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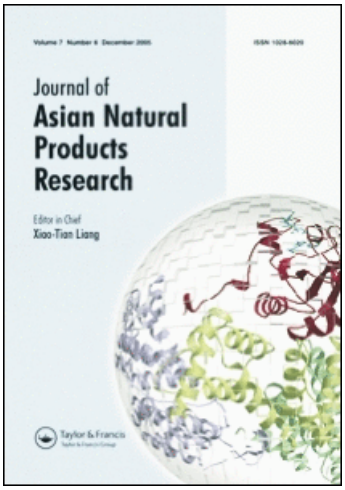

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
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Scutellarin protects against lipopolysaccharide-induced acute lung injury via inhibition of NF-κB activation in mice

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Online publication date: 26 March 2010

To cite this Article Tan, Zheng-Huai , Yu, Ling-Hong , Wei, Huai-Ling and Liu, Geng-Tao(2010) 'Scutellarin protects against lipopolysaccharide-induced acute lung injury via inhibition of NF-κB activation in mice', Journal of Asian Natural Products Research, 12: 3, 175 — 184
To link to this Article: DOI: 10.1080/10286020903347906
URL: http://dx.doi.org/10.1080/10286020903347906

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Journal of Asian Natural Products Research
Vol. 12, No. 3, March 2010, 175–184

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ORIGINAL ARTICLE

Scutellarin protects against lipopolysaccharide-induced acute lung injury via inhibition of NF-κB activation in mice

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(*Received 3 July 2009; final version received 16 September 2009*)

This paper investigates the effect of natural scutellarin on acute lung injury (ALI) induced by *Escherichia coli* endotoxin lipopolysaccharide (LPS) in mice and its mechanism of action. Mouse ALI was induced by the injection of LPS (15 mg/kg) via the tail vein, and mice were intraperitoneally injected with 50 and 25 mg/kg of scutellarin before the LPS injection. The lung index, serum NO_2^-/NO_3^-, and tumor necrosis factor-alpha (TNF-α) levels were determined using kits. The lung lesions were examined by light microscope. The mRNA levels of TNF-α, inducible nitric oxide synthase (iNOS), and FasL in pulmonary tissues were detected by RT-PCR. c-Fos, c-Jun, IκB, and iNOS proteins were detected by the western blotting method. Pretreatment with 25 and 50 mg/kg of scutellarin significantly reduced lung injury induced by LPS, which expressed in the decrease in lung morphological lesions, serum NO_2^-/NO_3^-, TNF-α levels, lactate dehydrogenase release, and total protein in the lavage fluid of bronchoalveolar of the lung. The mRNA level of TNF-α, iNOS, the protein content of c-Fos, iNOS, and the activation of NF-κB in pulmonary tissues were all inhibited, while the lung glutathione level increased. In conclusion, scutellarin has protective action against LPS-induced lung damage in mice, and its underlying mechanism might be the inhibition of IκBα degradation and the expression of TNF-α mRNA.

Keywords: scutellarin; LPS; iNOS; NF-κB; acute lung injury

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1. Introduction

During the combating with Severe Acute Respiratory Syndrome (SARS) in 2003 in China, some traditional Chinese medicines were applied for the treatment of SARS-induced lung injury in clinic, and were also screened in experimental lung injury models. In the authors' laboratory, some natural compounds from Chinese herbs were tested for experimental acute lung injury (ALI) induced by the injection of *Escherichia coli* endotoxin lipopolysaccharide (LPS) in mice. The reason forusing LPS-induced mouse ALI model for testing compounds was based on the facts that LPS is a commonly used endotoxin for reproducing model of acute lung inflammation and injury, and inducible nitric oxide synthase (iNOS) was found to play an important role in LPS-induced lung injury and mortality [1]. Furthermore, accumulated evidence supported that free radicals also play an important rule in ALI. The inflammatory

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ISSN 1028-6020 print/ISSN 1477-2213 online
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DOI: 10.1080/10286020903347906
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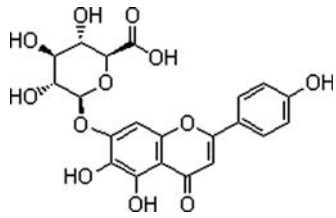

Figure 1. Chemical structure of **1** ($C_{21}H_{18}O_{12}$, MW: 462.4).

disease of the respiratory tract such as acute respiratory distress syndrome (ARDS) is commonly characterized by an increased expression of iNOS within respiratory epithelial and inflammatory immune cells, and a markedly elevated local production of nitric oxide (NO) [2–4]. The concentration of NO increased in the bronchoalveolar lavage fluid (BALF) of the ARDS patient. At the 3rd and 7th day, the concentration of NO in the BALF of the patient who died later was much higher than that of others [2].

