andrographolide sulfonate significantly increased the survival rate of mice in a dose-dependent manner. The survival rate of mice with the treatment of andrographolide sulfonate at the highest dose of 10 mg/kg (i.v.) was up to 62.5%, while that of andrographolide at the dose of 30 mg/kg (i.g.) was about 50%. These results suggest andrographolide sulfonate has an advantage of andrographolide for the amelioration of endotoxin shock in mice.

3.2. Andrographolide sulfonate attenuated lung and liver injury in mice with septic shock

LPS can cause multiple organ system failure. As shown in Fig. 3A, serum levels of ALT and AST were significantly elevated at 12 h after LPS injection. Against this, both andrographolide sulfonate and andrographolide treatment remarkably inhibited ALT and AST elevations. Comparing, the inhibitory rate of andrographolide (30 mg/kg, i.g.) on ALT or AST level was lower than that of andrographolide sulfonate (10 mg/kg, i.v.). The histological examination showed LPS treatment resulted in neutrophil infiltration, some extent of cell necrosis in liver and alveolar wall thickening, neutrophil infiltration in lung as shown in Fig. 3B. Against these changes, treatment with andrographolide sulfonate and andrographolide both markedly reduced the extent of tissue damage and neutrophil infiltration.

3.3. Andrographolide sulfonate inhibited serum levels of TNF-α and IL-1β in a time- and dose-dependent manner

Development of severe sepsis is thought to result from the overproduction of inflammatory cytokines such as TNF-α and IL-1β. As shown in Fig. 4A, serum TNF-α level peaked at 2 h after LPS challenge, and dropped to near basal level by 8 h. Against this, andrographolide sulfonate (10 mg/kg, i.v.) significantly reduced the peak level of TNF-α in mice with LPS-induced shock in a time- (Fig. 4A) and dose-dependent (Fig. 4C) manner. As to IL-1β, it elevated at 2 h, peaked later than after LPS challenge and dropped to near basal level by 8 h. (Fig. 4B). Against this, andrographolide sulfonate (10 mg/kg, i.v.) also markedly decreased IL-1β level in a time- (Fig. 4B) and dose-dependent (Fig. 4D) manner. It should be noted that the inhibitory effect of andrographolide (30 mg/kg, i.g.) on cytokine production was inferior to that of andrographolide sulfonate (10 mg/kg, i.v.).

3.4. Andrographolide sulfonate inhibited mRNA expressions of iNOS, IL-1β, TNF-α and IL-6 induced by LPS in the injured liver

Since andrographolide sulfonate significantly inhibited the serum levels of TNF-α and IL-1β, the mRNA expressions of various proinflammatory cytokines in the injured liver were examined. As shown in Fig. 5A, the mRNA expressions of iNOS, IL-1β, TNF-α and

