Protective Effects of the Ethanol Extract of *Viola tianshanica* Maxim against Acute Lung Injury Induced by Lipopolysaccharides in Mice

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Introduction

Acute lung injury (ALI), a common clinical complication of microbial infections, is the major cause of respiratory failure [1]. Intense parenchymal inflammatory processes are noted in ALI, since inflammatory cells gather and proinflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), are upregulated in lung tissue. The accumulation of inflammatory mediators triggers the pathophysiological cascades of the acute respiratory distress syndrome, which is characterized by diffuse alveolar damage, hemorrhage, etc. [2, 3]. The ALI model induced by intranasal administration of lipopolysaccharide (LPS) was widely accepted because LPS administration contributed to the inflammation of lung tissue [4–6].

*Viola tianshanica* Maxim, belonging to the Violaceae plant family, is traditionally used in Uighur medicine for treating pneumonia, headache, and fever. There is, however, a lack of basic understanding of its pharmacological activities. This study was designed to observe the effects of the ethanol extract (TSM) from *Viola tianshanica* Maxim on the inflammation response in acute lung injury (ALI) induced by LPS and the possible underlying mechanisms.

We found that TSM (200 and 500 mg/kg) significantly decreased inflammatory cytokine production and the number of inflammatory cells, including macrophages and neutrophils, in bronchoalveolar lavage fluid. TSM also markedly inhibited the lung wet-to-dry ratio and alleviated pathological changes in lung tissues. In vitro, after TSM (12.5–100 μg/ml) treatment to RAW 264.7 cells for 1 h, LPS (1 μg/ml) was added and the cells were further incubated for 24 h. TSM dose-dependently inhibited the levels of proinflammatory cytokines, such as NO, PGE₂, TNF-α, IL-6, and IL-1β, and remarkably decreased the protein and mRNA expression of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells. TSM also suppressed protein expression of p-IκBα and p-ERK1/2 and blocked nuclear translocation of NF-κB p65. The results indicate that TSM exerts anti-inflammatory effects related with inhibition on NF-κB and MAPK (p-ERK1/2) signaling pathways. In conclusion, our data demonstrate that TSM might be a potential agent for the treatment of ALI.

**Keywords:** *Viola tianshanica* Maxim, acute lung injury, anti-inflammation effect
swelling in mice and increased permeability of blood capillaries in mice induced by acetic acid. To date, however, the effects of TSM against ALI are not clear yet. The current study aimed to investigate the effect of TSM on LPS-induced ALI in mice and further to clarify the possible mechanisms. We found that TSM protected against lung injury by inhibiting inflammation and demonstrated that TSM played its protective role through inhibition of the NF-κB and MAPK signaling pathways.

Materials and Methods

Extraction of Plant Materials

Viola tianshanica Maxim was purchased from Uyghur Hospital of the Xinjiang Uyghur Autonomous Region, China. The plant material was identified by Associate Professor Jian He, Xinjiang Institute of Materia Medica. A voucher specimen was deposited at Xinjiang Institute of Materia Medica. After being crushed, the dry plant material (1 kg) was boiled in 10 L of 70% ethanol for 1 h, three times. The ethanol elution was collected and concentrated to obtain infusion-extract (TSM, 74 g). According to the method described in Chinese Pharmacopoeia [8], we confirmed the total flavonoid content of TSM was 38%.

Animals

Male BALB/c mice (18 to 20 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). Animals were kept under a SPF environment (temperature 24 ± 1°C, humidity 40–60%, 12 h/12 h light/dark cycle) for 3 days to adapt to these conditions. The animal protocol (No. 003428) was approved by the Animal Care & Welfare Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences.

Reagents

LPS (Escherichia coli 0111:B4), dexamethasone (DEX, D1756), indomethacin (INDO, 53-86-1), and Hoechst 33342 (B2261) were procured from Sigma-Aldrich Co., Ltd (USA). The Cyclooxygenase (COX) Inhibitor Screening Assay Kit and Prostaglandin E (PGE)

In Vivo Study

Animal model of ALI. The mice were randomly assigned into five groups (n = 12): control; LPS model; LPS + TSM (250 and 500 mg/kg); and LPS + DEX (5 mg/kg). The TSM and DEX groups were administered intragastric TSM and DEX, respectively; the control and LPS model mice were treated with the vehicle. After 1 h of treatment, the mice were administrated intranasally with 50 μL of LPS (300 μg formulated in PBS) to induce lung injury, whereas the control animals received only the equal volume of vehicle. Twenty-four hours later, the animals were sacrificed. Bronchoalveolar lavage fluid (BALF) samples were collected, and lung tissue were fixed in 4% paraformaldehyde and embedded in paraffin for histological examination and immediately frozen in liquid nitrogen.

