Protective effects of protocatechuic acid on acute lung injury induced by lipopolysaccharide in mice via p38MAPK and NF-κB signal pathways

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A B S T R A C T
The study aims to investigate the effects of protocatechuic acid (PCA) separated from Chinese herbs, on acute lung injury (ALI) induced by lipopolysaccharide (LPS) in mice. The mouse model was induced by intraperitoneal injection of LPS at the dose of 5 mg/kg body weight. Three doses of PCA (30, 15, 5 mg/kg) were administered to mice with intraperitoneal injection one hour prior to LPS exposure. Six hours later after LPS administration, the effect of PCA on ALI mice was assessed via histopathological examination by HE staining, inflammatory cytokine production by ELISA assay and RT-PCR, p38MAPK and NF-κB activation by Western blot analysis. We found that PCA administration significantly ameliorated lung histopathological changes and decreased protein concentration in the bronchoalveolar lavage fluid. Furthermore, the overproduction of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) was reduced by PCA. Additionally, PCA at the dose of 30 mg/kg could block the activation of p38MAPK and NF-κB signal pathways induced by LPS. In conclusion, our findings demonstrate that PCA possesses a protective effect on LPS-induced ALI in mice via suppression of p38MAPK and NF-κB signal pathways. Therefore, PCA may be useful in the therapy of lung inflammatory diseases, especially for ALI.

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1. Introduction

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), remains a major cause of high morbidity, mortality, and health care costs in critically ill patients [31]. ALI is characterized by disruption of the alveolar-capillary membrane barrier, accumulation of protein-rich fluid in the airspaces, protein-rich hyaline membrane formation, pulmonary edema, and overproduction of inflammatory cytokines [28,39]. Although many studies had explored the underlying mechanisms and improvement therapies of ALI, there has not established specific and effective treatment strategy for clinical application so far [30]. Therefore, the development of new medication and new strategies for the management of this disease is urgently needed. Lipopolysaccharide (LPS), a major outer membrane component of Gram-negative bacteria can induce a disturbance in immune and inflammatory responses, and is a key mediator of lung injury [7]. In this study, the LPS-induced mouse ALI model was used to evaluate the molecular mechanisms of protective effects of PCA on ALI.

Toll-like receptors (TLRs) are pathogen pattern recognition receptors that recognize viral and bacterial products and other pathogens and activate the innate immunity [37]. Among the TLRs, TLR4 is the specific receptor for LPS, triggers the activation of nuclear transcription factor kappa B (NF-κB) and mitogen activated protein kinase (MAPK) signaling cascades, which serve a key function in regulating immune and inflammatory responses, and modulates the transcription of multiple inflammatory factors and cytokines [10,26,43]. Abundant researches have confirmed that NF-κB signaling pathway participates in LPS-induced inflammatory injury [2,20]. Meanwhile, mitogen-activated protein kinase (MAPK) mediates the production of inflammatory mediators, and especially P38MAPK plays a vital role in LPS-induced inflammatory injury [33,34]. In addition, p38 MAPK has been proposed to regulate the activation of NF-κB [32]. MKK3/6 is the upstream kinases of p38MAPK, which is responsible for the phosphorylation and activation of p38MAPK. P38MAPK activates downstream activating transcription factors 2 (ATF-2) which bind and induce expression of target genes in nuclear, then together regulate inflammatory responses.

The traditional Chinese herb protocatechuic acid (PCA) is a major benzoic acid derivative and found in human diet, fruits, plants and spices, such as onion (Allium cepa L.), grapes (Vitis vinifera), melissa (Melissa officinalis L.), a medical rosemary (Rosmarinus officinalis L.) and so on [16]. The major active component of PCA shows biochemical and pharmacological activities including anti-oxidative properties, anti-apoptotic effects, anti-inflammatory activities, etc [19,36,45]. In our present study, we explored the effect of PCA on LPS-induced ALI in
mice and the underlying mechanism, attempting to provide a new potential treatment for ALI from traditional Chinese herbal medicine.