Scutellarin (**1**, 4′,5,6-trihydroxylflavone-7-*O*-glucuronoside; Figure 1) is an active ingredient extracted from *Erigeron breviscapine* (*vant*) Hand Mass. The injection made of **1** has been used in the treatment of cardio-cerebral vascular diseases in China [5]. Scutellarin was reported to have scavenging activity of hydroxyl radicals, superoxide anion radicals, and hydrogen peroxide *in vitro* [6], and reduce secondary liver injury elicited due to brain ischemia–reperfusion in rats [7]. Scutellarin was also shown to have hepatoprotective action against experimental liver toxicity of selenium in rats [8]. Our previous study found that **1** has protective action against concanavalin A-induced liver injury via inhibiting the NF-κB–tumor necrosis factor-alpha (TNF-α)–iNOS transduction pathway in mice [9]. Thus, the authors of the present paper presumed that **1** might have a therapeutic effect on ALI in mice. We found that **1** significantly protected against LPS-induced ALI in mice. The results are described in the present paper.

2. Results and discussion

2.1 Scutellarin reduced lung lesions induced by LPS in mice

Histological examination of the lung lesions was performed 24 h after the LPS injection. As shown in Figure 2, the wall of the alveolus became thicker in the lung of LPS-injected mice compared with normal control mice, and there was filtration of lymphocytes and monocytes in the lung. Pretreatment with **1** significantly ameliorated the lung injury induced by LPS (Figure 2).

2.2 Scutellarin improved the alternations of lung index, malondialdehyde, and glutathione levels in lung tissues, serum NO_2^-/NO_3^-, and TNF-α levels induced by LPS in mice

After the injection of LPS, the lung index and malondialdehyde (MDA) level in pulmonary tissues increased at 4 h, and they are still higher than normal control

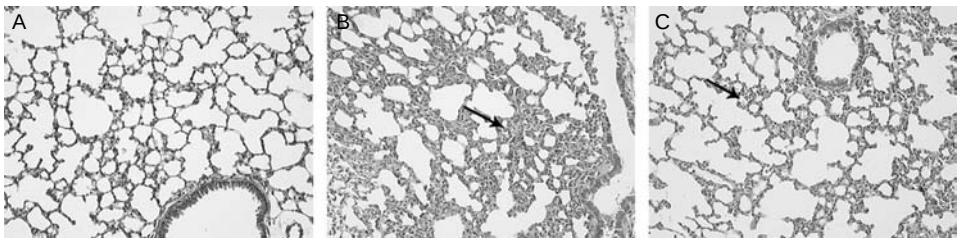

Figure 2. Effect of **1** on the pathology of lung lesions induced by LPS in mice. (A) Control; (B) LPS and (C) **1** + LPS. Hematoxylin–eosin staining.

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Table 1. Effects of LPS on the lung index and the levels of GSH and MDA in the lung of mice.

Time (h)	Dose (mg/kg)	No. of mice	MDA (nmol/l)	GSH (nmol/l)	Lung index (mg/10 g body weight)
0	NS	8	5.5 ± 0.7	3.5 ± 0.5	70.8 ± 6.5
4	15	8	4.5 ± 0.6*	3.9 ± 0.5	75.1 ± 5.3
24	15	7	6.1 ± 0.7	2.4 ± 0.4**	90.3 ± 8.3**
48	15	8	6.9 ± 0.7**	3.3 ± 0.5	91.8 ± 8.4**

Notes: *$P < 0.01$, **$P < 0.001$ vs. controls (0 h).

mice at 48 h. The content of lung glutathione (GSH) increased a little at 4 h, then decreased sharply at 24 h, and restored to the normal level at 48 h (Table 1). The level of NO_2^-/NO_3^- in the serum gradually increased, reached the peak at 8 h, and then decreased slowly. The serum TNF-α level increased maximum at 1 h, and then decreased sharply, but it is still higher than that of normal control mice at 8 h (Table 2).