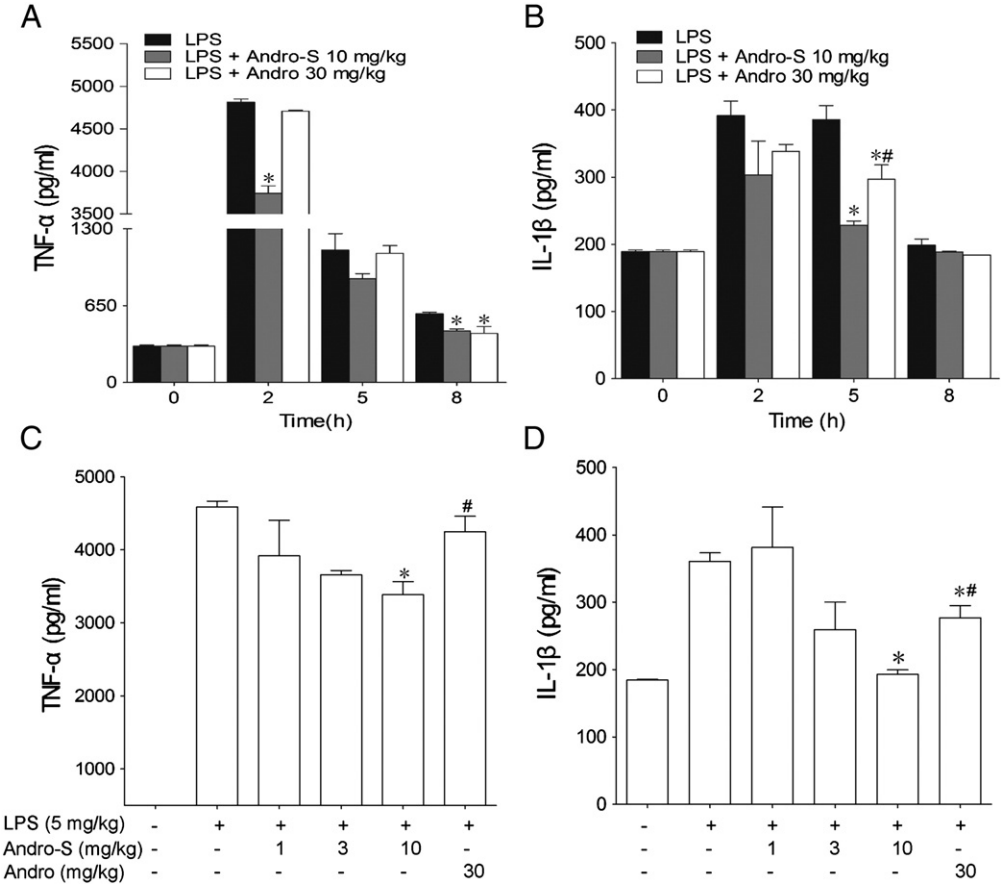

Fig. 4. Andrographolide sulfonate inhibited serum levels of TNF-α and IL-1β in mice with septic shock. Time course of TNF-α (A) and IL-1β (B) released into serum were measured by ELISA. Values were shown as the means ± SEM of three mice at each time point. $^{*}P<0.05$ vs. LPS group at the same time point; $^{\#}P<0.05$ vs. LPS + Andro-S group at the same time point. Dose-dependent inhibition on serum levels of TNF-α (C) and IL-1β (D) at 2 h after LPS injection. Values were shown as the means ± SEM of eight mice in each group. $^{*}P<0.05$ vs. LPS group; $^{\#}P<0.05$ vs. LPS + Andro-S group. Andro: Andrographolide; Andro-S: Andrographolide sulfonate.

