Original Article
Effects of polydatin on oleic acid-induced acute respiratory distress syndrome in rats

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Received May 28, 2018; Accepted August 4, 2018; Epub November 15, 2018; Published November 30, 2018

Abstract: The aim of this study was to investigate the protective effects of polydatin (PD) against oleic acid (OA) induced acute respiratory distress syndrome (ARDS). PD was injected into rat femoral veins simultaneously with OA administration. H&E staining was used to observe pathological changes. Neutrophil numbers, protein concentrations, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) levels, and lung wet to dry (W/D) ratios were determined. Protein expression of Na+/K+-ATPase α1, Na+/K+-ATPase β1, Bax, Bcl-2, caspase-3, NF-κB-p65, phosphorylated mitogen-activated protein kinases (MAPKs), and phosphorylated-PI3-kinase p85/p55 (p-PI3K) in lung tissues was assessed. PD treatment obviously alleviated pathological changes in lung injuries and inhibited increased levels of W/D ratios, neutrophil numbers, total protein levels, TNF-α levels, and IL-1β levels, induced by OA. PD treatment also upregulated expression of caspase-3 and Bax and downregulated expression of Bcl-2, induced by OA administration. Moreover, PD treatment reversed MAPKs, PI3K, and NF-κB-p65 expression in lung tissues from OA-administered rats. In conclusion, PD exhibits protective effects against OA-induced acute lung injuries in rat with ARDS, acted on through inhibition of MAPKs/NF-κB or PI3K/NF-κB signaling pathways. PD may be a potential candidate for prevention and treatment of ARDS.

Keywords: Polydatin (PD), acute respiratory distress syndrome (ARDS), oleic acid (OA), inflammation

Introduction

Acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (ALI), is a syndrome with an elevated overall incidence [1]. The mortality rate of ARDS remains very high. The main characteristic of ARDS is increased pulmonary capillary permeability, which may cause the accumulation of protein-rich fluid in the alveoli. Subsequently, the capillary endothelium and alveolar epithelium will be damaged, causing the release of cytokines and formation of pulmonary edema [2]. Therefore, ARDS can lead to permeability-type lung edema. Currently, treatment for ARDS is mainly mechanical ventilation and fluid removal. Only a few drugs have been found effective for treatment of ARDS [3].

Na+/K+-ATPase (NKA) is located on the basolateral surface of the alveolar epithelial cells. It is indispensable for the transport of ions by consuming ATP and pumping Na+ out of the cells in exchange for potassium influx, maintaining Na+ and K+ gradients across the plasma membrane [4]. The basolateral membrane location of NKA is crucial for alveolar fluid reabsorption where the vectorial Na+ transport is followed by water in an isosmolar manner [5]. Upregulation of NKA increases active Na+ transport, increasing the clearing of edema by the lungs. Damage to NKA function has been associated with lung injuries [6]. Oleic Acid (OA) is an NKA inhibitor and an Na+ channel inhibitor in the lungs. It can induce significantly increased endothelial permeability, which mimics occurrence and development of ARDS [7]. Thus, OA has been commonly used to induce ARDS.

Polydatin (3, 4’, 5-trihydroxystibene-3-β-mono-D-glucoside; PD), a monocrystalline compound extracted from the herb Polygonum cuspidatum, has many biological functions, including protecting against hemorrhagic shock [8], anti-
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inflammatory and anti-oxidant activity [9], reducing ischemia/reperfusion injuries [10], and alleviating alcohol-induced acute liver injuries [11]. Previous studies have demonstrated that PD could inhibit p38 MAPK/NF-κB signaling pathways [12]. In IgE-mediated passive systemic anaphylaxis models, PD suppressed mast cell degranulation via downregulating MAPK, PI3K/AKT, and NF-κB signaling pathways [13]. In addition, PD can protect the kidneys from ischemia/reperfusion injuries via regulating PI3K/Akt pathways [14]. Thus, the functions of PD are partly mediated by MAPK, PI3K/AKT, and NF-κB signaling pathways. However, whether PD is involved in OA-induced ARDS through regulating MAPK, PI3K/AKT, and NF-κB signaling pathways has not been fully elucidated.