Cell counting. The BALF samples were centrifuged at 3,000 rpm for 10 min at 4°C. The cell pellets were resuspended in PBS. A hemacytometer was used to detect the total cell counts. Cytospins were stained with the Wright Giemsa staining method for neutrophil and macrophage counts.

Lung wet-to-dry weight (W/D) ratio. In brief, the excised left lung was blotted dry and weighed to obtain the “wet” weight, and then the “dry” weight was obtained after the left lung was placed in an oven at 80°C for 48 h. The lung tissue edema was expressed as the ratio of the wet lung weight to the dry lung weight.

Cytokine assays. The levels of TNF-α and IL-6 in the BALF were evaluated according to the manufacturer’s instructions for ELISA kits.

Histological changes in lung tissues. Histopathological evaluation was performed. Lung tissue sections (4 μm thick) was cut and stained with hematoxylin and eosin (H&E). Thereafter, light microscopy was performed to evaluate the histological changes.

In Vitro Study

Cell culture. The RAW 264.7 murine macrophage cell line was cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in humidified air at 37°C with 5% CO2.

Cell viability. To determine cytotoxicity, the RAW 264.7 cells (2 × 105 cells per well) were seeded in a 96-well plate. The cells were exposed to 12.5–100 μg/ml TSM for 24 h after serum starvation for 12 h. At the end of incubation, MTT (5 mg/ml) was added and incubated for the next 4 h. The medium was then removed, and dimethyl sulfoxide (150 μl per well) was added to dissolve the produced formazan crystals. Absorbance was measured at 570 nm using a microplate reader (M5; Molecular Devices, USA).

Nitrite production and cytokine assays. After being exposed to TSM (12.5–100 μg/ml) for 1 h, RAW 264.7 cells were incubated with LPS (1 μg/ml) for 24 h. NO production in the culture medium was estimated by the Griess assay [9]. TNF-α, IL-6, and IL-1β production levels were assessed using ELISA assay kits, and the PGE2 content was measured by a PGE2 Express EIA Kit.
Western blotting. RAW 264.7 cells were incubated with TSM (12.5–100 μg/ml) for 1 h. Except for the control group, the cells were then incubated with LPS (1 μg/ml) for an additional 24 h. After incubation, the cells were lysed in ice-cold cell lysis buffer (C1053; Applygen Technologies Inc., China) and blotting was performed. In brief, the lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (Millipore, USA). After blocking, the membranes were incubated with primary antibodies diluted at 1:1,000 in diluent buffer (5% BSA in TBST) at 4°C overnight. Thereafter, the membranes were washed four times and incubated with goat anti-rabbit IgG antibody at 1:400 dilution at 4°C for the next 12 h. After washing 3 times, the membranes were incubated with goat anti-rabbit IgG for 1 h at 37°C. The cell nuclei were stained with Hoechst 33342 (50 ng/ml) for 15 min. Live cell imaging was analyzed by Cellomics ArrayScan VTI (Thermo Scientific, USA).

**COX activity analysis.** In vitro, the inhibitory effects of various concentrations (3.125–100 μg/ml) of TSM on COX-1 and COX-2 enzyme activity were measured using a COX inhibitor screening assay kit according to the manufacturer’s protocol. The IC_{50} of COX-1 and COX-2 were calculated using a nonlinear regression algorithm, respectively.

**Statistical analysis.** The experimental results were presented as the means ± SD. Statistical evaluation was carried out by one-way analysis of variance, followed by Tukey’s test using IBM Statistics SPSS 22.0. Statistical significance was accepted at p < 0.05.