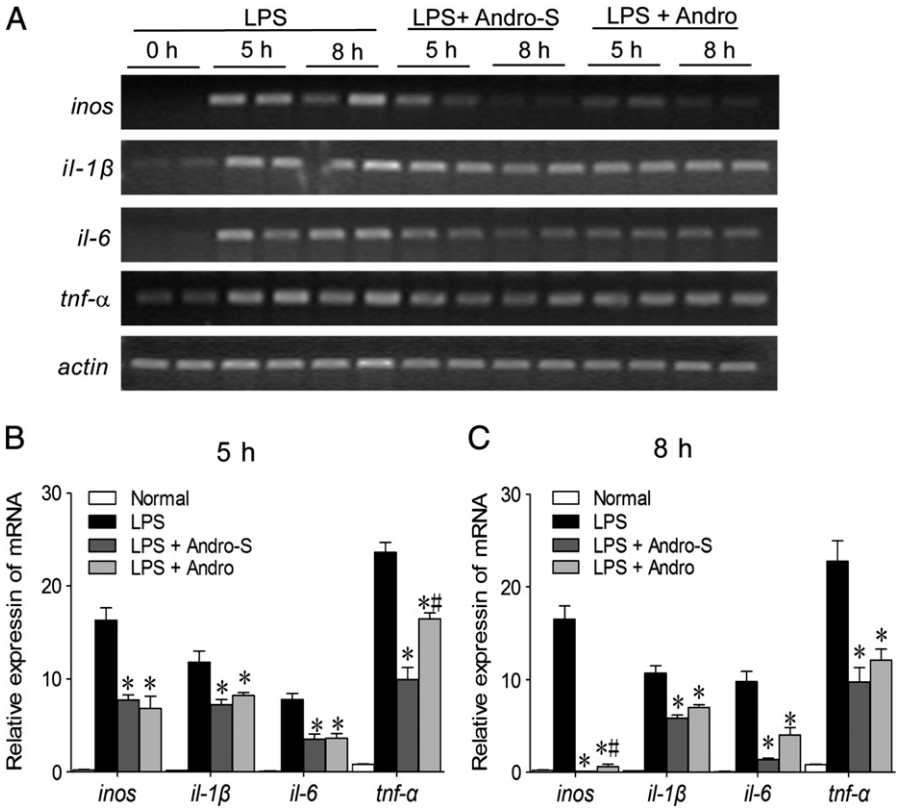

Fig. 5. Andrographolide sulfonate inhibited LPS-induced proinflammatory cytokine expression. RNA of liver tissue was extracted form the treated mice and mRNA levels of iNOS, TNF-α, IL-1β, IL-6 were examined by RT-PCR (A, representation of 3 mice) and real-time PCR (B and C, with 3 mice in each group). $^{*}P<0.05$ vs. LPS group; $^{\#}P<0.05$ vs. LPS + Andro-S group. Andro: Andrographolide; Andro-S: Andrographolide sulfonate.

IL-6 were remarkably increased after LPS challenge. Both andrographolide sulfonate and andrographolide significantly inhibited the elevated expression of cytokines both at 5 h (Fig. 5B) and 8 h (Fig. 5C) after LPS challenge.

3.5. Andrographolide sulfonate reduced LPS-induced activations of p38 MAPK, STAT3 and NF-κB signaling

For LPS signaling, activations of mitogen-activated protein kinases (MAPKs), STAT3 and NF-κB all play essential roles in transcriptional induction of various genes involved in inflammation, such as iNOS, COX-2, TNF-α, IL-1β and IL-6 [22]. Previously, andrographolide has been reported to attenuate inflammation by inhibition of NF-κB and STAT3 activation [23–25]. As shown in Fig. 6, LPS treatment caused different phosphorylations of p38, ERK, JNK, p65 as well as STAT3 in the injured liver from mice with septic shock. Both andrographolide sulfonate and andrographolide treatment markedly reduced the phosphorylations of p38, STAT3 and NF-κB but has little effect on activations of ERK and JNK.

4. Discussion

Andrographis paniculata has shown efficacy in the treatment of common colds or uncomplicated upper respiratory tract infection with doses of 1200 mg/day [26,27]. The drug significantly reduced the intensity of symptoms such as tiredness, sleeplessness, sore throat, earache, nasal secretion cough, and headache. Its clinical efficacy has been associated to the anti-inflammatory properties [28]. Despite these, low oral bioavailability of andrographolide has remained a major hurdle. The solubility of andrographolide in water at 37 °C is 207 μmol/L [29]. In our study, we prepared a water-soluble form of andrographolide by reacting with sulfuric acid. As shown in Fig. 1, there were more then ten components in the production and main four of them have been indicated on the graph. The concentration of the clinically used andrographolide sulfonate injection is nearly 52300 μmol/L. In addition, the injection of andrographolide sulfonate can also avoid the effluxes by P-glycoprotein in the intestine [17].

In order to compare the effect of andrographolide sulfonate and andrographolide, the mice sepsis model was chosen. The present study showed that andrographolide sulfonate has a quick effect and is better on alleviating endotoxic shock via intravenous injection verses andrographolide by intragastric administration. First, andrographolide sulfonate significantly decreased the mortality induced by a lethal dose of LPS and prolonged the survival time dose dependently at a range from 1 to 10 mg/kg in mice. The survival rate of 10 mg/kg andrographolide sulfonate (i.v.) was higher than 30 mg/kg andrographolide (i.g.). Second, andrographolide sulfonate decreased the level of TNF-α and IL-1β. In addition, andrographolide sulfonate (10 mg/kg) significantly reduced the peak level of TNF-α 2 h after LPS injection while andrographolide did not manifest significant inhibition effect till 8 h after treatment. Third, both andrographolide sulfonate and andrographolide prevented organ damage induced by LPS, as exemplified by results in the liver and lung.

Inflammatory stimuli such as LPS led to the activation of mitogen-activated protein kinases (MAPKs) the transcription factor NF-κB, which mediated the expression of several pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 [30], as well as other inflammatory mediators, including nitric oxide (NO) and PGE2, which are synthesized by inducible nitric oxide synthase (iNOS) and cyclooxygenase 2. The anti-inflammatory activity of andrographolide has been observed both in vitro and in vivo. Many previous studies showed that andrographolide exerted anti-inflammatory activity by inhibiting activation of NF-κB via different pathways. For example,

