Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

International Immunopharmacology 16 (2013) 139–147

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Prime-O-glucosylcimifugin attenuates lipopolysaccharide-induced acute lung injury in mice

Na Chen [a,1], Qianchao Wu [a,1], Gefu Chi [b], Lanan Wassy Soromou [c], Jinli Hou [d], Yanhong Deng [a], Haihua Feng [a,*]

[a] College of Animal Science and Veterinary Medicine, Jilin University, Changchun 130062, PR China
[b] Department of Outpatient Clinic, the Affiliated Hospital of Inner Mongolia University for the Nationalities, Tongliao 028000, PR China
[c] Département de Médecine Vétérinaire, Institut Supérieur des Sciences et de Médecine Vétérinaire (ISSMV) de Dalaba, Guinée
[d] Key Laboratory of Zoonosis, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, PR China

ARTICLE INFO

Article history:
Received 26 January 2013
Received in revised form 7 April 2013
Accepted 11 April 2013
Available online 24 April 2013

Keywords:
Prime-O-glucosylcimifugin
Lipopolysaccharide
Acute lung injury

ABSTRACT

Prime-O-glucosylcimifugin is an active chromone isolated from *Saposhnikovia* root which has been reported to have various activities, such as anti-convulsant, anticancer, anti-inflammatory properties. The purpose of this study was to evaluate the effect of prime-O-glucosylcimifugin on acute lung injury (ALI) induced by lipopolysaccharide in mice. BALB/c mice received intraperitoneal injection of Prime-O-glucosylcimifugin 1 h before intranasal instillation (i.n.) of lipopolysaccharide (LPS). Concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and interleukin (IL)-6 in bronchoalveolar lavage fluid (BALF) were measured by enzyme-linked immunosorbent assay (ELISA). Pulmonary histological changes were evaluated by hematoxylin–eosin, myeloperoxidase (MPO) activity in the lung tissue and lung wet/dry weight ratios were observed. Furthermore, the mitogen-activated protein kinases (MAPK) signaling pathway activation and the phosphorylation of IκBα protein were determined by Western blot analysis. Prime-O-glucosylcimifugin showed promising anti-inflammatory effect by inhibiting the activation of MAPK and NF-κB signaling pathway.

1. Introduction

The acute respiratory distress syndrome (ARDS), a clinically important complication of severe acute lung injury (ALI) in humans, highly associated with sepsis pneumonias and severe acute respiratory syndrome (SARS) is a significant cause of morbidity and mortality in critically ill patients [1–3]. Inflammatory stimuli from microbial pathogens, such as endotoxin (lipopolysaccharide [LPS]), are well recognized for their ability to induce pulmonary inflammation, and experimental administration of LPS, has been used to induce pulmonary inflammation in animal models of ALI [4–6]. The development of an ALI model by way of i.n. LPS instillation is well suited for preliminary pharmacological studies of new drugs or other therapeutic agents because i.n. instillation of LPS into mice can produce a controlled ALI without causing systemic inflammation and multi-organ failure [4,7]. LPS-induced ALI is considered a neutrophil-dependent ALI that contributes to local recruitment and activation of neutrophils [8]; the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6; and the formation of reactive oxygen and nitrogen species [9–11]. Neutrophil recruitment in the lungs is regarded as a histological hallmark in the progression of ALI [12]. Several candidate therapy strategies such as fluid management, surfactants, glucocorticoids, and stem cells have been applied to treat acute lung injury and acute respiratory distress syndrome in the last decade [13,14]. However, the mortality resulting from these conditions remains high [15].

Fangfeng, the root of *Saposhnikovia divaricata* (Turcz) Schischk, is widely applied for headache, febrility, vertigo and arthralgia as an important member of traditional Chinese medicines (TCMs). It has been proved that there are numerous pharmacologic effects of the extract from Fangfeng, such as suppression of adjuvant arthritis, inhibitory effects on the peptic ulcers and analgesic, anti-convulsant, anticancer, anti-inflammatory and anticoagulant activities, etc in modern pharmacological experiments [16]. Abundant compounds were isolated from it, such as chromones, conmarins, and polyacetylenes [17,18]. Prime-O-glucosylcimifugin is a major active chromone isolated from Fangfeng. In recent years, it has been identified that chromones are the main active components which contribute most to its pharmacological efficacy [19], but so far, the anti-inflammatory effects of Prime-O-glucosylcimifugin on ALI has not yet been studied. Therefore, with a mouse model of acute lung inflammation, the present study was undertaken to examine the effect of Prime-O-glucosylcimifugin on acute lung injury induced by intranasal instillation of LPS in BALB/c mice and investigate its possible mechanisms.

* Corresponding author. Tel.: +86 431 8783 6161; fax: +86 431 8783 6160.
E-mail address: fhh70@163.com (H. Feng).
[1] These authors contributed equally to this work.

1567-5769/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.intimp.2013.04.014

2. Materials and methods

2.1. Reagents

Prime-O-glucosylcimifugin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Jilin, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin for cell culture were purchased from Invitrogen-Gibco (Grand Island, NY, USA). 3-(4, 5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and lipopolysaccharide (LPS) (*Escherichia coli* 055:B5) were purchased from Sigma Chemical Co. (San Diego, CA, USA). Rabbit polyclonal anti-p44 ERK, mouse monoclonal phospho-specific p42-p44 ERK antibodies, rabbit polyclonal anti-p54 JNK, mouse monoclonal phospho-specific p46-p54 JNK antibodies, rabbit polyclonal anti-p38, mouse monoclonal phospho-specific p38 antibodies, rabbit mAb IκBα and mouse mAb phospho-IκBα were purchased from Cell Signaling Technology Inc. (Beverly, MA). HRP-conjugated goat anti-rabbit and goat–mouse antibodies were provided by GE Healthcare (Buckinghamshire, UK). Mouse TNF-α, IL-6 and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend (CA, USA). The myeloperoxidase (MPO) determination kit was purchased from Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu Province, China). All other chemicals were of reagent grade.

2.2. Animals

BALB/c male mice, 8 weeks old and weighing approximately 18 to 20 g, were purchased from the Center of Experimental Animals of Baiqiuen Medical College of Jilin University (Jilin, China), and maintained at an animal facility under pathogen free conditions. The mice were fed a standard diet and water ad libitum and housed in microisolator cages under standard conditions (temperature: 24 ± 1 °C, relative humidity: 40%–80%). The mice were allowed to adapt themselves to their environment for 2–3 days before experimentation. All animal experiments were performed in accordance with the National Institutes of Health (NIH) guide for the Care and Use of Laboratory Animals and approved by the Jilin University animal administration committee.

2.3. In vitro study

2.3.1. Cell culture and sample treatment

The RAW 264.7 mouse macrophage cell line was obtained from the China Cell Line Bank (Beijing, China). Cells were cultured in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 3 mM glutamine and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin)) at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. Cells were treated with various concentrations of Prime-O-glucosylcimifugin for 1 h followed by stimulation with LPS (1 mg/L).