**Results**

**Effects of TSM on Inflammatory Cell Count in BALF**

Compared with the control group, LPS significantly increased the number of total cells, neutrophils, and macrophages in BALF of ALI mice (Figs. 1A–1C). Pretreatment with TSM (200 and 500 mg/kg) and DEX (5 mg/kg) was found to obviously decrease the number of these inflammatory cells in BALF.

**Effects of TSM on Lung W/D Ratio**

The lung W/D ratio was evaluated for analysis of edema formation. The ratio was significantly increased in the LPS group compared with the control group (Fig. 1D). However, this increase was effectively inhibited in the TSM (250 and 500 mg/kg) + LPS group or DEX (5 mg/kg) + LPS group.

**Effects of TSM on TNF-α and IL-6 Levels in BALF**

The production levels of TNF-α and IL-6 in BALF were analyzed using ELISA assay kits. Compared with the control group, LPS caused an elevation of TNF-α and IL-6 levels in BALF (Fig. 2). Treatment with TSM and DEX appeared to lessen these changes.

**Effects of TSM on Histological Changes in Lung Tissues**

As shown in Fig. 3, lung histological changes were visualized using H&E stain, with remarkably increased inflammatory cell infiltration, interstitial edema, alveolar wall thickening, and alveolar hemorrhage observed in the LPS group. Normal lung tissue structure and clear pulmonary alveoli were observed in the control group. Compared with the LPS group, however, TSM (250 and 500 mg/kg) treatment and DEX (5 mg/kg) treatment obviously alleviated LPS-induced pathological changes respectively.

**Cell Viability**

After RAW 264.7 cells were exposed to TSM (12.5–100 μg/ml) for 24 h, no significant difference in cell viability
Fig. 1. Effects of Viola tianshanica Maxim extract (TSM) on the number of total cells (A), macrophages (B), and neutrophils (C) in the bronchoalveolar lavage fluid (BALF) and on pulmonary edema (D) in LPS-induced acute lung injury mice. Mice were pretreated with TSM (250 and 500 mg/kg) or dexamethasone (DEX) (5 mg/kg) 1 h before an intranasal administration of LPS. BALF was collected at 24 h after LPS administration to measure the number of total cells, macrophages, and neutrophils. The lung wet/dry weight ratio was determined at 24 h after LPS challenge. The values presented are the means ± SD (n = 6 in each group). ##p < 0.01 versus the control group, *p < 0.05 and **p < 0.01 versus the LPS group.

Fig. 2. Effects of Viola tianshanica Maxim extract (TSM) on levels of TNF-α and IL-6 in bronchoalveolar lavage fluid (BALF) of LPS-induced acute lung injury in mice. Mice were pretreated with TSM (250 and 500 mg/kg) or dexamethasone (DEX) (5 mg/kg) 1 h before an intranasal administration of LPS. BALF was collected at 24 h following LPS challenge to analyze the inflammatory cytokines TNF-α (A) and IL-6 (B). The values presented are the means ± SD (n = 6 in each group). ##p < 0.01 versus the control group; **p < 0.01 versus the LPS group.
was observed compared with the control group (Fig. 4A). The concentrations of TSM were no more than 100 \( \mu \text{g/ml} \) in in vitro experiments.

**NO and Cytokine Assay**

To assess NO production, nitrite in the culture medium of LPS-treated RAW264.7 cells was determined with the Griess reagent. Meanwhile, the levels of proinflammatory cytokines and PGE\(_2\) were measured using ELISA kits. The results indicated that the production of NO, TNF-\(\alpha\), IL-6, IL-1\(\beta\) and PGE\(_2\) were markedly increased in response to LPS stimulation (Figs. 4B–4F). However, the release of NO, TNF-\(\alpha\), IL-6, IL-1\(\beta\) and PGE\(_2\) were dose-dependently reduced by TSM at concentrations of 12.5, 25, 50 and 100 \(\mu\text{g/ml}\).