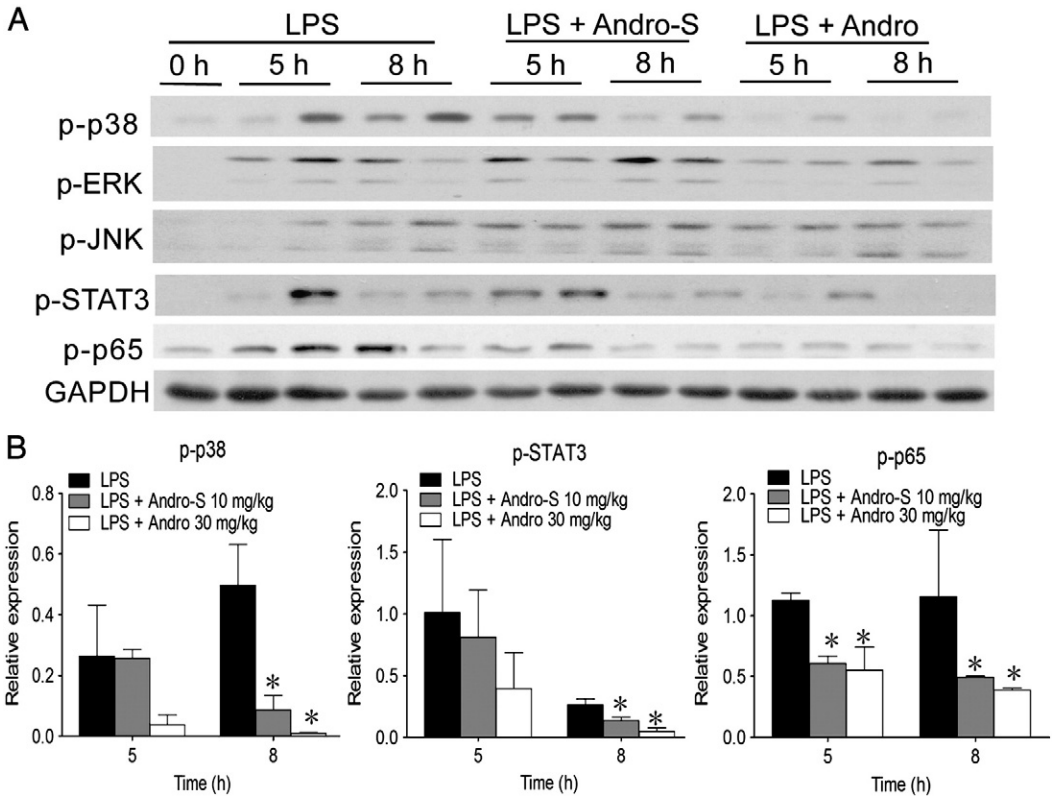

Fig. 6. Andrographolide sulfonate reduced LPS-induced activations of p38 MAPK, STAT3 and NF-κB. (A) Liver tissue protein was extracted form the treated mice and protein levels of p-p38, p-ERK, p-JNK, p-p65 and p-STAT3 were examined by Western blot. (B) The bands were measured and quantified by ImageJ analysis and values are shown as mean ± SEM from three mice. *$P<0.05$ vs. LPS group. Andro: Andrographolide; Andro-S: Andrographolide sulfonate.

andrographolide formed a covalent adduct with reduced cysteine 62 of p50, thus blocking the binding of NF-κB oligonucleotide to nuclear proteins [25]. In HL-60 cells, andrographolide inhibited NF-κB binding to DNA and thus reduced the expression of pro-inflammatory proteins [31]. And in vascular smooth muscle cells, andrographolide enhanced the dephosphorylation of NF-κB p65 subunit at Ser536 through activation of protein phosphatase 2A [23]. Our study showed that andrographolide sulfonate can inhibit phosphorylation of NF-κB and p38 as same as andrographolide. STAT3 is another important transcription factor for production of pro-inflammatory cytokines such as IL-1β and IL-6 which play a major role in inflammatory disease [32]. Our results here demonstrated that both andrographolide sulfonate and andrographolide can attenuate STAT3 activation, which is in agreement with previous reports [33,34].

Taken together, these data suggest that the anti-inflammatory effect of andrographolide sulfonate can oppose LPS-mediated cytokines production by negative regulation involving p38 MAPK, STAT3 and NF-κB activation and serves to control and resolve inflammation quicker and better than andrographolide.

Conflict interest statement

The authors have declared that no competing interests exist.

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