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Platycodon grandiflorum extract attenuates lipopolysaccharide-induced acute lung injury via TLR4/NF-κBp65 pathway in rats

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Abstract: The traditional Chinese medicine *Platycodon grandiflorum* (PG) is often used for the treatment of a number of chronic inflammatory diseases. In Chinese veterinary clinic, PG is always extracted by decoction and taken orally, however, the molecular mechanism of PG extract (PE) to reduce LPS-induced inflammation, especially acute lung injury (ALI) *in vivo*, are not known. Thus, we have studied the anti-inflammatory effects of PE on lipopolysaccharide (LPS)-induced acute lung injury via TLR4/NF-κBp65 pathway in rat. Sprague-Dawley rats were randomly divided into 4 groups: control group, LPS group, LPS+PE low dose group and LPS+PE high dose group. All rats were given corresponding PE solution or the same amount of normal saline by intragastric administration for 7 days. On the 7th day, 1 h after the last administration, 500 μg of LPS were introduced intratracheally to establish ALI rat model, and the same volume of normal saline was given to control group. The results showed that PE reduced the levels of LPS-induced pro-inflammatory mediators including IL-6, PGE2, and TNF-α, alleviated the lung injury histologically, and down-regulated LPS-induced mRNA and protein levels of TLR4/NF-κBp65 in lung tissue. This study demonstrated that PE has the anti-inflammatory effects on LPS-induced ALI in rats through TLR4/NF-κBp65 signaling pathway, indicating that PE is an effective suppressor for anti-inflammatory activities.

Keywords: *Platycodon grandiflorum* extract, acute lung injury, lipopolysaccharide, inflammation, TLR4/NF-κBp65 pathway

INTRODUCTION

Acute lung injury (ALI) is a serious health disorder associated with a high mortality rate of 30–40% (Matthay *et al*., 2003). Lipopolysaccharide (LPS), a major outer membrane constituent of gram negative bacteria, is the major pathogenic factor that induces inflammatory reactions, and ultimately leading to ALI (Chen *et al*., 2010). Administration of LPS has been used to prepare ALI animal models in numerous studies (Li *et al*., 2018).

Toll-like receptors (TLRs) are transmembrane receptors that recognize pathogen-associated molecular patterns and damage-associated molecular patterns (Deng *et al*., 2017). Various studies have confirmed that LPS mainly activates Toll-like receptor 4 (TLR4) signaling pathway and triggers an inflammatory response in ALI (Imai *et al*., 2008). TLR4 can activate LPS-mediated nuclear factor-κB (NF-κB), subsequently lead to the synthesis and release of several pro-inflammatory mediators (Wan and Lenardo, 2010; O'Banion, 1999).

Platycodon grandiflorum (PG) is a perennial plant growing in China, Korea and Japan (Wang *et al*., 2004). The root of PG is usually been used as an expectorant and a remedy for conditions such as coughing, asthma, pulmonary tuberculosis and inflammation (Jang *et al*., 2013). Platycodin D and radix platycodonis polysaccharide are the main active constituents of PG. Several studies have reported that platycodin D exhibit anti-inflammatory activity (Wang *et al*., 2004; Jang *et al*., 2013; Chung *et al*., 2008; Zhang *et al*., 2015). In addition, polysaccharides extracted from PG could activate various cell types of the innate and adaptive immune systems and exert significant immunological activity (Zhao *et al*., 2017; Yoon *et al*., 2003). In Chinese veterinary clinic, PG is always extracted by decoction and taken orally. However, up to now, rarely attention has been paid in the molecular mechanism of PG extract (PE) to attenuate LPS-induced inflammation, especially ALI *in vivo*. In this research, we have established the ALI rat model by LPS, evaluated the effects of PE on the expression levels of TLR4/NF-κBp65 and its downstream signaling molecules and investigated the underlying molecular mechanisms of its anti-inflammation.