2.3.2. MTT assay for cell viability

Cytotoxicity studies induced by Prime-O-glucosylcimifugin were evaluated by the MTT assay. RAW 264.7 macrophages were seeded in 96-well plates at a density of 4×10^5 cells/mL in complete medium and incubated for 1 h (100 μL/well). Then the cells were treated with different concentrations of Prime-O-glucosylcimifugin (0–100 μg/mL, 50 μL/well) for 1 h, followed by stimulation with LPS (1 mg/L, 50 μL/well) for 18 h. After 18 h, 10 μL MTT (5 g/L) was added to each well and the cells were further incubated for 4 h. The supernatant was removed and the cells were lysed with 150 μL/well DMSO. The optical density was measured at 570 nm on a microplate reader.

2.3.3. Measurement of cytokine production

RAW 264.7 cells were seeded in 24-well plates (4×10^5 cells/mL) and treated with 12.5, 25 or 50 μg/mL of Prime-O-glucosylcimifugin for 1 h prior to stimulation of 1 mg/L LPS for 24 h in a 37 °C, 5% CO_2 incubator. Cell-free supernatants were collected and assayed. The concentrations of TNF-α, IL-1β and IL-6 in the supernatants of RAW 264.7 cell cultures were measured by using an ELISA kit, according to the manufacturer's instructions (BioLegend, Inc., Camino Santa Fe, Suite E, San Diego, CA, USA).

2.3.4. Western blot analysis

RAW 264.7 cells (4×10^5 cells/mL) plated onto 6-well plates were incubated for 24 h and treated with 12.5, 25 or 50 μg/mL of Prime-O-glucosylcimifugin for 1 h and then stimulated with 1 mg/L of LPS for 30 min. The cells were collected and washed three times with ice-cold PBS. The cells were treated with a cell lysis buffer [50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.6% NP-40, 1 mM Na3VO4, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 mM p-nitrophenyl phosphate, and 1:25 Complete Mini Protease Inhibitor cocktail (Boehringer, Mannheim, Germany)] and kept on ice for 30 min. The cell lysates were centrifuged (12,000 *g* at 4 °C) for 5 min to obtain a cytosolic fraction. The protein concentration was determined by BCA protein assay kit (Beyotime, Haimen, China). Aliquots of the lysates were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The blots were blocked with 5% (w/v) non-fat dry milk for 2 h at 37 °C, followed by incubation with specific primary antibody at 4 °C overnight. Blots were washed with Tween 20/Tris-buffered saline [TTBS, 20 mM Tris–HCl buffer, pH 7.6, containing 137 mM NaCl and 0.05% (vol/vol) Tween 20] and incubated with a peroxidase-conjugated secondary antibody for 1 h. Blots were again washed with TTBS and the immunoactive proteins were detected using ECL plus (Thermo, USA).

2.4. In vivo study

2.4.1. Establishment of LPS-induced ALI model

The mice were randomly divided into five groups: Control group; LPS group; LPS + Prime-O-glucosylcimifugin (2.5, 5 or 10 mg/kg bodyweight). Prime-O-glucosylcimifugin was given intraperitoneally. One hour later, LPS group and LPS + Prime-O-glucosylcimifugin group mice were given 50 μL LPS intranasally (i.n) (200 mg/L) to induce acute lung injury. Control mice were given 50 μL PBS intranasally (i.n) without LPS.

2.4.2. Collecting BALF and cytokine assays

All the mice were alive after 7 h LPS stimulation. Collection of BALF was performed three times through a tracheal cannula with 0.5 mL of autoclaved PBS, instilled up to a total volume of 1.3 mL. BALF was centrifuged (4 °C, 3000 rpm, 10 min) to pellet the cells. The concentrations of TNF-α, IL-1β and IL-6 in the BALF were measured using sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the protocol recommended by the manufacturer.

2.4.3. Inflammatory cell counts of BALF

The cell pellets were resuspended in PBS, and the total cell number was counted using a standard hemocytometer. Differences in cell numbers were examined by counting on a smear prepared by Wright–Giemsa staining.

2.4.4. Lung wet-to-dry weight (W/D) ratio

Seven hours after intranasal instillation of LPS, the mouse lungs were excised, and immediately weighed to obtain the wet weight. The dry weight was determined after heating the lungs at 80 °C for 48 h. The W/D ratio was calculated by dividing the wet weight by the dry weight.

2.4.5. Pulmonary myeloperoxidase activity in ALI mice

MPO activity, which reflects the parenchymal infiltration of neutrophils and macrophages, was measured as described previously

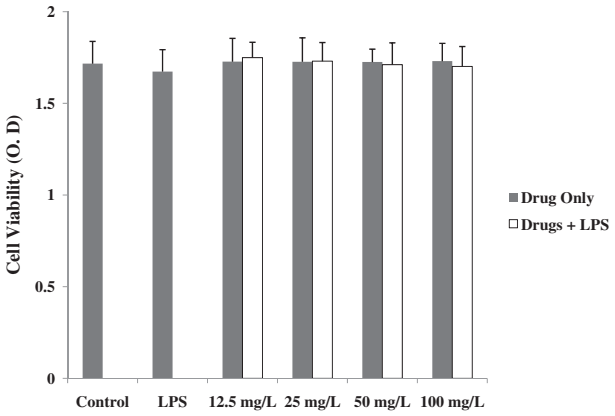

Fig. 1. Effect of Prime-O-glucosylcimifugin on macrophage toxicity. Cells were cultured with Prime-O-glucosylcimifugin (0–100 mg/L) in the absence or presence of 1 mg/L LPS for 18 h. Cell viability was assessed by MTT reduction assays. Data are presented as mean ± SEM of three independent experiments.

[20,21]. Mice were killed 7 h after LPS administration under diethyl ether anesthesia. Lung tissues were homogenized in 50 mM hydroxyethyl piperazine ethanesulfonic acid (HEPES) (pH 8.0) containing 0.5% cetyltrimethyl ammonium bromide (CTAB) and subjected to three freeze–thaw cycles. The homogenate was centrifuged at 13,000 ×*g* for 30 min at 4 °C, and the cell-free extracts were stored at −20 °C until further use. The MPO activity was assayed using a mouse MPO ELISA kit. Samples were diluted in phosphate citrate buffer (pH 5.0).

2.4.6. Histopathologic evaluation of the lung tissue

Histopathologic examination was performed on mice that were not subjected to BALF collection. The lungs were excised and fixed in 10% buffered formalin. Then the tissues were dehydrated with graded alcohol, embedded in paraffin and sliced. The sections were stained with hematoxylin and eosin (H&E) and pathological changes of lung tissues were observed under a light microscope.