Based on the above results, the effects of **1** on MDA, GSH, NO_2^-/NO_3^-, and TNF-α levels were determined at different time points. MDA, GSH, NO_2^-/NO_3^-, and TNF-α were measured at 4, 24, 8, and 1 h, respectively**.**

As shown in Tables 3–5, pretreatment with 50 mg/kg of **1** significantly increased the lung GSH level, and decreased the lung MDA (Table 3) and serum NO_2^-/NO_3^- levels (Table 4) in mice after the LPS injection. The effect of **1** on the lung index was not statistically significant.

For examining the effect of **1** on TNF-α level in LPS-injected mice, the TNF-α level in the serum and BALF was assayed. One hour after the injection of LPS, the levels of TNF-α in the serum and BALF all increased significantly. The serum TNF-α level increased from 169 pg/ml of normal control to 5203 pg/ml of LPS-injected mice. Meanwhile, the level of TNF-α in the BALF also increased from 12 pg/ml of normal control to 1083 pg/ml of LPS-injected mice. When mice were pretreated with 50 mg/kg of **1**, the elevated TNF-α in the BALF and serum all decreased significantly (Table 5).

The data of the effect of **1** on the levels of lactate dehydrogenase (LDH) and proteins in the BALF of mice injected with LPS are listed in Table 6. Also, the levels of LDH and proteins in the BALF significantly increased at 4 h after the injection of LPS. Pretreatment with 50 mg/kg of **1** significantly decreased the LDH and protein levels in the BALF in LPS-injected mice (Table 6).

2.3 Scutellarin decreased expressions of TNF-α mRNA and iNOS mRNA in lung tissues of LPS-injected mice

At first, the expressions of TNF-α, interferon-gamma (IFN-γ), FasL, and iNOS mRNA in pulmonary tissues of

Table 2. Effects of LPS on the levels of TNF-α and NO_2^-/NO_3^- in the serum in mice.

Time (h)	Dose (mg/kg)	No. of mice	NO_2^-/NO_3^- (μmol/l)	TNF-α (pg/ml)
0	NS	5	18.5 ± 5.1	45.9 ± 4.3
1	15	5	11.8 ± 5.8	2625.9 ± 22.7**
2	15	5	40.6 ± 14.0*	1739.8 ± 471.1**
4	15	5	114.8 ± 11.2**	500.4 ± 187.1**
8	15	5	251.3 ± 38.1**	218.7 ± 46.9**
24	15	5	113.7 ± 71.7*	

Notes: *$P < 0.05$, **$P < 0.001$ vs. controls (0 h).

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Table 3. Effect of **1** on the lung index and levels of GSH and MDA in the lung of LPS-injected mice.

Groups	Dose (mg/kg)	No. of mice	Lung index (mg/10 g body weight)	GSH (nmol/g lung tissue)	MDA (nmol/g lung tissue)
Controls		10	74.1 ± 4.4*	0.43 ± 0.06*	0.49 ± 0.10**
LPS		10	82.1 ± 7.4	0.38 ± 0.04	0.61 ± 0.07
Scutellarin	25	9	79.5 ± 5.6	0.42 ± 0.04	0.56 ± 0.10
Scutellarin	50	9	77.6 ± 7.4	0.45 ± 0.04*	0.45 ± 0.07***

Notes: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. LPS.

Table 4. Effect of **1** on the level of NO_2^-/NO_3^- in the serum and lung tissues in mice injected with LPS.

Groups	Dose (mg/kg)	No. of mice	Serum NO_2^-/NO_3^- (μmol/l)	Lung tissue NO_2^-/NO_3^- (μmol/mg protein)
Controls		10	37.9 ± 33.4***	2.5 ± 1.8
LPS		11	329.2 ± 110.4	2.6 ± 2.2
Scutellarin	25	11	287.7 ± 92.2	2.6 ± 3.1
Scutellarin	50	10	238.3 ± 78.1*	0.5 ± 0.5**

Notes: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. LPS.