MATERIALS AND METHODS

Chemicals and reagents

The herbal medicine was purchased from Tongrentang Pharmacy Products (Chengdu, PR China) certified by China Food and Drug Administration (CFDA). The preparation method of PE was modified based on a study

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of Liang (2009): *Platycodon grandiflorum* (10 kg) was extracted twice with 30% ethanol (each time refluxed for 2 hrs). The ethanol extract was concentrated with a vacuum evaporator, filtered, lyophilized and then stored at 4ºC until use. The freeze-dried powder was approximately 22.0% (w/w). Previous studies (Zhang *et al*., 2017; Huang *et al*., 2013) have demonstrated that both platycodin D and polysaccharides are the main active constituents in PE. In the present study, the levels of platycodin D and polysaccharides were used as chemical markers for the quality control of PE. The final freeze-dried powder contained 8.25 mg/g platycodin D and 27.96 mg/g polysaccharides.

LPS (*Escherichia coli* 055:B5L2880) was bought from the Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits (IL-6, TNF-α and PGE2) were from Nanjing KeyGEN Biotech Co., Ltd. (Nanjing, PR China). Primary antibodies for NF-κBp65, TLR4, COX-2 and GAPDH were from Wuhan Sanying Biotechnology Co., Ltd. (Wuhan, P.R.China). Secondary antibodies (Goat Anti-Rabbit IgG (H+L); HRP-conjugated Goat Anti-Mouse IgG) were bought from BBI Life Science Co., Ltd. (Shanghai, PR China). All other chemicals were of analytical grade or better.

Animals

Male Sprague-Dawley (SD) rats (n=32) weighing 200±20 g were obtained from Chengdu Dashuo Experimental Animal Co., Ltd. (Chengdu, PR China) (License number: SCXK2015-030). All animals were maintained at the laboratory animal research center of Sichuan Animal Science Academy in house cages under standard laboratory conditions (temperature of 22 ± 2ºC, and a natural light-dark cycle). The rats were fed a regular diet and allowed access to water *ad libitum*. All experimental procedures used for this study were performed in accordance with the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996) and prospectively reviewed and approved by the Ethics Committee of Sichuan Animal Science Academy.

Experimental design

SD Rats were randomly divided into four groups (n=8, each): control group; LPS group (8 mg/kg); LPS (8 mg/kg)+PE low dose group (10 mg/kg) and LPS (8 mg/kg)+PE high dose group (50 mg/kg) (table 1). All rats were given corresponding PE solution or the same amount of normal saline by intragastric administration once each morning for 7 days. On the 7th day, 1 h after the last administration, 1600 μg of LPS (8 mg/kg body weight) were introduced intratracheally to establish the ALI rat model, and the same volume of normal saline water was received in control group (Hu *et al*., 2018).

After 6 h, all rats were killed under anesthetic ether. Blood samples were centrifuged at 4000 rpm for 5 min, and separated plasma was stored at -80ºC. Each lung sample was removed quickly, the upper lobe and the middle lobe of the right lung tissues were fully fixed and stored with 4% paraformaldehyde, the remaining lung tissues were washed with ice-cold normal saline, blotted dry, and frozen at -80ºC.

Table 1: Administration of reagents in each group

	Control group	LPS group	LPS+PE low dose group	LPS+PE high dose group
LPS	-	8 mg/kg	8 mg/kg	8 mg/kg
PE	-	-	10 mg/kg	50 mg/kg

Pathological observation of the lung (H&E)

After fixed and stored with 4% paraformaldehyde at least 24 h, the lung tissues were embedded in paraffin and cut into 5 μm section for conventional hematoxylin and eosin (H&E) staining. A light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) were used to observe the pathological changes of the lung tissues.

Enzyme-linked immunosorbent assay of IL-6, PGE2, and TNF-α

*The l*evels of pro-inflammatory mediators (IL-6, PGE2, and TNF-α) in plasma samples were quantified in accordance with the manufacturer's instructions (Nanjing Jiancheng Bioengineering institute, Nanjing, P.R.China).