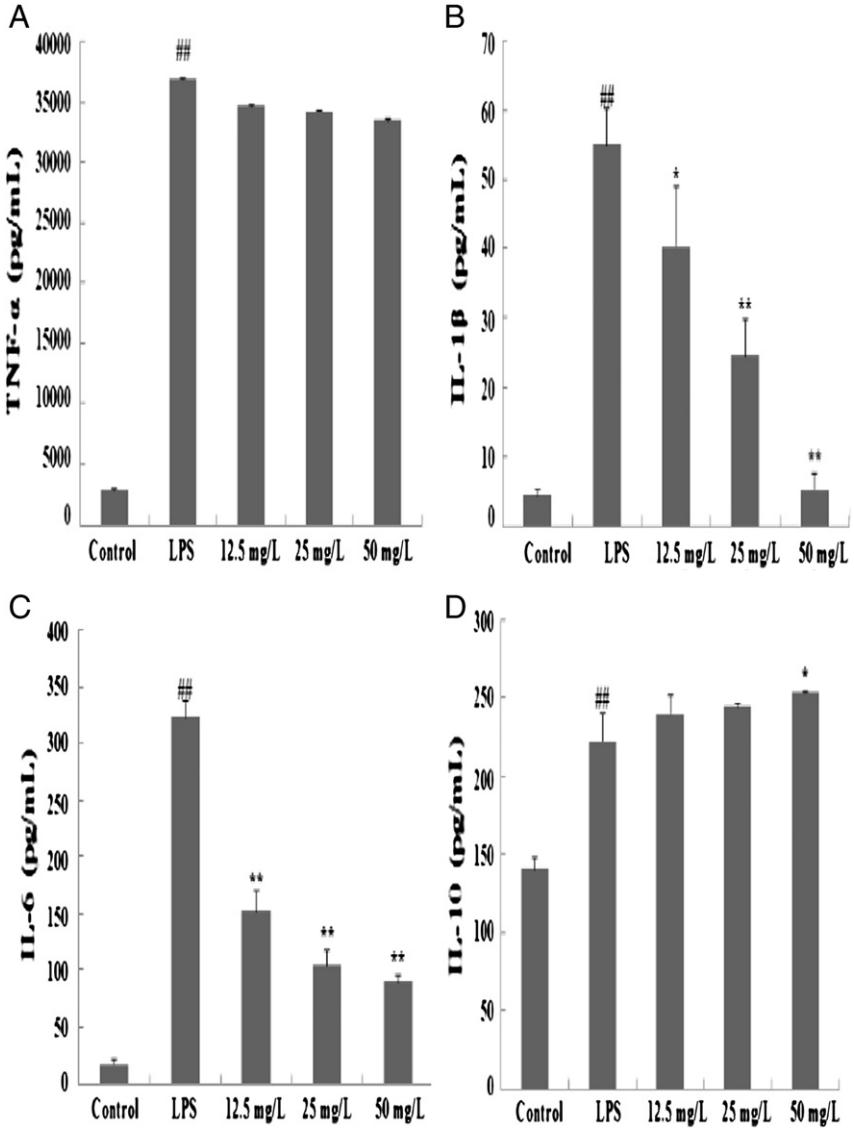

Fig. 2. Effects of Prime-O-glucosylcimifugin on LPS-induced cytokine (TNF-α, IL-1β, IL-6 and IL-10) production in vitro. The cells were treated with LPS alone or LPS plus different concentrations (12.5, 25 or 50 mg/L) of Prime-O-glucosylcimifugin for 24 h. The production of TNF-α, IL-1β and IL-6, and IL-10 in the culture supernatant of macrophages was measured by ELISA kits. The values represent mean ± SEM of three independent experiments and differences between mean values were assessed by Student's *t*-test. $^{\#\#}P < 0.01$ indicates significant differences from the unstimulated control group, $^{*}P < 0.05$ vs. LPS, $^{**}P < 0.01$ vs. LPS.

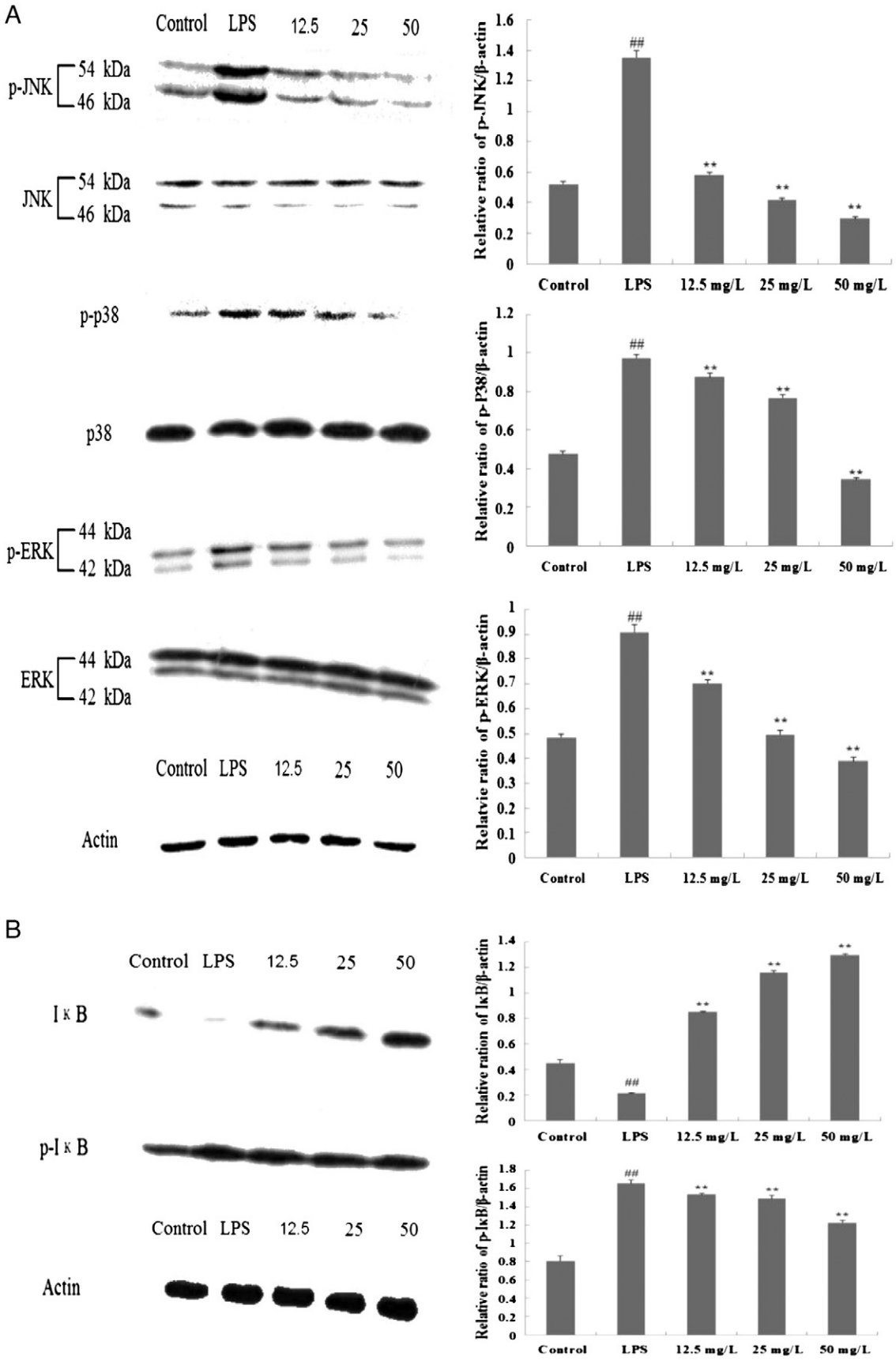

Fig. 3. Effect of Prime-O-glucosylcimifugin on MAPKs and NF-κB signaling pathways in LPS stimulated RAW cells. RAW 264.7 cells were treated with different concentrations (12.5, 25 or 50 mg/L) of Prime-O-glucosylcimifugin for 1 h before stimulating them with LPS (1 mg/L) for 30 min. Protein samples were analyzed by Western blot with antibodies specific for the phosphorylated forms of ERK, JNK, p38 and IκB. Quantification of protein expression was normalized to β-actin using a densitometer (Imaging System). (A) The phosphorylation of ERK, JNK and p38 after pretreatment with Prime-O-glucosylcimifugin and LPS challenge. (B) The phosphorylation of IκB after pretreatment with Prime-O-glucosylcimifugin and LPS challenge. The data are representative of three independent experiments and expressed as mean ± SEM. $^{\#\#}P < 0.01$ indicates significant differences from the unstimulated control group, $^{*}P < 0.05$ vs. LPS, $^{**}P < 0.01$ vs. LPS.