The present study investigated the effects of PD on ARDS in OA-induced ARDS rats. The roles of MAPKs, PI3K, and NF-κB pathways in PD-mediated effects on ARDS were analyzed and discussed.

Materials and methods

Animals

Male Sprague-Dawley rats (n = 30), weighing 180-220 g, were purchased from the Experimental Animal Center at Yanbian University. Animals were acclimatized for a week and had free access to chow and water. All experiments were approved by Institutional Animal Care and Use Committee of Yanbian University School of Medical Sciences.

Animal grouping

The rats were randomly divided into five groups, including the control group, OA+normal saline (NS) group, OA+small dose PD (PD_s) group, OA+medium dose PD (PD_m) group, and OA+large dose PD (PD_l) group, with 6 rats in each group. For OA+PD groups, rats received OA (100 μl/kg; Sangon Biotech, Shanghai, China) injections and PD (1 mg/kg; Sigma-Aldrich, St. Louis, USA) injections, simultaneously, via femoral veins. Doses of PD in OA+PD_s, OA+PD_m, and OA+PD_l groups were 15 mg/kg, 30 mg/kg, and 45 mg/kg, respectively. Rats in the OA+NS group received OA injections and equal volumes of NS injections, while rats in the control group received equal volumes of NS injections.

Collection of bronchoalveolar lavage fluid (BALF) and cell counting

Rats were sacrificed at 3 hours after exposure to OA and median sternotomies were performed. The right main bronchus was ligated and a catheter was inserted into the trachea. Double-lavage procedures were performed using 0.5 mL of phosphate-buffered saline (PBS) (pH 7.2) to pass through the catheter twice. The fluid recovery rate was more than 90%. Next, lavage samples were centrifuged at 1,500 g for 10 minutes at 4°C. Sediment cells were resuspended in PBS and subjected to cell counting. Cells on the slides were visualized with Wright-Giemsa (Sigma, USA) and polymorphonuclear neutrophils (PMNs) were counted in a double-blind manner. Total protein concentrations in the BALF were detected with Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China).

H&E staining

Lungs were harvested at 3 hours after OA injections. The right middle lobes of the lungs were fixed with 4% paraformaldehyde and embedded in paraffin. Specimens were cut into 4-μm sections using a rotary microtome (2165; Leica Biosystems Nussloch Gmbh, Nussloch, Germany). After deparaffinization and rehydration, the microsections were stained with H&E (Richard-Allan Scientific, Kalamazoo, MI, USA). The slides were examined by microscopy under a magnification of × 200.

Lung injury scores were calculated with a semi-quantitative scoring system, as previously described. Pathologic findings, including alveolar congestion, alveolar hemorrhages, infiltration or aggregation of neutrophils in the airspace or vessel walls, and thickness of alveolar wall/hyaline membrane formation and inflammatory cell infiltration, were evaluated. The grading scale to score pathologic findings was as follows: 0 = no injury; 1 = slight injury (25%); 2 = moderate injury (50%); 3 = severe injury (75%); and 4 = very severe injury (almost 100%). Scores of each pathologic finding were added up to obtain lung injury scores (total score: 0-16).

Wet/Dry (W/D) ratio

Lung edema was assessed by W/D weight. The remaining right lungs were harvested and the
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“wet” weights of lungs were measured immediately. The “dry” weights of lungs were measured after incubating at 60°C for 48 hours. W/D ratios were then calculated.

**ELISA**

Levels of TNF-α and IL-1β in BALF were measured using ELISA kits (Elabscience, Wuhan, China), according to manufacturer instructions. Absorbance at 450 nm was read with an automatic microplate reader (SPECTROstar Nano, BMG LABTECH, Germany).