Real-time PCR analysis

Total RNA from each sample was extracted by use of TRIzol reagent (Invitrogen Corporation and Applied Biosystems, Inc., Carlsbad, CA, USA) according to the manual. RNA concentration, purity and integrity was measured in accordance with our previous study (Li *et al*., 2017). RNA samples (5 μl) were reverse transcribed to cDNA using RevertAid Premium Reverse Transcriptase (Thermo Fisher Scientifc Inc., Chelmsford, MA, USA) according to the manual. The cDNA products were frozen and stored at -80ºC until assay.

Real-time PCR was carried out by ABI StepOne RT-PCR (Applied Biosystems, Foster City, CA, USA) in a 20 μl final volume that contained 10 μl High RoxSybrGreen qPCR Master Mix (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, P.R.China), 2 μl cDNA, 0.4 μl of each oligonucleotide primer (10 μM), and 7.2 μl diethyl pyrocarbonate-treated autoclaved distilled water on the StepOne RT-PCR instrument. Real-time PCR amplification was performed under the following conditions: initial denaturation at 95ºC for 3 min, followed by 45 cycles of denaturation at 95ºC for 3 sec, annealing and extension at 60ºC for 30 sec. GAPDH house-keeping gene was used as the control. The Real-time PCR data was calculated by the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen TD, 2001). The sequences of the forward and reverse primers are presented in table 2.

Table 2: Sequences of the forward and reverse primers used for real-time RT-PCR

Gene	Forward	Reverse
TLR4	CAGAATGAGGAC TGGGTGAGA	TTGGCAGCAATGGCTA CAC
NF-κBp65	TCAATGGACCAAC TGAACCC	ATGTTGAAAAGGCATA GGGC
COX-2	GTATGCTACCATC TGGCTTCG	GTGTTGCACGTAGTCT TCGATC
GAPDH	CAAGTTCAACGG CACAGTCAA	CGCCAGTAGACTCCAC GACA

Western blot analyses

The total protein of the lung tissues was extracted (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, P.R.China) and the protein concentrations were quantified by the bicinchoninic acid (BCA) method (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, P.R.China). Protein extracts (40 μg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Sangon Biotech (Shanghai) Co., Ltd., Shanghai, P.R.China) and then electrotransferred to polyvinylidene difluoride membranes (PVDF; Guangzhou Ewell Bio-technology Co., Ltd., Guangzhou, P.R.China). The immunoblot was incubated with TBST (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20) containing 5% skim milk for 1 h at room temperature, followed by incubation overnight with an appropriate dilution of primary antibodies at 4ºC. Membranes were washed e four times in Tween 20/Tris-buffered saline (TBST) and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein immunoreactive bands were visualized with an enhanced chemiluminescence solution (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, P.R.China) and their densities were measured by Image J software.

STATISTICAL ANALYSIS

All data were presented as mean ± SD from 3 independent experiments at least. Statistical significant values were evaluated by One-way ANOVA, using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). In all tests, P-values less than 0.05 were considered to be statistically significant.

RESULTS

Effect of PE on LPS-induced rat lung histopathological changes

As shown in fig. 1A, no histopathological changes were observed in the lung tissues of rats in control group. While, in LPS group, the lung tissues displayed several obvious inflammatory changes. These changes included severe inflammatory cells infiltration, and thickening of the alveolar wall (fig. 1B). In LPS+PE groups (10 mg/kg; 50 mg/kg), LPS-induced pathological damage was significantly attenuated (fig. 1C and fig. 1D).