2.5. Statistical analysis

All values are presented as mean ± SD. Data were entered into a database and analyzed using SPSS software (SPSS for Windows version 13.0, Chicago, USA) and comparison between groups was made with one-way ANOVA (Dunnett's t-test) and Student's t-test. P-values of 0.05 or less were considered statistically significant.

3. Results

3.1. In vitro study

3.1.1. Effects of Prime-O-glucosylcimifugin on macrophage toxicity

To assess the suitable concentration of Prime-O-glucosylcimifugin for the study, RAW 264.7 cells were incubated with Prime-O-glucosylcimifugin at concentrations ranging from 12.5 to 100 mg/L in the absence or presence of LPS and cell viability was measured by MTT test 18 h later. The results showed that Prime-O-glucosylcimifugin at concentrations from 12.5 to 100 mg/L had no cytotoxic effect on RAW 264.7 cells (Fig. 1).

3.1.2. Effects of Prime-O-glucosylcimifugin on LPS-induced cytokine production in vitro

TNF-α, IL-1β, IL-6 and IL-10 concentrations in the culture supernatant of RAW 264.7 macrophages were measured by ELISA kits (Fig. 2). RAW 264.7 macrophages treated with LPS alone produced significant amounts of TNF-α, IL-1β, IL-6 and IL-10 compared to the control group. However, the production of TNF-α was slightly decreased while the levels of IL-1β and IL-6 were significantly inhibited in a dose-dependent manner when the cells were treated with 12.5, 25 or 50 mg/L of Prime-O-glucosylcimifugin ($^{*}P < 0.05$, $^{**}P < 0.01$). In contrast, the concentrations of IL-10 was significantly increased when the cells were treated with 50 mg/L of Prime-O-glucosylcimifugin ($P < 0.05^{*}$).

3.1.3. Effects of Prime-O-glucosylcimifugin on MAPKs and NF-κB signaling pathways in LPS stimulated RAW 264.7 cells

In order to investigate the mechanism by which Prime-O-glucosylcimifugin inhibits LPS-induced cytokine production, we examined the levels of LPS-induced phosphorylation of ERK1/2, JNK and p38 MAPK in the cytoplasm by Western blotting analysis using three different phosphor-special antibodies. In our study, Fig. 3A showed that LPS stimulation significantly increased the phosphorylation of ERK1/2, JNK and p38. However, Prime-O-glucosylcimifugin inhibited the phosphorylation of ERK1/2, JNK and p38 in LPS-induced cells. There were no changes in the expression of non-phosphorylated MAPKs among groups. Furthermore, we examined the effect of Prime-O-glucosylcimifugin on IκBα phosphorylation and degradation. The results showed that LPS-induced IκBα degradation was inhibited after pretreatment with Prime-O-glucosylcimifugin in a dose-dependent manner (Fig. 3B).

3.2. In vivo study

3.2.1. Effects of Prime-O-glucosylcimifugin on cytokines in BALF of LPS-induced ALI mice

BALF was collected at 7 h after LPS administration and the cytokine levels in BALF were measured by ELISA according to the manufacturer's instructions and as described in the Materials and methods section. The levels of TNF-α, IL-1β and IL-6 in BALF were increased dramatically compared with control group (Fig. 4). However, pretreatment with Prime-O-glucosylcimifugin (2.5, 5 or 10 mg/kg) significantly down-regulated the levels of TNF-α, IL-1β and IL-6 in a dose-dependent manner ($^{*}P < 0.05$, $^{**}P < 0.01$).

3.2.2. Effects of Prime-O-glucosylcimifugin on inflammatory cell count in the BALF of LPS-induced ALI mice

Seven hours after LPS administration, the BALF was collected to evaluate the total cell counts and the number of inflammatory cells in BALF, such as macrophages and neutrophils. As shown in Fig. 5, LPS challenge markedly increased the number of total cells, neutrophils, and macrophages compared to the control group ($P < 0.01$). In addition, pretreatment with Prime-O-glucosylcimifugin was found to significantly decrease the number of total cells, neutrophils and macrophages ($^{*}P < 0.05$, $^{**}P < 0.01$).

3.2.3. Effects of Prime-O-glucosylcimifugin on lung W/D ratio in LPS-induced ALI mice

To evaluate LPS-induced changes in pulmonary vascular permeability to water, we evaluated the wet weight to dry weight ratio of the lungs. The lung W/D ratio was evidently higher at 7 h after LPS administration compared with the control mice as illustrated ($^{**}P < 0.01$). Pretreatment of mice with Prime-O-glucosylcimifugin significantly reduced the water gain (Fig. 6) ($^{*}P < 0.05$, $^{**}P < 0.01$).