**p-ERK1/2, p-I\(\kappa\)B\(\alpha\), TNF-\(\alpha\) and IL-6 Expression**

Western blot analysis was used to characterize the effects of TSM on the expression of p-ERK1/2, I\(\kappa\)B\(\alpha\) phosphorylation, TNF-\(\alpha\), and IL-6 in LPS-stimulated RAW 264.7 cells. The results showed that treatment with TSM (12.5–100 \(\mu\text{g/ml}\)) attenuated LPS-induced phosphorylation of ERK1/2 (Fig. 5B1) and I\(\kappa\)B\(\alpha\) (Fig. 5B2). Consistent with several previous studies [11], the expression levels of TNF-\(\alpha\) and IL-6 were strongly upregulated in RAW 264.7 cells stimulated with LPS; however, the protein expression levels of TNF-\(\alpha\) and IL-6 were significantly inhibited by treatment with TSM (12.5–100 \(\mu\text{g/ml}\)) in a dose-dependent manner (Figs. 5B3 and 5B4).

**TNF-\(\alpha\) and IL-6 mRNA Expression**

As shown in Fig. 6, stimulation with LPS strongly upregulated the mRNA expression of TNF-\(\alpha\) and IL-6. Treatment with TSM at the concentrations of 12.5, 25, 50, and 100 \(\mu\text{g/ml}\) inhibited the mRNA expression of TNF-\(\alpha\) and IL-6 in a dose-dependent manner.

**Expression and Nuclear Translocation of NF-\(\kappa\)B p65**

Translocation of NF-\(\kappa\)B to the nucleus is required for NF-\(\kappa\)B-dependent transcription following LPS stimulation. To further clarify the molecular mechanism, the effect of TSM on LPS-induced NF-\(\kappa\)B nuclear translocation was investigated. In the control group, fluorescence was shown as a ring and located mainly in the cytoplasm. The shape of fluorescence in the LPS-treated cells was different, and fluorescence was mainly located in the nucleus. Compared with the control group, the expression of NF-\(\kappa\)B p65 was obviously induced in the nucleus of RAW 264.7 cells treated with LPS.
Cytoplasmic NF-κB p65 protein was significantly translocated from the cytosol to the nucleus within 30 min. TSM therefore, dose-dependently inhibited NF-κB p65 translocation in LPS-induced RAW 264.7 cells (Fig. 7).

COX-1 and COX-2 Activities

The results of Table 1 showed that TSM dose-dependently inhibited COX-1 and COX-2 activities. The IC₅₀ of COX-1 and COX-2 were 1.2 μg/ml and 18.8 μg/ml, respectively.
Discussion

Since LPS was a major activator of an inflammatory response that leads to ALI [12], the mice model of ALI was therefore established by intranasal administration of LPS in our study. Glucocorticoids can cause neutrophilic granulocyte and alveolar macrophages and are used to treat ALI/ARD [13, 14]. Thus, DEX was chosen as a positive control in the

**Fig. 5.** Effects of *Viola tianshanica* Maxim extract (TSM) on expression of p-ERK (B1), p-IκBα (B2), TNF-α (B3), and IL-6 (B4) in LPS-stimulated RAW 264.7 cells.

The cells were pretreated with TSM (12.5–100 μg/ml) or INDO (15 μg/ml) 1 h before stimulation with LPS (1 μg/ml) for 24 h. Proteins extracted from RAW 264.7 cells using the Cytoplasmic Protein Extraction Kit (Beyotime, China) were analyzed by western blot analysis. Gel results are presented in Fig. 5A. Quantification of protein expression was normalized to GAPDH using Quantity One software. The values are the means ± SD of three independent experiments. *p < 0.01 versus the control group; *p < 0.05, **p < 0.01 versus the LPS-alone group.
During ALI, the predominant inflammatory cells, such as neutrophils and macrophages, contribute to the development of ALI [15]. The present study first reported that TSM remarkably reduced the massive infiltration of inflammatory cells and the number of neutrophils and macrophages in the lung tissues (Figs. 1A–1C). Pulmonary edema is a typical characteristic of ALI [16, 17]. We found that the
lung W/D ratio was significantly decreased in the TSM groups, which suggests that treatment with TSM effectively ameliorated pulmonary edema (Fig. 1D).