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Table 5. Effect of **1** on the level of TNF-α in the serum and BALF in LPS-injected mice.

Groups	Dose (mg/kg)	No. of mice	Serum TNF-α (ng/ml)	TNF-α in BALF (ng/ml)
Control		5	0.169 ± 0.076**	0.012 ± 0.008**
LPS	15	8	5.203 ± 0.281	1.083 ± 0.564
Scutellarin	50	8	4.372 ± 0.796*	0.471 ± 0.413*

Notes: *$P < 0.05$, **$P < 0.001$ vs. LPS.

Table 6. Effect of **1** on the levels of LDH and protein in the BALF of mice injected with LPS.

Groups	Dose (mg/kg)	No. of mice	LDH (mmol/l)	Protein (g/l)
Control		9	0.70 ± 0.09***	1.02 ± 0.24**
LPS	15	9	1.09 ± 0.22	84.3 ± 17.3
Scutellarin	50	10	0.91 ± 0.14*	1.07 ± 0.27*

Notes: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. LPS.

mice injected with LPS were determined. As can be seen from Figure 3, all TNF-α, IFN-γ, FasL, and iNOS mRNA in pulmonary tissues of normal mice were very low. After the injection of LPS, the expression of TNF-α increased rapidly at 1 h and iNOS mRNA at 4 h, while the IFN-γ and FasL mRNA expressions were not enhanced by the injection of LPS (Figure 3).

Based on the above evidence, the effects of **1** on the expressions of TNF-α and iNOS mRNA in the lungs of LPS-injected mice were selectively determined. Again, the LPS injection induced the expressions of TNF-α mRNA at 1 h and iNOS mRNA at 6 h in the lungs of

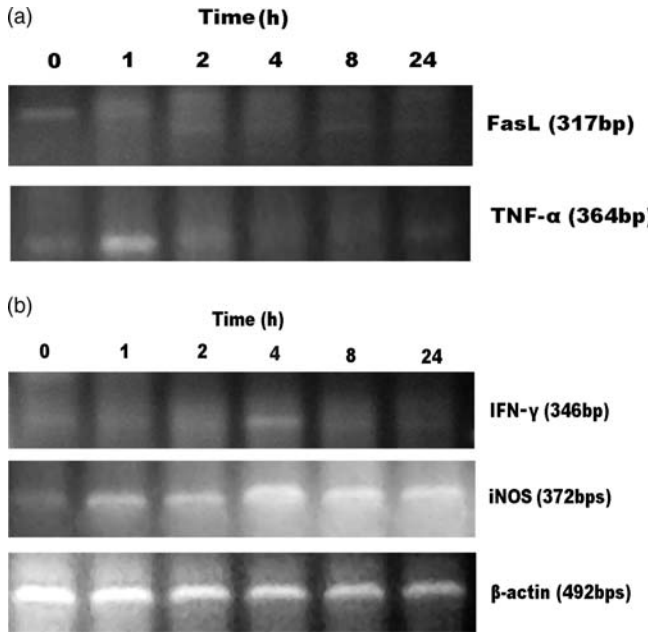

Figure 3. Expression of (a) TNF-α, FasL and (b) IFN-γ, iNOS, and β-actin mRNA in the lung at different times after the injection of LPS in mice.

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mice. Pretreatment with **1** significantly decreased the expressions of TNF-α mRNA and iNOS mRNA in the lungs of LPS-injected mice (Figure 4).

2.4 Scutellarin inhibited expressions of c-Fos, iNOS, and IκBα in the lungs of LPS-induced mice

The protein expression of c-Fos, c-Jun, and IκBα in the lung at 1 h after the LPS injection, and iNOS at 8 h after the LPS treatment was detected by the western blotting method. From the visualized protein bands in Figure 5, it may be seen that the levels of c-Fos, c-Jun, and iNOS proteins in the lungs of normal group mice were low. After the LPS injection, the protein bands of c-Fos, c-Jun, and iNOS in the lung were visualized. Prior administration of 50 mg/kg of **1** reduced the expression of c-Fos and iNOS proteins in the lung stimulated by the LPS injection, but **1** showed no effect on the c-Jun protein expression. The IκBα protein level in the