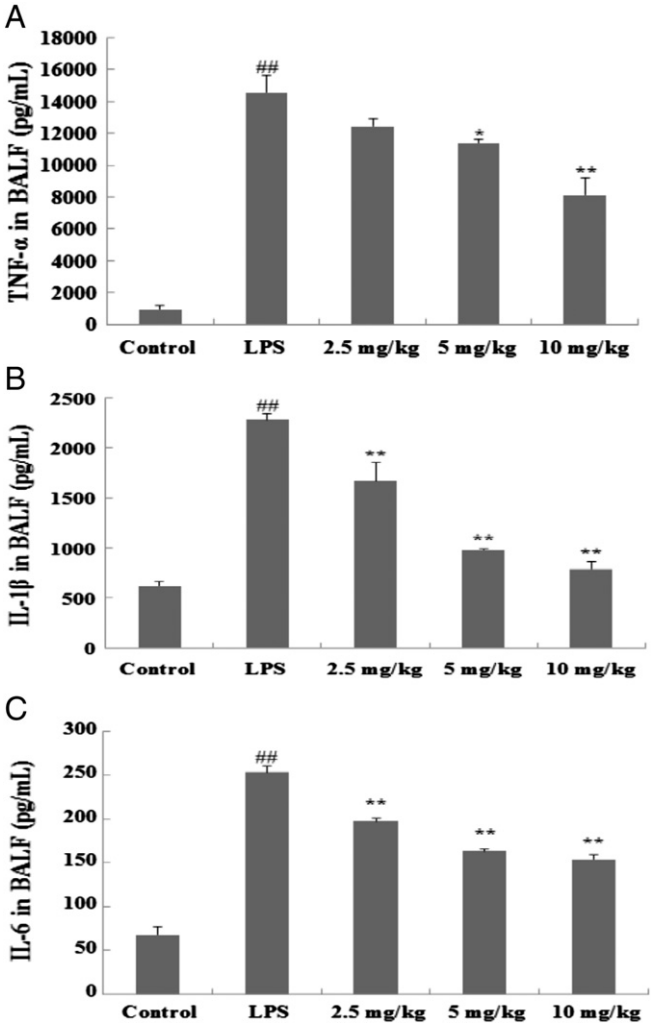

Fig. 4. Effect of Prime-O-glucosylcimifugin on the production of TNF-α, IL-1β and IL-6 in BALF of LPS-induced ALI mice. Mice were given an intraperitoneal injection of Prime-O-glucosylcimifugin (2.5, 5 or 10 mg/kg) 1 h prior to administration of LPS. BALF was collected at 7 h following LPS challenge to analyze the inflammatory cytokines TNF-α (Fig. 4A), IL-1β (Fig. 4B) and IL-6 (Fig. 4C). The values presented are the mean ± SEM (n = 6 in each group). $^{\#\#}P < 0.01$ vs. control group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS group.

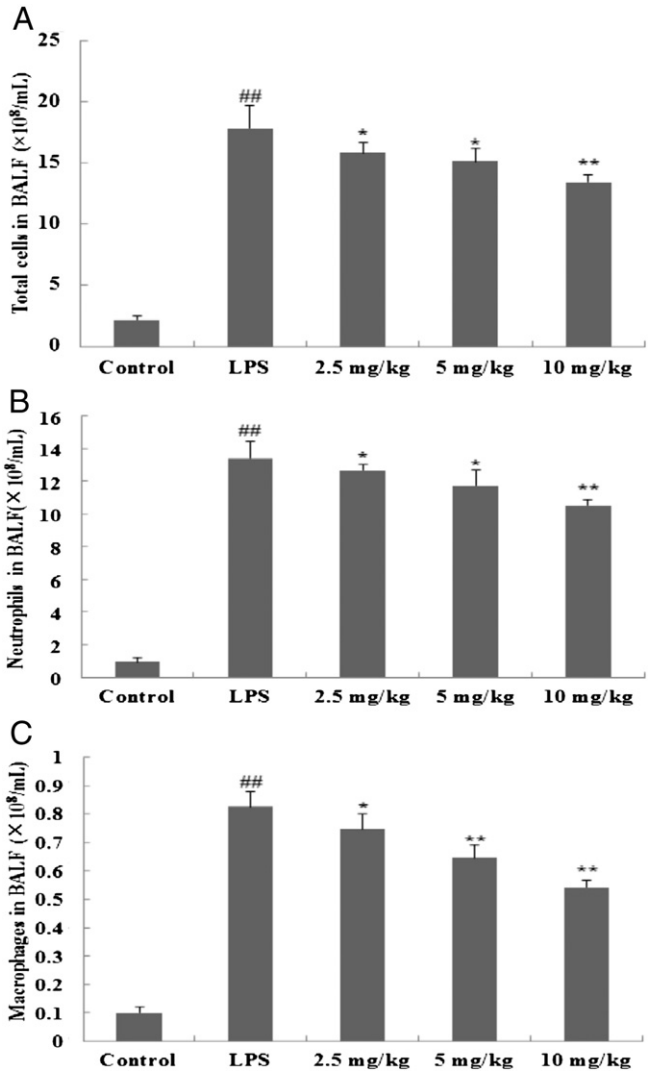

Fig. 5. Effects of Prime-O-glucosylcimifugin on the number of total cells, neutrophils, and macrophages in the BALF of LPS-induced ALI mice. Mice were given an intraperitoneal injection of Prime-O-glucosylcimifugin (2.5, 5 or 10 mg/kg) 1 h prior to an i.n. administration of LPS. BALF was collected at 7 h following LPS challenge to measure the number of total cells (A), neutrophils (B), and macrophages (C). The values presented are the mean ± SEM (n = 6 in each group). $^{\#\#}P < 0.01$ vs. control group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS group.

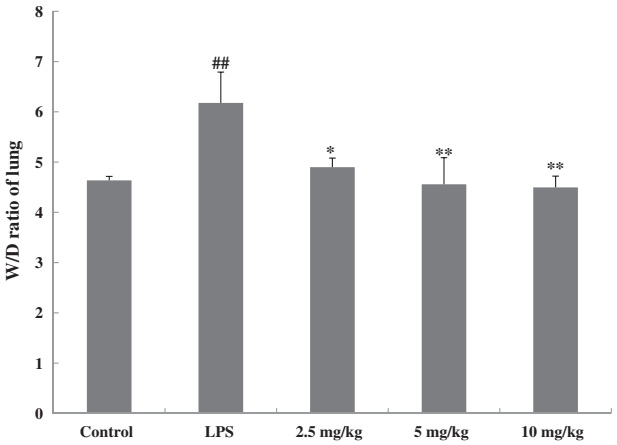

Fig. 6. Effects of Prime-O-glucosylcimifugin on the lung W/D ratio of LPS-induced ALI mice. Mice were given an intraperitoneal injection of Prime-O-glucosylcimifugin (2.5, 5 or 10 mg/kg) 1 h prior to an i.n. administration of LPS. The lung W/D ratio was determined at 7 h after LPS challenge. The values presented are the mean ± SEM (n = 6 in each group). $^{\#\#}P < 0.01$ vs. control group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS group.

4. Discussion

The results of this study indicate that Prime-O-glucosylcimifugin had a promising anti-inflammatory activity. Treatment with Prime-O-glucosylcimifugin before LPS challenge can attenuate LPS-induced inflammatory responses in RAW 264.7 cells and significantly protect mice against LPS-induced ALI.

The study primarily focused on anti-inflammatory effects of Prime-O-glucosylcimifugin on LPS-stimulated RAW 264.7 cells. LPS, a glycolipid that constitutes the major portion of the outermost membrane of Gram-negative bacteria, can bind to the cell membrane receptor of the monocytes/macrophages and endothelial cells, then activate the signal-transduction system, thus resulting in the synthesis and release of cytokines and inflammatory mediators [22]. Excessive production of pro-inflammatory cytokines not only enhances immune responses by fighting invading pathogens, but also has deleterious effects like perturbing regular hemodynamic and metabolic balances [23]. Thus, it is an important target in the treatment of inflammatory diseases to inhibit the pro-inflammatory mediator. As shown in Fig. 2, the levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 were down-regulated while the level of IL-10 was up-regulated in the Prime-O-glucosylcimifugin group. IL-10, an anti-inflammatory, is well known to down-regulate the production

3.2.4. Effects of Prime-O-glucosylcimifugin on MPO activity in LPS-induced ALI mice

The MPO activity (Fig. 7) was determined to assess the effects of Prime-O-glucosylcimifugin on neutrophil accumulation within pulmonary tissues. LPS challenge resulted in significantly increased lung MPO activity compared with the control group ($^{**}P < 0.01$). However, this increase was reduced by Prime-O-glucosylcimifugin ($^{*}P < 0.05$, $^{**}P < 0.01$).