2. Materials and methods

2.1. Animals

Adult male Kuiming mice, weighting approximately 18 to 20 g, were supplied from Yan Tai Iyue Pharmaceutical Co. (Yantai, China). Mice were housed in micro-isolator cages at a constant temperature of 24 ± 1 °C and relative humidity of 40–80%. They were given standard rodent chow and water ad libitum. The experiments were performed in accordance with the guidelines of the National Institutes of Health for the Care and Use of Laboratory.

2.2. Regants

PCA was purchased from Nanjing for Mr. Yao Institute of Traditional Chinese Medicine (TCM). Lipopolysaccharide (LPS, *Escherichia coli* O111:B4) was obtained from Sigma Co. Dexamethasone (sodium phosphate injection) (Dex, purity: > 99.6%) was purchased from Jinan and Pharmaceutical Co. (Jinan, China). SB203580, a p38 MAPK-specific inhibitor, was purchased from Selleckchem. Mouse TNF-α and IL-1β enzyme linked immunosorbent assay (ELISA) kits were provided from Shanghai Lang's Biological Technology Co. (Shanghai, China). The primary antibodies including phosphorylated and non-phosphorylated forms of p38MAPK and p65, phosphorylated MKK3/6, phosphorylated ATP2, TLR4, NF-κB p65 and the horseradish peroxidase-labeled IgG secondary antibodies and β-actin were purchased from Bioworld Technology, Inc. (Louis Park, USA). The Protein Extraction Kit and Cell Lysis Buffer for Western were obtained from Beyotime Institute of Biotechnology. Transcriptor First Strand cDNA Synthesis Kit and LightCycler FastStart DNA Master SYBR Green I kit were purchased from Roche Technology. Transcriptor First Strand cDNA Synthesis Kit and LightCycler technology. The gene-specific primer sequences used in real-time qPCR were IL-1β-gene (FORWARD: 5′-CTCAACAAGGAGCACAGAAGC-3′ and REVERSE: 5′-TCCAGCCCATATCTAGGAAAG-3′), TNF-α-gene (FORWARD: 5′-CACCCACATCAAGGCTC-3′ and REVERSE: 5′-GAGACA GGAGAACCCGAGG-3′), β-actin gene (FORWARD: 5′-GTGCTATGTTGCTCCTATCC-3′ and REVERSE: 5′-ATGCCACAGGATCCATC-3′). Individual PCR products were analyzed using melting-point analysis. Real-time qPCR product was analyzed using the comparative Ct method according to the manufacturer’s instructions. All of the assays included β-actin, an internal control, and sample variation was corrected by subtracting the β-actin.

2.3. Experimental protocol

LPS-induced ALI in mice was performed as described in previous studies [9]. Eighty mice were randomly divided into 8 groups, the control group, LPS group, PCA (30, 15, 5 mg/kg) + LPS group, SB203580 + LPS group, Dex + LPS group, and PCA + SB203580 + LPS group. All samples were centrifuged at 3000 rpm for 20 min at 4 °C. We measured TNF-α and IL-1β cytokine levels in the blood and BALF by commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s protocols.

2.3.3. Histopathologic evaluation

Lung tissues which were not subjected to BALF collection were harvested for further histopathologic evaluation. The right upper lobes were harvested at 6 h after LPS administration and were fixed in 4% buffered paraformaldehyde for more than 24 h. Then the lung tissues were dehydrated with graded alcohol and embedded in paraffin; 5 μm sections were prepared and stained with hematoxylin–eosin (H&E). Histopathology was observed under a light microscope. The lung damage score was performed by a histologist who was blinded to the experimental protocol complying with the histologic examination criteria [23] (Table 1). The final injury score was derived from the following calculation: Score = (20 × A + 14 × B + 7 × C + 7 × D + 2 × E) / (number of field × 100).