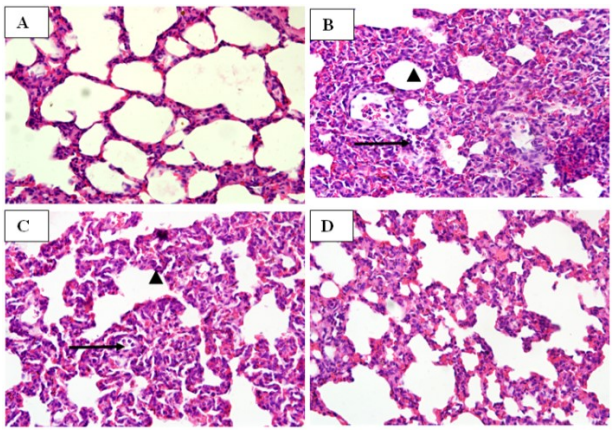

Fig. 1: Rat lung photomicrographs (H & E, 400×) from: (A) No pathological changes observed in control group; (B) LPS group showing marked tissue damage, thickening of the alveolar wall, inflammatory cell infiltration; (C) LPS + PE low dose group showing marked improvement in tissue damage, thickening of the alveolar wall, and inflammatory cell infiltration; (D) LPS + PE high dose group showing marked improvement in the histological picture with minimal damage. The black triangle indicates thickening of the alveolar wall; the black arrow indicates inflammatory cell infiltration.

Effect of PE on the levels of LPS-induced pro-inflammatory mediators

The effects of PE on the levels of IL-6, PGE2, and TNF-α were presented in fig. 2. The results revealed the levels of IL-6, PGE2, and TNF-α in LPS group were significantly increased compared with corresponding values in control group ($P<0.05$). However, after intragastric administration of PE, the levels of IL-6, PGE2, and TNF-α in LPS+PE low and high dose groups were significantly inhibited compared with the levels in LPS group, respectively ($P<0.05$).

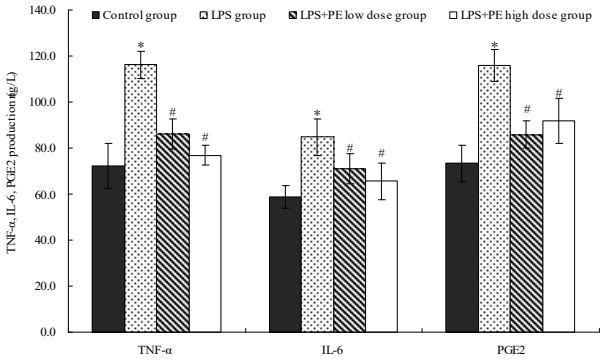

Fig. 2: Effect of PE (LPS + PE low dose group: 10 mg/kg BW for 7 days; LPS + PE high dose group: 50 mg/kg BW for 7 days) on the levels of LPS-induced TNF-α, IL-6 and PGE2 in plasma (n=8). *Significantly different from control group, $P < 0.05$. # Significantly different from LPS group, $p < 0.05$.

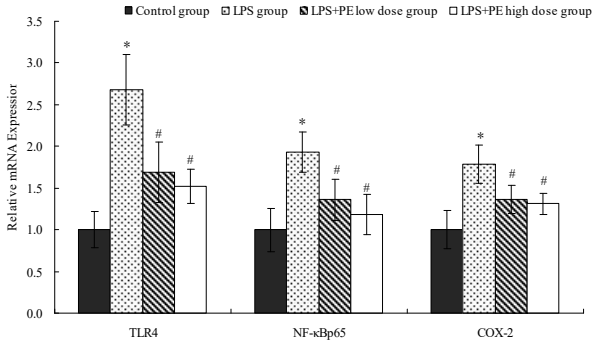

Figure 3: Effect of PE (LPS + PE low dose group: 10 mg/kg BW for 7 days; LPS + PE high dose group: 50 mg/kg BW for 7 days) on mRNA expression of LPS-induced TLR4, NF-κBp65 and COX-2 in the lung (n=8). Results are calculated from three independent experiments performed in triplicate measurements. *Significantly different from control group, $p < 0.05$. # Significantly different from LPS group, $p < 0.05$.