3.2.5. Effects of Prime-O-glucosylcimifugin on histological changes in the lung tissue of LPS-induced ALI mice

To evaluate the effect of Prime-O-glucosylcimifugin on ALI, we observed histological changes after Prime-O-glucosylcimifugin treatment in LPS-treated mice. In the LPS group, the lungs were significantly damaged with inflammatory cell infiltration, alveolar wall thickening and interstitial edema. In contrast, Prime-O-glucosylcimifugin (2.5, 5 or 10 mg/kg) was found to decrease these histopathological changes (Fig. 8).

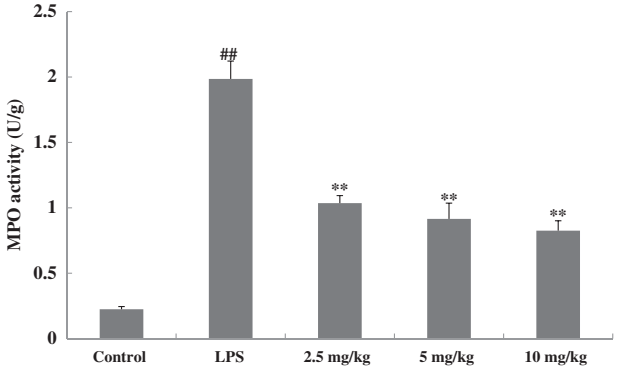

Fig. 7. Effects of Prime-O-glucosylcimifugin on MPO activity in lungs of LPS-induced mice. Seven hours after LPS instillation, lung homogenates were prepared for determination of MPO activity. MPO activity in the lungs was determined with a kit by measurement of the H_2O_2-dependent oxidation of an o-dianisidine solution. Data are presented as the mean ± SEM (n = 6 in each group). $^{\#\#}P < 0.01$ vs. control group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS group.

of TNF-α, IL-1β, IL-6, IL-12 and NO [24–26]. A number of cytokines in combination with endotoxin can cause expression of inducible nitric oxide synthase (iNOS) in macrophages [27]. Inducible NOS is an important pharmacological target in inflammatory and mutagenesis research. It has been proved that Prime-O-glucosylcimifugin had inhibitory effect on the synthesis of NO induced by LPS in RAW 264.7 cells [28,29]. Combined with the finding of our study, it seems like Prime-O-glucosylcimifugin inhibits the synthesis of NO by producing IL-10 in RAW 264.7 cells. The anti-inflammatory actions of IL-10 can interfere with the production of pro-inflammatory cytokines through the suppression of NF-κB activation by preserving the expression of IκB protein. NF-kB is a key transcriptional factor involved in regulating the expression of proinflammatory mediators, including cytokines, chemokines, and adhesion molecules, thereby playing a critical role in mediating inflammatory responses [30,31]. Under resting conditions, NF-κB is held inactive by IκB. However, NF-κB can be activated by some stimulation of various receptors including TNF receptor, Toll-like receptors (TLRs) and T-cell receptor (TCR). Persistent activation of NF-κB is central to the pathogenesis of many inflammatory lung disorders including chronic obstructive pulmonary, asthma, pneumonia, and acute lung injury [32]. The activation of NF-κB is implicated in the MAPK signaling pathway. Inhibition of MAPK family pathway, such as ERK, p38, and JNK, alleviates the production of pro-inflammatory cytokines [33]. Therefore, we investigate the possibility that Prime-O-glucosylcimifugin inhibits the production of TNF-α, IL-1β, and IL-6 via interfering with the activation of MAPK and NF-κB. The results showed that Prime-O-glucosylcimifugin obviously not only inhibited NF-κB activation, but also inhibited LPS-induced phosphorylation of ERK1/2, JNK and p38 in RAW 264.7 cells (Fig. 3). Based on the above observations, our results suggest that Prime-O-glucosylcimifugin had anti-inflammatory ability by suppressing the expression of pro-inflammatory