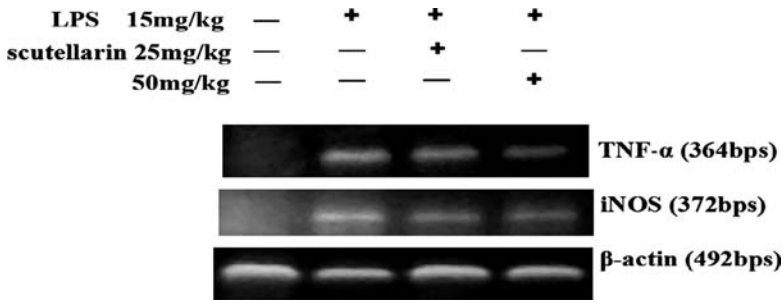

Figure 4. Effects of **1** on the expression of TNF-α mRNA and iNOS mRNA in the lung of LPS-injected mice.

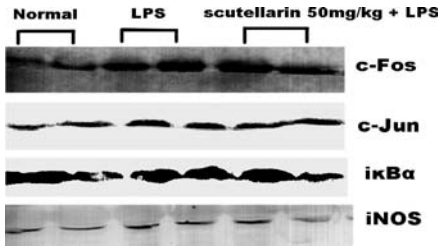

Figure 5. Effects of **1** on the expression of IκBα, iNOS, c-Jun, and c-Fos in the lung of LPS-injected mice.

lungs of normal mice was higher. While after the injection of LPS, the IκBα protein level in the lung decreased, indicating that the IκBα degradation from NF-κB increased. The IκBα protein in the lung of mice treated with 50 mg/kg of **1** was near to that of normal mice. In the western blotting detection of the above biomarkers, two lungs from each group were measured, and the determination of every sample was repeated twice. Because there were only two samples per group, the protein bands were not measured quantitatively by densitometry (Figure 5).

2.5 Discussion

It was reported that the increase in protein content in the BALF and lung edema was the main characteristic pathology of ALI and ARDS [10]. Administration of LPS induced high level of TNF-α production in animal models and reproduced many common features of septic shock with severe pro-inflammatory reactions [11]. Inflammation is believed to be the key event of TNF-α-dependent pathophysiological events. Deregulated recruitment of leukocytes and lymphocytes at the inflammed foci leads to injury. TNF-α also depletes cellular GSH, a cellular antioxidant [12]. Overexpression of TNF-α in transgenic mice induces differential changes in redox status and GSH-regulating enzymes by depleting the total cellular GSH levels [13]. Both *in vitro* and *in vivo* studies show that TNF-α stimulates the reactive oxygen species generation from pulmonary and non-pulmonary tissues [14]. In the present study, we found that the injection of LPS increased the levels of TNF-α and NO_2^-/NO_3^- in the serum, MDA in the lung and protein content in the BALF mice. Scutellarin (**1**) significantly decreased the MDA content and increased the GSH level, which may be related to the inhibition of the TNF-α expression.

The inflammatory response of cells to TNF-α is mediated, in part, by the regulation of gene expression by the AP-1 and NF-κB groups of transcription factors [15]. In turn, AP-1 and NF-κB participated in starting the inflammatory effect of TNF-α, and mediated the synthesis of TNF-α induced by LPS. Full LPS response of the TNF gene requires both NF-κB and non-NF-κB nuclear proteins [16].

AP-1 is a complex which composes proteins of the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) proto-oncogene families. Fos and Jun family proteins function as dimeric transcription factors that bind to AP-1 regulatory elements in the promoter and enhancer regions of genes [17–19]. NF-κB is a family of dimeric transcription factors involved in immune and inflammatory responses and apoptosis. In unstimulated cells, IκB protein localizes NF-κB dimers in the cytoplasm by masking the nuclear localization sequence of NF-κB. The activation of NF-κB can be induced by various stimulations, including cytokines and oncogenic signals. Once IκB is degraded, the nuclear localization sequence of NF-κB is unmasked, allowing nuclear accumulation, DNA binding, and transcriptional activation of target genes [20]. Several genes associated with the inflammatory process, including iNOS, cyclooxygenase-2 (COX-2), TNF-α, and others, contain putative NF-κB binding sites within their promoters [21]. NF-κB decoy markedly reduced the expression levels of iNOS, COX-2, and others in the