2.3.4. Western blot analysis of p38 MAPK and NF-κB expression in the lung tissue

The middle lobes of the mice’ right lungs were harvested and frozen in liquid nitrogen immediately until homogenization. Sample were lysed in RIPA buffer on ice for 30 min containing 50 mmol/L Tris·HCl (pH 8.0), 1% Triton X-100, 0.150 mmol/L NaCl, and 100 μg/mL PMSF. Samples were centrifuged and protein concentrations in the supernatant were assayed using a BCA protein assay kit. Equal amounts of protein (50 μg) were loaded per lane on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and separated by electrophoresis. After that, the protein was transferred onto polyvinylidene difluoride (PVDF) membranes. The non-specific protein binding was blocked with 5% nonfat dry milk in PBS supplement with 0.05%Tween for 2 h at the room temperature. After blocking, the membranes were incubated with the specific primary antibodies, including respectively overnight at 4 °C. Then the membranes were washed with TBST for three times. Secondary antibody (diluted 1:3000) was used to detect the membranes for 1 h at the room temperature. The blots were probed using ECL Western blotting regents, quantified by densitometry scanning, and the protein bands were determined with a computer image analysis system. β-actin was used as the internal control.

2.3.5. RT-PCR analysis of TNF-α and IL-1β level in the lung tissue

Total RNA of the lung tissue were extracted using Trizol reagent. Then the cDNA was generated by extracted total RNA using Transcriptor First Strand cDNA Synthesis Kit. Real-time PCR was performed to amplify the IL-1β and TNF-α transcripts using LightCycler FastStart DNA Master SYBR Green I kit. The gene-specific primer sequences used in real-time qPCR were IL-1β-gene (FORWARD: 5′-CTCAACAAGGAGCACAGAAGC-3′ and REVERSE: 5′-TCCAGCCCATATCTAGGAAAG-3′), TNF-α-gene (FORWARD: 5′-CACCCACATCAAGGCTC-3′ and REVERSE: 5′-GAGACA GGAGAACCCGAGG-3′), β-actin gene (FORWARD: 5′-GTGCTATGTTGCTCCTATCC-3′ and REVERSE: 5′-ATGCCACAGGATCCATC-3′). Individual PCR products were analyzed using melting-point analysis. Real-time qPCR product was analyzed using the comparative Ct method according to the manufacturer’s instructions. All of the assays included β-actin, an internal control, and sample variation was corrected by subtracting the β-actin.

Table 1 Lungs injury scoring system.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score per field</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>A Neutrophils in alveolar space</td>
<td>None</td>
</tr>
<tr>
<td>B Neutrophils in interstitial space</td>
<td>None</td>
</tr>
<tr>
<td>C Hyaline membranes</td>
<td>None</td>
</tr>
<tr>
<td>D Proteinaceous debris filling airspaces</td>
<td>None</td>
</tr>
<tr>
<td>E Alveolar septal thickening</td>
<td>2–4</td>
</tr>
<tr>
<td>Score = (20 × A + 14 × B + 7 × C + 7 × D + 2 × E) / (number of field × 100)</td>
<td></td>
</tr>
</tbody>
</table>
2.3.6. Statistical analysis

Data were presented as means ± standard deviations. Multiple groups were compared by using one-way analysis of variance (ANOVA). Statistical differences between groups were considered as significant when p < 0.05. All statistical analyses were performed using SPSS version 15.0 software.

3. Results

3.1. Effects of PCA on histopathological changes in mice challenged with LPS

To evaluate the histological changes in the lung tissue after PCA treatment in LPS-induced mice, lung sections obtained 6 h after administration of LPS were dyed with H&E. As shown in Fig. 1, in the normal group, slight histological alteration was observed in lung specimens. However, in the LPS group, the lung showed obviously pathologic changes, such as infiltration of inflammatory cells, alveolar wall thickening and alveolar hemorrhage. Conversely, pathological improvement was observed in PCA + LPS group as PCA (30, 15, 5 mg/kg) ameliorated many of the symptoms of LPS-induced ALI in mice, as well as in SB203580 + LPS group, Dex + LPS group and PCA + SB203580 + LPS group.

3.2. Effects of PCA on protein concentration in BALF in LPS-induced ALI mice

Six hours after LPS administration, the total protein concentration in the BALF was evaluated in order to test the permeability of lung endothelial and epithelial. As shown in Fig. 2, treatment with LPS caused a
markedly increase in the total protein concentration in the BALF compared to those of the normal group ($p < 0.01$), while pretreatment with PCA (30 mg/kg) significantly ameliorates protein leakage. Furthermore, SB203580 also obviously reduced the protein concentration. Additionally, Dex developed a comparable reduction in the levels of total protein in BALF.