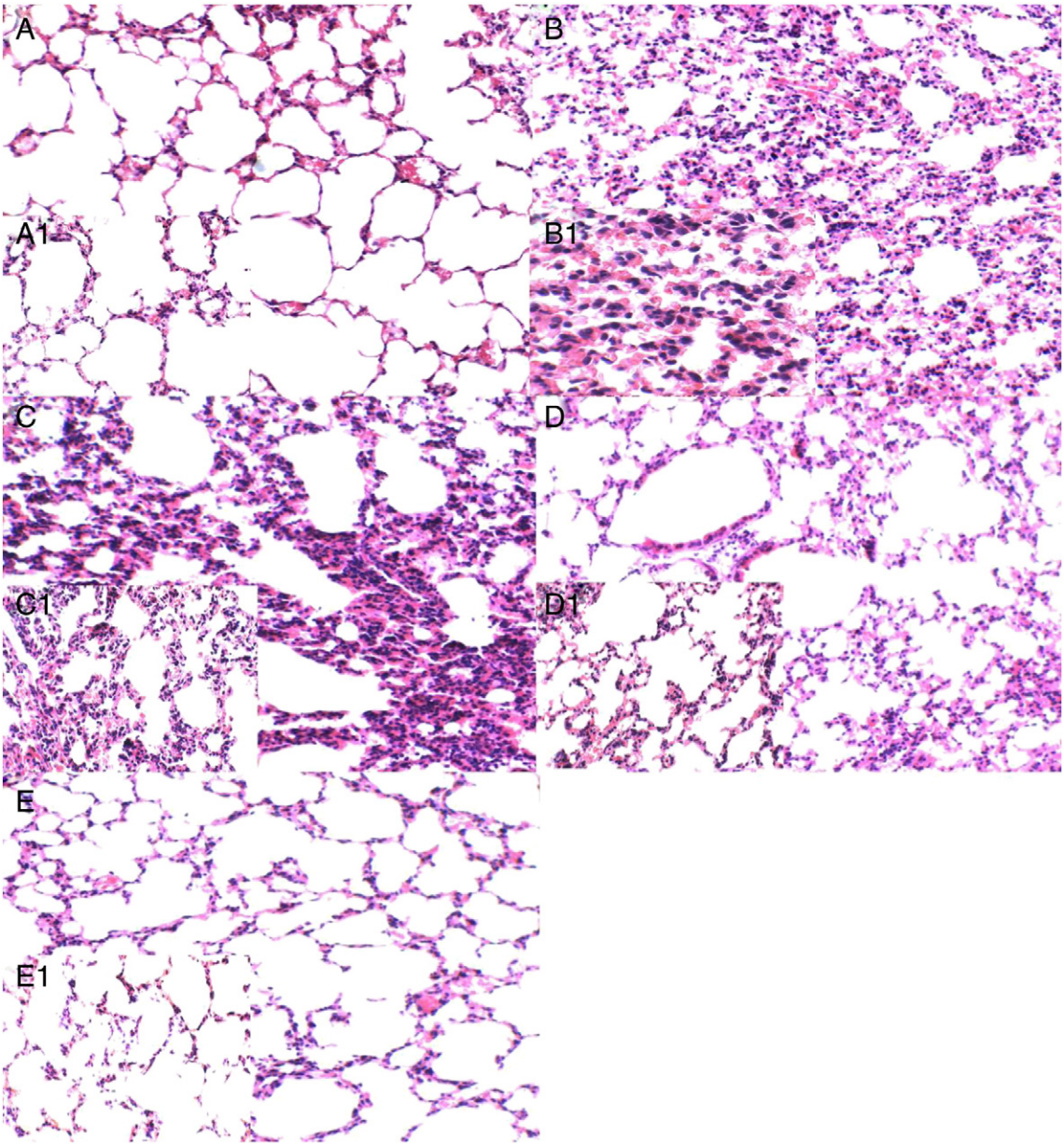

Fig. 8. Effect of Prime-O-glucosylcimifugin on histopathological changes in lung tissues in LPS-induced ALI mice (A, B, C, D, E: ×100; A1, B1, C1, D1, E1: ×400). Mice were given an intraperitoneal injection of Prime-O-glucosylcimifugin (2.5, 5, 10 mg/kg) 1 h prior to an i.n. administration of LPS. Lungs (n = 3) from each experimental group were processed for histological evaluation at 7 h after LPS challenge. To confirm pathologic changes in lung tissues, we performed hemotoxylin–eosin staining. (A) Normal mice; (B) LPS treated mice; (C, D, E) LPS + Prime-O-glucosylcimifugin-treated mice.

cytokines through blocking the activation of MAPK and NF-κB pathways in vitro.

Acute lung injury is characterized by systemic airway inflammatory response including cytokines (e.g., TNF-α, IL-6, IL-8), chemokines, pro-inflammatory mediators and a variety of cells, which regulate the migration and pulmonary infiltration of neutrophils into the interstitial tissue [34]. Neutrophils are an important component of the inflammatory response that characterizes acute lung injury (ALI) [35]. Once an inflammatory response is initiated, neutrophils are the first cells to be recruited to sites of infection or injury. Although neutrophils have beneficial actions in eradicating microbial infections, excessive neutrophil activation, with resultant release of cytokines and other pro-inflammatory mediators, results in tissue injury and contributes to the development of organ dysfunction, such as ALI [9].

High levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, perform a central role in the initiation and propagation of the inflammatory cascade in LPS-induced ALI [36]. Cytokines, such as TNF-α, IL-1β, IL-6, IL-8, and IL-10, that are secreted by alveolar macrophages stimulate more chemotaxis and attract more neutrophils to injured lungs [37–39]. In our study, the levels of TNF-α, IL-1β and IL-6 in BALF were lower in the Prime-O-glucosylcimifugin group than in the LPS group. These reductions may have contributed to the decreased neutrophil count in BALF in the LPS-induced ALI model treated with Prime-O-glucosylcimifugin.

Edema is a typical symptom of inflammation both in systemic inflammation and in local inflammation [40]. Permeability edema which accompanies acute lung injury, severe pneumonia and acute respiratory distress syndrome (ARDS) is associated with alveolar fluid clearance capacity reduction, increase of capillary endothelial permeability, and alveolar epithelial barrier disruption [41]. To quantify the magnitude of pulmonary edema, we determined the W/D ratio of the lung tissue and Prime-O-glucosylcimifugin was shown to inhibit this ratio. On the other hand, MPO is an enzyme located mainly in the primary granules of neutrophils, thus MPO activity in the parenchyma reflects the adhesion and margination of neutrophils in the lung [42]. In the LPS-induced ALI model, a large amount of PMN is recruited from peripheral blood into the lung, producing a substantial amount of MPO and reactive oxygen derivatives, and finally resulting in a cascade-like response and tissue damage [12,43]. By contrast, administration of Prime-O-glucosylcimifugin markedly suppressed LPS-induced BALF neutrophilia (Fig. 6) and MPO activity (Fig. 7), as well as ameliorated the histopathologic changes in lung tissue produced by LPS challenge (Fig. 8). Recent studies that have been conducted on pravastatin [44], ceftiofur [45], and pinocembrin [46] showed the same regulatory effects as Prime-O-glucosylcimifugin, suggesting that this agent may be an important regulator of inflammatory responses. Taken together, these results indicate that the protective effects of Prime-O-glucosylcimifugin on acute lung injury in a mouse model induced by LPS may be due to its inhibition of inflammatory mediators and limitation of inflammatory response in the lung.

In conclusion, the findings of this study showed that Prime-O-glucosylcimifugin has a promising anti-inflammatory effect and a protective effect against LPS-induced ALI. Pretreatment with Prime-O-glucosylcimifugin inhibited the release of in vitro and in vivo inflammatory responses by counteracting MAPK and NF-κB activation. These findings strongly suggest that Prime-O-glucosylcimifugin has a potent anti-inflammatory activity and may represent a novel strategy for the modulation of inflammatory responses. Further studies are warranted to investigate the clinical usefulness of Prime-O-glucosylcimifugin.

Acknowledgments

This work was supported by grants from the National Nature Science Foundation of China (No. 30972212) and the China Postdoctoral Science Foundation (No. 20090461034).