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septic lung tissue [22]. Our results showed that the injection of LPS significantly enhanced the production of c-Jun and c-Fos, reduced the degradation of IκBα protein, enhanced the expression of iNOS mRNA and protein in the lungs, and increased the level of NO_2^-/NO_3^- in the serum of mice. Prior administration of **1** reduced the expression of c-Fos protein and the degradation of IκBα from NF-κB, expressed as the increase in IκBα protein in the lungs of LPS-injected mice. There is a possibility that **1** downregulates the activation of AP-1 (although not direct assay) and NF-κB by decreasing the expression of c-Fos and degradation of IκBα, and thereby inhibited the expressions of TNF-α and iNOS mRNA, decreased the levels of NO_2^-/NO_3^- in the serum and BALF, LDH activity and total protein content in the BALF, and, finally, reduced the lung lesions induced by LPS in mice.

In summary, **1** has protective action against lung damage induced by LPS in mice, and its active mechanism might be related to blocking IκBα degradation.

3. Materials and methods

3.1 Reagents

Scutellarin (**1**, purity > 95%) was purchased from Kunming Longjin Pharmaceutical Co. Ltd (Kunming, China). LPS was obtained from Sigma Chemical Co. (St Louis, MO, USA). TRIZOL reagent was a product of Invitrogen (Carlsbad, CA, USA). Access RT-PCR System was obtained from Promega Co. (Madison, WI, USA). Quantikine Mouse TNF-α kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). NO_2^-/NO_3^- kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

3.2 Animals

Male Institute of Cancer Research mice weighing 22–24 g were obtained from Beijing Weitong Lihua Animal Company (Beijing, China). The mice were housed five per cage in a thermoregulated environment (23 ± 1°C, 50 ± 5% humidity) under a 12 h light–dark cycle. The mice accessed food and water *ad libitum.* All animal experiments were performed in accordance with the instructions of the Ethical Committee for Care and Welfare of Laboratory Animals from Beijing municipal government.

3.3 Methods

3.3.1 Time course of LPS-induced lung injury in mice

Mice were injected with a dose of LPS (15 mg/kg) or a corresponding volume of normal saline via the tail vein, respectively. The mice were killed at 1, 2, 4, 8, and 24 h after the LPS injection, respectively, for different measurements. The concentration of NO_2^-/NO_3^- and TNF-α in the serum, activity of LDH and total protein in the BALF were measured using kits. The wet lung was weighed and divided by the body weight as the lung index. The contents of GSH and MDA, and the expressions of iNOS, TNF-α, FasL, and IFN-γ mRNA in the pulmonary tissues were measured with different methods.

3.3.2 Effect of *1* on LPS-induced ALI in mice

At 13, 7, and 1 h prior to the LPS injection, 25 and 50 mg/kg of **1** (dissolved in normal saline) were intraperitoneally administered for three times. The control group received the same volume of normal saline instead of **1**. The mice were killed at 1, 4, 6, 8, and 24 h after the LPS injection, respectively, for different measurements.

3.3.3 RT-PCR detection of TNF-α, IFN-γ, FasL, and iNOS mRNA in lung tissues

Mice were killed at 1, 2, 4, 8, and 24 h after the injection of LPS. The lungs were

removed and placed in liquid nitrogen for the detection of TNF-α, IFN-γ, FasL, and iNOS mRNA by RT-PCR. The total RNA of the lung was extracted using Trizol reagent according to the manufacturer's instructions (Gibco Industries Inc., Langley, OK, USA). The primers were used as follows: 5′-GAC AGC AGT GCC ACT TCA TC-3′ and 5′-TTA AGG CTT TGG TTG GTG AA-3′ (317 bps) for FasL; 5′-GGC GGT GCC TAT GTC TCA G-3′ and 5′-GGG CAG CCT TGT CCC TTG A-3′ (364 bps) for TNF-α; 5′-CTC AAG TGG CAT AGA TGT GG-3′ and 5′-ACT CCT TTT CCG CTT CCT GA-3′ (346 bps) for IFN-γ; 5′-GCC TCA TGC CAT TGA GTT CAT CAA CC-3′ and 5′-GAG CTG TGA ATT CCA GAG GCC TGA AG-3′ (372 bps) for iNOS; and 5′-CTC CTA CCA CAC CCA TTC TCA TCC-3′ and 5′-GCA ATG CCT GGG TAC ATG GTG G-3′ (492 bps) for β-actin. The β-actin gene was amplified as an internal control. PCR conditions were 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 45 s. PCR products were separated by electrophoresis through 2% agarose containing 0.5 μg/ml ethidium bromide. The DNA bands were imaged using a Kodak digital imaging system (Kodak DC120, Digital Science 1D system, Rochester, NY, USA).