3.3. Effects of PCA on cytokine production in BALF and blood of LPS-induced ALI mice

TNF-α and IL-1β are important inflammation mediators in LPS-induced ALI, so we investigate cytokine production in BALF and blood. BALF and blood of the mice were harvested 6 h after administering LPS for the analysis of cytokine concentration by ELISA. It was found that IL-1β (Fig. 3A) and TNF-α (Fig. 3B) levels were significantly elevated in the LPS-induced ALI group, while treatment with PCA and SB203580 effectively prevented this elevation.

3.4. Effects of PCA on LPS-induced p38MAPK signaling pathways in mice

The p38MAPK family is a critical pathway associated with inflammatory response to LPS-induced ALI in mice. We investigated whether or not p38MAPK was suppressed by PCA using Western blot analysis and explore the mechanism of PCA inhibits pro-inflammatory cytokine production. In the present study, we measured the expression levels of phosphorylated MKK3/6, phosphorylated p38 MAPK, non-phosphorylated p38MAPK and phosphorylated ATF-2 in mice with LPS-induced ALI. Compared with the normal control group, the LPS group had significantly higher phosphorylation levels of MKK3/6, p38 MAPK and ATF-2 after 6 h of stimulation with LPS (Fig. 4). The phosphorylation levels of MKK3/6, p38 MAPK and ATF-2 were considerably inhibited by the treatment of PCA and SB203580 shown in Fig. 4.

3.5. Effects of PCA on NF-κB signaling pathways in mice with LPS-induced ALI

We investigated whether or not NF-κB signaling pathways was suppressed by Western blot to explore the mechanism by which PCA inhibits pro-inflammatory cytokine production. As shown in Fig. 5, the expression of TLR4 was increased in the LPS group, at the same time, the LPS group showed significant IκB-α phosphorylation in cytoplasm protein extracts compared with the normal control group. PCA treatment prior to LPS induction inhibited the increased TLR4 expression and phosphorylation of IκB-α in a dose-dependent manner. In addition,
The activation of NF-κB signaling pathways was significantly blocked by Dex and SB203580.

3.6. Effects of PCA on the TNF-α and IL-β mRNA levels in the lung tissue

The p38MAPK and NF-κB signaling pathways could activate ATF-2, then regulate the inflammation factor synthesis, so we investigated the TNF-α and IL-β mRNA levels in lung by RT-PCR. It was found that IL-1β (Fig. 6A) and TNF-α (Fig. 6B) levels were remarkably elevated in the LPS-induced ALI group, while treatment with PCA and SB203580 effectively prevented this elevation.

4. Discussion

Sepsis has been referred to be an important risk factor for ALI [27]. Intraperitoneal administration of LPS has gained wide acceptance as a model of severe lung injury [24]. LPS can act as endotoxin and bind to its receptor on cell membrane, initiating a series of innate immune responses, inducing the development of ALI, which is characterized by the infiltration of neutrophils into the lung and the release of inflammatory mediators, cytokines and chemotactic factors [14,22,29]. Subsequently, alveolar space and epithelial endothelial barrier is disrupted by inflammation, resulting in lung injury [5,44]. Thus, inhibition of lung inflammation may be an approach for the treatment of ALI.

Protocatechuic acid (PCA), which is isolated from vegetables, nuts, brown rice, fruits, and herbal medicines, has exerted anti-inflammatory effects. Sung-Won Min et al. [25] have reported that PCA could inhibit LPS-stimulated TNF-α and IL-1β secretion in RAW264.7 cells. Miaomiao Wei et al. [40] have demonstrated that PCA suppressed ovalbumin-induced airway inflammation in a mouse allergic asthma model. In this study we examined the protective effect of this agent on LPS-induced ALI in mice.