References

[1] Gu J, Korteweg C. Pathology and pathogenesis of severe acute respiratory syndrome. Am J Pathol 2007;170:1136–47.
[2] Mendez JL, Hubmayr RD. New insights into the pathology of acute respiratory failure. Curr Opin Crit Care 2005;11(1):29–36.
[3] Mei SHJ, McCarter SD, Deng Y, Parker CH, Liles WC, Stewart DJ. Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin. PLoS Med 2007;4(9):269.
[4] Kitamura Y, Hashimoto S, Mizuta N, Kobayashi A, Kooguchi K, Fujiwara I, et al. Fas/FasL-dependent apoptosis of alveolar cells after lipopolysaccharide-induced lung injury in mice. Am J Respir Crit Care Med 2001;163(3 Pt 1):762–9.
[5] Rojas M, Woods CR, Mora AL, Xu J, Brigham KL. Endotoxin-induced lung injury in mice: structural, functional, and biochemical responses. Am J Physiol Lung Cell Mol Physiol 2005;288(2):333–41.
[6] Gharib SA, Liles WC, Matute-Bello G, Glenny RW, Martin TR, Altemeier WA. Computational identification of key biological modules and transcription factors in acute lung injury. Am J Respir Crit Care Med 2006;173(6):653–8.
[7] Wu Y, Singer M, Thouron F, Alaoui-El-Azher M, Touqui L. Effect of surfactant on pulmonary expression of type IIA PLA(2) in an animal model of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2002;282(4):743–50.
[8] Abraham E. Neutrophils and acute lung injury. Crit Care Med 2003;31(4 Suppl.): 195–9.
[9] Schutte H, Lohmeyer J, Rosseau S, Ziegler S, Siebert C, Kielisch H, et al. Bronchoalveolar and systemic cytokine profiles in patients with ARDS, severe pneumonia and cardiogenicpulmonary oedema. Eur Respir J 1996;9(9):1858–67.
[10] Matthay MA, Geiser T, Matalon S, Ischiropoulos H. Oxidant-mediated lung injury in the acute respiratory distress syndrome. Crit Care Med 1999;27(9):2028–30.
[11] Nys M, Deby-Dupont G, Habraken Y, Legrand-Poels S, Ledoux D, Canivet JL, et al. Bronchoalveolar lavage fluids of patients with lung injury activate the transcription factor nuclear factor-kappaB in an alveolar cell line. Clin Sci (Lond) 2002;103(6):577–85.
[12] Grommes J, Soehnlein O. Contribution of neutrophils to acute lung injury. Mol Med 2011;17(3–4):293–307.
[13] Cribbs SK, Matthay MA, Martin GS. Stem cells in sepsis and acute lung injury. Crit Care Med 2010;38(12):2379–85.
[14] Diaz JV, Brower R, Calfee CS, Matthay MA. Therapeutic strategies for severe acute lung injury. Crit Care Med 2010;38(8):1644–50.
[15] Matthay MA, Goolaerts A, Howard JP, Lee JW. Mesenchymal stem cells for acute lung injury: preclinical evidence. Crit Care Med 2010;38(10 Suppl.):569–73.
[16] Yen KY. Illustrated Chinese Materia Medica Crude and Prepared. Taiwan: SMC Publishing; 1992;1–340.
[17] Sasaki H, Taguchi H, Endo T, Yosioka I. The constituents of *Ledebouriella seseloides* Wolff. I. Structures of three new chromones. Chem Pharm Bull 1982;30(10): 3555–62.
[18] Baba K, Tabata Y, Kozawa M, Kimura Y, Arichi S. Studies on Chinese traditional medicine Fang-Feng (I): structures and physiological activities of polyacetylene compounds from *Saposhnikoviae radix*. Jpn J Pharmacol 1987;41(3):189–94.
[19] Wang JH. Pharmacognostical Studies of Chinese Drug Fang Feng. [PhD Thesis] Beijing Medical University; 1986.
[20] Ysebaert DK, De Greef KE, Vercauteren SR, Ghielli M, Verpooten GA, Eyskens EJ, et al. Identification and kinetics of leukocytes after severe ischaemia/reperfusion renal injury. Nephrol Dial Transplant 2000;15(10):1562–74.
[21] Peng X, Hassoun PM, Sammani S, McVerry BJ, Burne MJ, Rabb H. Protective effects of sphingosine1-phosphate in murine endotoxin-induced inflammatory lung injury. Am J Respir Crit Care Med 2004;169(11):1245–51.
[22] Fenton MJ, Golenbock DT. LPS-binding proteins and receptors. J Leukoc Biol 1998;64(1):25–32.
[23] Lin WJ, Yeh WC. Implication of Toll-like receptor and tumor necrosis factor alpha signaling in septic shock. Shock 2005;24(3):206–9.
[24] Ge'rard C, Bruyns C, Marchant A, Abramowicz D, Vandenabeele P, Delvaux A, et al. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. J Exp Med 1993;177(2):547–50.
[25] Ertel W, Keel M, Steckholzer U, Ungethüm U, Trentz O. Interleukin-10 attenuates the release of proinflammatory cytokines but depresses splenocyte functions in murine endotoxemia. Arch Surg 1996;131(1):51–6.
[26] Grütz G. New insights into the molecular mechanism of interleukin-10-mediated immunosuppression. J Leukoc Biol 2005;77(1):3–15.
[27] Cochran FR, Selph J, Sherman P. Insights into the role of nitric oxide in inflammatory arthritis. Med Res Rev 1996;16(6):547–63.
[28] Wang CC, Chen LG, Yang LL. Inducible nitric oxide synthase inhibitor of the Chinese herb I. *Saposhnikovia divaricate* (Turcz.) Schischk. Cancer Lett 1999;145(1–2):151–7.
[29] Zhao B, Yang XB, Yang XW, Liu JX. Biotransformation of prim-O-glucosylcimifugin by human intestinal flora and its inhibition on NO production and DPPH free radical. J Asian Nat Prod Res 2012;14(9):886–96.
[30] Pantano C, Ather JL, Alcorn JF, Poynter ME, Brown AL, Guala AS, et al. Nuclear factor-kappaB activation in airway epithelium induces inflammation and hyperresponsiveness. Am J Respir Crit Care Med 2008;177(9):959–69.
[31] Duan W, Wong WS. Targeting mitogen-activated protein kinases for asthma. Curr Drug Targets 2006;7(6):691–8.
[32] Ci X, Chu X, Wei M, Yang X, Cai Q, Deng X. Different effects of farrerol on an OVA-induced allergic asthma and LPS-induced acute lung injury. PLoS One 2012;7(4):34634.
[33] Chon H, Choi B, Jeong G, Lee E, Lee S. Suppression of proinflammatory cytokine production by specific metabolites of *Lactobacillus plantarum* 10hk2 via inhibiting NF-κB and p38 MAPK expressions. Comp Immunol Microbiol Infect Dis 2010;33(6):e41–9.

[34] Shields CJ, Winter DC, Redmond HP. Lung injury in acute pancreatitis: mechanisms, prevention, and therapy. Curr Opin Crit Care 2002;8(2):158–63.
[35] Zhao X, Zmijewski JW, Lorne E, Liu G, Park YJ, Tsuruta Y, et al. Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2008;295(3):497–504.
[36] Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2008;295(3):379–99.
[37] Strieter RM, Kunkel SL. Acute lung injury: the role of cytokines in the elicitation of neutrophils. J Investig Med 1994;42(4):640–51.
[38] Kobayashi A, Hashimoto S, Kooguchi K, Kitamura Y, Onodera H, Urata Y, et al. Expression of inducible nitric oxide synthase and inflammatory cytokines in alveolar macrophages of ARDS following sepsis. Chest 1998;113(6):1632–9.
[39] Shinbori T, Walczak H, Krammer PH. Activated T killer cells induce apoptosis in lung epithelial cells and the release of pro-inflammatory cytokine TNF-alpha. Eur J Immunol 2004;34(6):1762–70.
[40] Zhang X, Li J, Chen C, Ci X, Yu Q, Zhang X, et al. Protective effect of abamectin on acute lung injury induced by lipopolysaccharide in mice. Fundam Clin Pharmacol 2010;25(6):700–7.
[41] Lucas R, Verin AD, Black SM, Catravas JD. Regulators of endothelial and epithelial barrier integrity and function in acute lung injury. Biochem Pharmacol 2009;77(12):1763–72.
[42] Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol 2005;77(5):598–625.
[43] Razavi HM, Wang le F, Weicker S, Rohan M, Law C, McCormack DG, et al. Pulmonary neutrophil infiltration in murine sepsis: role of inducible nitric oxide synthase. Am J Respir Crit Care Med 2004;170(3):227–33.
[44] Yeo CD, Rhee CK, Kim IK, Kang HH, Lee SH, Lee SY, et al. Protective effect of pravastatin on lipopolysaccharide-induced acute lung injury during neutropenia recovery in mice. Exp Lung Res 2013;39(2):99–106.
[45] Chu X, Song K, Xu K, Zhang X, Song Y, Wang D, et al. Ceftiofur attenuates lipopolysaccharide-induced acute lung injury. Int Immunopharmacol 2010;10(5):600–4.
[46] Soromou LW, Chu X, Jiang L, Wei M, Huo M, Chen N, et al. In vitro and in vivo protection provided by pinocembrin against lipopolysaccharide-induced inflammatory responses. Int Immunopharmacol 2012;14(1):66–74.