3.3.4 Detection of the expression of IκBα, c-Jun, c-Fos, and iNOS in lung tissues by the western blotting method

One hour after the injection of LPS, the mice were killed, and the lung was removed and placed in liquid nitrogen for the detection of the expression of IκB, c-Fos, c-Jun, and iNOS. The lung tissues were homogenized in RIPA lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 1 mM ethylenediaminetetraacetic acid, 1% TritonX-100, 0.5% Nonidet P40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF) at 4°C, and centrifuged at 10,000 *g* at 4°C for 30 min. The supernatants were mixed in Laemmli loading buffer, boiled for 4 min, and then subjected to SDS-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose membranes and blotted against primary antibodies for 2 h. The membranes were washed with tris-buffered NaCl solution with Tween-20 (20 mM Tris base, 150 mM NaCl, 0.05% Tween-20), and incubated with a 1:300 dilution of horseradish peroxidase-conjugated secondary antibodies for 2 h. The protein bands were visualized by the diaminobenzidine system and were scanned.

3.3.5 Lavage of lung bronchoalveolar

The mice were killed at 1, 2, 4, 8, and 24 h after the LPS injection. Moreover, their tracheas were exposed. The lavage of bronchoalveolar was performed by the injection of 1.0 ml of phosphate buffer solution (PBS) at pH 7.4 through a plastic catheter, and the fluid was collected by gentle suction. The BALF was centrifuged for 10 min at 1800 rpm, and the supernatant was collected for the measurement of the LDH, TNF-α, total protein, and NO_2^- /NO_3^- levels.

3.3.6 TNF-α assay

The TNF-α in the serum and BALF was measured by ELISA using Quantikine Mouse TNF-α kit according to the manufacturer's instructions (R&D Systems).

3.3.7 Measurement of LDH activity

The activity of LDH in the BALF was measured using the biochemical kits according to the manufacturer's instruction.

3.3.8 Measurement of GSH

The lung tissues from mice were homogenized in PBS at pH 7.4. The lung homogenate (0.5 ml) was mixed with the same volume of 4% sulfosalicylic acid

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dehydrate, and centrifuged at 2500 rpm at 4°C for 10 min. The 0.5 ml supernatant was mixed with 4.5 ml of 0.004% dithionitrobenzoic acid, and placed at room temperature for 10 min. The absorbance was measured at 412 nm on a spectrophotometer [23].

3.3.9 Measurement of MDA

The lung tissues were homogenized in PBS at pH 7.4 as above. The lung homogenate (0.2 ml) was added to 3% SDS (0.5 ml), 20% acetic acid buffer (pH 3.5, 1.5 ml) and 1% 2-thiobarbituric acid (1.5 ml), mixed and heated at 95°C for 60 min, and then centrifuged at 3500 rpm for 15 min after cooling in water. Absorbance of the aliquots of the supernatant was measured at 535 nm by spectrophotometry [24].

3.3.10 Measurement of NO_2^-/NO_3^- and total protein

The concentration of NO_2^-/NO_3^- in the serum and BALF was measured using related biochemical kits according to the manufacturer's instruction. Protein content in the BALF was measured by the Lowry method [25].

3.3.11 Examination of lung lesions by light microscope

Twenty-four hours after the injection of LPS, the mice were killed. The lung was removed and processed into sections of 5 μm thick, and stained with hematoxylin–eosin. The lung lesions were examined by a pathologist who was blinded.

3.3.12 Statistical analysis

All the data are expressed as means ± SD. The differences in the biomarkers between groups were analyzed using one-way analysis of variance test, and then the individual differences between groups were evaluated using Dunnett's test.

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Acknowledgement

This work was financially supported by a grant (2003CB514128) from the Ministry of Science and Technology of China.

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