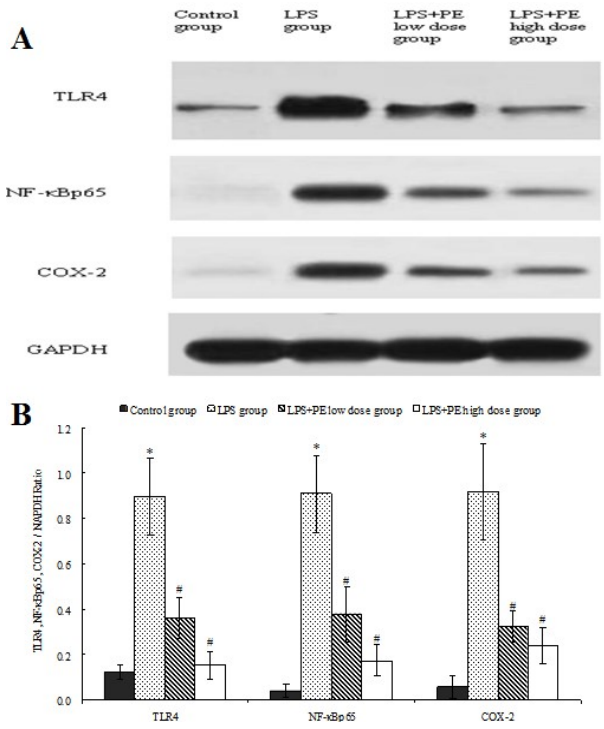

Figure 4: Effect of PE (LPS + PE low dose group: 10 mg/kg BW for 7 days; LPS + PE high dose group: 50 mg/kg BW for 7 days) on protein expression levels of LPS-induced TLR4, NF-κBp65 and COX-2 in the lung (n=8). (A) The mRNA expression of TLR4, NF-κBp65 and COX-2 was determined by western blot. GAPDH was used as the internal control. Images are representatives of three independent experiments. (B) The relative protein band intensities were quantifed by densitometric analyses and normalized to GAPDH. *Significantly different from control group, $p < 0.05$. # Significantly different from LPS group, $p < 0.05$.

Effect of PE on mRNA expression levels of LPS-induced TLR4, NF-κBp65 and COX-2 in the lung

The effect of PE on the mRNA expression levels of TLR4, NF-κBp65 and COX-2 in the lung were presented in fig. 3. The mRNA expression levels of TLR4, NF-κBp65 and COX-2 in LPS group were significantly up-regulated compared with corresponding values in control group ($P<0.05$). In addition, after intragastric administration of PE, the mRNA expression levels of TLR4, NF-κBp65 and COX-2 in LPS+PE low and high dose groups were significantly decreased compared with the corresponding values in LPS group, respectively ($P<0.05$).

Effect of PE on protein expression levels of LPS-induced TLR4, NF-κBp65 and COX-2 in the lung

The effect of PE on the protein expression levels of TLR4, NF-κBp65 and COX-2 in the lung were presented in fig. 4. The protein expression levels of TLR4 and NF-κBp65 and COX-2 in LPS group were significantly increased compared with the corresponding values in controls ($P < 0.05$). However, after intragastric administration of PE, the protein expression levels of TLR4 and NF-κBp65 and COX-2 in LPS+PE low and high dose groups were significantly decreased compared with the levels in LPS group, respectively ($P < 0.05$).

DISCUSSION

From the histological damage of lung, LPS prompted ALI, in which the quantity of inflammatory cells expanded essentially, which was reliable to the past investigations (Zhang *et al*, 2017). Moreover, the pretreatment with PE reduced the histological damage induced by LPS, which indicates that PE can alleviate the acute lung injury induced by LPS, and these phenomena should be related to some changes in the molecular mechanism.