It has been proved that the activation of inflammatory cells induced by LPS can result in elevated production of proinflammatory cytokines and mediators, such as TNF-α, IL-6, eicosanoids, and NO [18, 19]. Previous studies demonstrated that proinflammatory cytokines and mediators can regulate inflammatory responses through the NF-κB pathway [20]. Neutrophil response to inflammatory cytokines is also regulated by NF-κB activation [21]. Suppression of NF-κB activation could lessen the secretion of TNF-α, IL-6, eicosanoids, and NO, and protect against ALI in animals [22]. In this study, we found TSM could dose-dependently lower the level of TNF-α and IL-6 in BALF (Fig. 2), which suggested TSM could alleviate the inflammatory response and lessen lung damage. We speculate that the protection mechanism of TSM against ALI, at least in part, is related with its inhibition of the NF-κB signaling pathway.

In addition, lung histopathological analysis also showed that TSM exerted a significant anti-inflammatory activity. In accordance with a previous study, histopathological analysis by H&E staining showed that inflammatory cells clustered, and edema formation and alveolar wall thickness increased in lung tissue of LPS-induced ALI in mice. As shown in Fig. 3, pretreatment with TSM markedly dose-dependently ameliorated the pulmonary injury, which further indicated that TSM could have a protective effect on LPS-induced ALI.

It is known that various inflammatory cytokines and mediators could be overproduced by LPS-stimulated macrophages. Like previous studies [23, 24], the production and secretion of NO, PGE₂, TNF-α, IL-1β, and IL-6 were significantly upregulated in LPS-stimulated RAW 264.7 cells. In the current study, the production of these cytokines and mediators could be significantly reduced by TSM dose-dependently (Fig. 4), which further confirmed the anti-inflammatory effects of TSM. In addition, TSM could obviously inhibit the activity of COX-1 and COX-2 in a dose-dependent manner, where IC₅₀ values were 1.2 μg/ml and 18.8 μg/ml, respectively (Table 1). It has been reported that COX-2 is involved in the development of inflammation in response to inflammatory stimuli, such as LPS, and meanwhile, as a reference gene, COX-1 is widely expressed in tissues [24]. To some extent, we think inhibition of COX-2 might be one of the anti-inflammatory mechanisms of TSM.

As an important transcription factor, NF-κB transcriptionally activates various inflammatory cytokines, and reversely NF-κB can be activated by inflammatory cytokines to amplify the inflammatory responses [25–27]. IkBα phosphorylation and degradation led to activation of NF-κB followed by NF-κB nuclear translocation [28]. In addition, MAPK is of importance in inducing cytokine production [29]. MAPK pathways regulate the synthesis and secretion of pro-inflammatory cytokines and mediators via activating macrophages [30]. The LPS stimulation has been demonstrated to induce phosphorylation and activation of ERK1/2, JNK, and p38 MAPKs in murine macrophages [31]. In this study, we confirmed that TSM could markedly suppress the expression of p-IκBα, p-ERK1/2, TNF-α, and IL-6 at the protein level (Fig. 5). TSM also could remarkably inhibit mRNA expression of TNF-α and IL-6 (Fig. 6); meanwhile, TSM could notably inhibit nuclear translocation of NF-κB p65 in LPS-stimulated RAW 264.7 (Fig. 7). It is concluded that TSM suppressed NF-κB and the MAPK signaling pathway, which might be the underlying mechanism of its

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<th><strong>Table 1. Inhibitory effect of <em>Viola tianshanica</em> Maxim extract (TSM) on COX enzyme activity.</strong></th>
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<td><strong>Group</strong></td>
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<td>100% activity</td>
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<td>COX-1 inhibitor, SC-560</td>
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IC₅₀ = 1.2 μg/ml | IC₅₀ = 18.8 μg/ml
anti-inflammatory effects.

In summary, pretreatment with TSM could obviously decrease inflammatory cytokines production and the lung W/D ratio, inhibit neutrophil infiltration in the lungs, and alleviate pulmonary histopathological changes subjected to ALI. Furthermore, in vitro, TSM ameliorated the production of NO, PGE$_2$, TNF-α, IL-6, and IL-1β in LPS-stimulated RAW 264.7 cells. We also confirmed that TSM could dose-dependently suppress the protein and mRNA expression of TNF-α and IL-6. These effects were largely owing to its inhibition of the phosphorylation of IkBα and ERK1/2. These data collectively suggest that TSM might be considered as a potential agent for the treatment of ALI, but this still needs further study.

Acknowledgments

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