Inflammatory mediators such as TNF-α and IL-1β are mainly produced in macrophages activated by bacterial products, including LPS [12,18]. TNF-α and IL-1β are thought to cause induction and progression of ALI/ARDS [38]. Inhibiting the overproduction of TNF-α and IL-1β showed lessening pulmonary injuries in LPS-induced ALI model [8,41]. Increased levels of TNF-α and IL-1β in BALF have been noted in ALI and ARDS patients [1]. Our findings are in agreement with previous studies, and our results showed that the LPS group had higher production of TNF-α and IL-1β in the BALF and plasma than the normal control group. We found that the overproduction of pro-inflammatory mediators by LPS, such as TNF-α and IL-1β was markedly decreased by PCA and PCA intervention prevented the release of those cytokines in a dose-dependent manner.

It appears that the effects of PCA on ALI induced by LPS may be related to the inhibition of inflammatory factors TNF-α and IL-1β. Transcription of pro-inflammatory mediators such as TNF-α and IL-1β is regulated by activation of the transcription factor NF-κB [6]. Conformably, recent evidence suggests that LPS has been shown to initiate multiple intracellular signaling events, including the activation of MAPK and NF-κB, which ultimately leads to the synthesis and release of a number of pro-inflammatory mediators, including TNF-α and IL-1β. So we estimated the expression of TNF-α and IL-1β in the RNA level, and the results were consistent with the previous study [15,42]. Therefore, it seems likely that it is possible for PCA to exert anti-inflammation effect due to regulate p38MAPK and NF-κB activation.
Nuclear factor kappa B (NF-κB), which serves a key function in regulating immune and inflammatory responses, modulates the transcription of multiple inflammatory factors and cytokines [21]. NF-κB is usually localized to the cytosol and bound to its inhibitor Inhibitory kappa B (IκB). Upon TLR4-mediated activation, NF-κB is released from IκB and translocates to the nucleus where it activates the target genes.
and induces expression of many proteins, including acute phase response proteins, cell adhesion molecules, and cytokines to regulate inflammatory responses [3]. Meanwhile, IκB is phosphorylated by IκB kinase and degraded [4]. NF-κB comprises a family of transcription factors, including the subunit members p50, p52, p65, RelB and c-Rel, and the p65 protein is one of the most abundant subunits of NF-κB [13]. In this study Western blot analysis showed that PCA could substantially block the activation of NF-κB signal pathways by inhibiting TLR4, phospho-IκB, and phospho-p65 expression levels.

In addition to the NF-κB signaling pathway, the p38MAPK signaling pathway is another signaling pathway related to inflammation. p38MAPK is one subgroup of MAPK family which is an important enzyme for the pathogenesis of LPS-induced lung injury [35]. One major target of p38 MAPK is the activating transcription factor 2 (ATF-2). In conjunction with the activation of NF-κB, p38MAPK activation induces the expression of multiple genes that together regulate the inflammatory response [11,17]. To further detail the LPS-induced MAPK intra-cellular signaling, the p38MAPK inhibitor SB203580 was introduced into the experiments. We designed B + LPS and H + B + LPS group in experimental animals. SB203580 as a bolus directly inhibits the activation of p38 MAPK and NF-κB signal pathways by blocking the Akt/NF-κB pathway in acute lung injury induced by lipopolysaccharide in mice, Evid. Based Complement. Alternat. Med. 2012 (2011).}

In summary, we report that PCA prior to the administration of LPS effectively ameliorates lung histopathological changes and decreases protein concentration in the bronchoalveolar lavage fluid. Furthermore, the level of pro-inflammatory mediators induced by LPS, such as TNF-α and IL-1β, was markedly reduced by PCA. Additionally, Western blot analysis showed that phospho-p38MAPK, p38MAPK protein and phospho-ATF-2 expression levels decreased remarkably by the treatment of SB203580. The effect of PCA (30 mg/kg) associated with SB203580 on the suppression of p38 MAPK activation was stronger than the SB203580 used alone. According to these results, we speculated that NF-κB participates along with p38MAPK in the activation of ALI by LPS. In conclusion, our findings demonstrate that PCA possesses a protective effect on LPS-induced ALI in mice mainly via suppression of p38 MAPK and NF-κB signal pathways which can inhibit pulmonary inflammatory process.

Acknowledgments

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