TNF-α and IL-6 are critical pro-inflammatory cytokines involved in the inflammatory process of ALI (Wei and Huang, 2014; Liu *et al*, 2014). TLR4 serves as a cell-surface co-receptor for CD14, and is well known as the unique receptor for LPS, leading to LPS-mediated NF-κB pathway activation and triggering the release of these pro-inflammatory cytokines (Chow *et al*., 1999; Wang *et al*., 2017). Previous studies demonstrated that the injury of ALI could be attenuated by the inhibition of these cytokines (Zhang *et al*., 2017; Yi *et al*., 2019). In this research, compared with the LPS group, the concentrations of TNF-α and IL-6 in LPS+PE (10, 50 mg/kg) groups were significantly decreased, indicating that PE has the ability to improve LPS-induced lung injury by inhibiting the concentrations of TNF-α and IL-6. By detecting the mRNA and protein expression levels, It is observed that PE pretreatment significantly decreased mRNA and protein expressions of TLR4 and NF-κBp65

induced by LPS in lung tissues. It is proposed that the decreased concentrations of TNF-α and IL-6 partly resulted from PE inhibiting the mRNA and protein expressions of TLR4 and NF-κBp65 on LPS-induced inflammatory rats.

COX-2 is also a downstream substrate of TLR4/NF-κB pathway (Cao *et al*., 2018), and is responsible for catalyzing the synthesis of PGE2. COX-2 and PGE2 play a key role in the expansion of inflammatory response (Verma *et al*., 2017). In this study, the results demonstrated that PE pretreatment down-regulated LPS-induced mRNA and protein expression levels of COX-2 and the concentration of PGE2, which was roughly consistent with previous study on Platycodin D (Sun *et al*., 2020). We speculate that this down-regulation may be partially associated with the decreased mRNA and protein expressions of TLR4/NF-κBp65. Above all, this observation raises the possibility that one of the main anti-inflammatory mechanism of PE on LPS-induced inflammatory rats may be the reduction of the concentrations of IL-6, PGE2, and TNF-α through the inhibition of TLR4/NF-κBp65 signal pathway, which finally alleviates the lung injury histologically in the inflammation.

The main active constituents of PG include platycodin D and radix platycodonis polysaccharide. The anti-inflammatory effect of PE may be accomplished jointly by these active constituents. Hu *et al* (2017) have reported that platycodin D could inhibit LPS-induced NF-κB activation, significantly improve lung pathological changes, myeloperoxidase activity, and the concentrations of TNF-α, IL-1β and IL-6. However, Hu *et al* (2017) have also mentioned that platycodin D did not act through affecting the expression level of TLR4. Therefore, in combination with our study, we considered that platycodin D may be one of the major active ingredient involved in anti-inflammation in PE, and there may be other active constituent(s) in PE involved in the regulation of TLR4 expression. Besides platycodin D, radix platycodonis polysaccharide may also have the effect of anti-inflammation. Huang *et al* (2013) have reported that radix platycodonis polysaccharide can inhibit the airway inflammation in asthma, decreased the expression levels of IL-4 and IL-5 in the bronchoalveolar lavage fluid (BALF) and NF-κB in rat lung tissue. Park *et al* (2014) have suggested that TLR4 might be one of the platycodon grandiflorum polysaccharide receptors in dendritic cell. Yoon *et al* (2003) have reported that the polysaccharide isolated from *P. grandiflorum* activated macrophages via TLR4.

In this study, we only studied the anti-inflammatory effects of PE on LPS-induced ALI rat model via TLR4/NF-κBp65 signal pathway, the anti-inflammatory mechanism of PE via other signaling pathways has not been studied yet, thus, further studies need to be done to evaluate the effects through other signaling pathways.

CONCLUSION

This study showed that PE reduced the levels of LPS-induced pro-inflammatory mediators including IL-6, PGE2 and TNF-α, alleviated the lung injury histologically, and down-regulated LPS-induced mRNA and protein levels of TLR4/NF-κBp65 in lung tissue. The present study demonstrated that PE has the anti-inflammatory effects on ALI induced by LPS in rats through TLR4/NF-κBp65 signaling pathway, indicating that PE is an effective suppressor for anti-inflammatory activities.

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