**Western blotting**

Lung tissues were homogenized in RIPA lysis buffer (Beyotime, Shanghai, China). Protein concentrations were detected by Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). After separation by SDS-PAGE, proteins were transferred to PVDF membranes. After blocking, the membranes were incubated with primary Abs to NKA α1 (1:1000; Ruiyingbio, Suzhou, China), NKA β1 (1:1000; Ruiyingbio), β-actin (1:500; Elabscience), Bax (1:1500; Elabscience), Bcl-2 (1:500; Elabscience), Caspase-3 (1:1000; Elabscience), phospho (p)-p38 (1:1000; Elabscience), p-JNK1/2/3 (1:1000; Elabscience), p-ERK1/2 (1:1000; Elabscience), p-PI3K (1:1000; Elabscience), NF-kB-p65 (1:1000; Elabscience), p38 (1:1000; Elabscience), JNK (c-Jun NH2-terminal kinases) 1/2/3 (1:1000; Elabscience), ERK1/2 (1:1000; Elabscience), or PI3K (1:1000; Elabscience) overnight at 4°C. After rinsing, the membranes were reacted with HRP-conjugated secondary Ab (1:1000; Bioss, Beijing, China) at room temperature for 2 hours. After development with ECL Western Blotting Detection Kit (Solarbio, Beijing, China), the Western blot images were analyzed using Quantity One software (Bio-Rad Laboratories, Inc, USA). β-actin was used as an internal control. All experiments were repeated at least three times.

**Immunohistochemistry**

Lung tissue paraffin specimens were dewaxed and rehydrated. Endogenous peroxidase was inactivated by 3% hydrogen peroxide at RT for 10 minutes. The antigen was retrieved at 95°C for 20 minutes by placing the slides in 0.01 M sodium citrate buffer (pH 6.0). Mouse anti-rat NF-kB-p65 monoclonal antibody (1:200; Elabscience) was added as the primary antibody at 4°C overnight. Following incubation with the biotinylated secondary antibody (ZSGB-Bio, Beijing, China) at RT for 20 minutes, the slides were incubated with streptavidin-peroxidase complex at RT for 20 minutes. Immunostaining was developed using 3, 3’-diaminobenzidine and Mayer’s hematoxylin was used for counterstaining. Areas with

*Figure 1.* PD attenuates OA-induced lung histopathological changes and pulmonary edema. OA was injected from femoral veins and the control group was injected with the same amount of vehicle. PD (15 mg/kg, 30 mg/kg, and 45 mg/kg) was injected from femoral veins at the same time of OA administration. Rats were sacrificed at 3 hours post-OA stimulation. (A) H&E staining of lung tissues (200 × magnification), Scale bars: 50 μm. (B) Acute lung injury scores and (C) wet to dry weight ratios of the lungs. Data are presented as mean ± SD (n = 6 in each group). **p<0.01 versus control group; *p<0.05 versus OA+NS group.
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brown-yellow granules were positively stained with NF-κB-p65. Positive areas were counted from 5 random fields at 200 × magnification. Positive rate was defined as the ratio of NF-κB-p65 positive area to the total area of vision.

Statistical analysis

Unless otherwise stated, all experiments were performed at least three times, independently. All variables are presented as mean ± SD and were analyzed using SPSS v20.0 (IBM, Armonk, NY, USA). Differences were determined using one-way ANOVA with LSD multiple comparison test and Student’s t-test, as needed. P<0.05 is considered statistically significant.

Results

PD alleviates OA-induced ALI and pulmonary edema

To investigate the effects of PD administration on lung injuries induced by OA, pathological changes were observed by H&E staining. Under light microscopy, lung tissues in the control rats were intact. The alveolar space was clear and there was no congestion in the alveolar wall (Figure 1A). Diffuse thickening of the alveolar wall, inflammatory cells infiltration, partial alveolar hemorrhages, and structural damage were observed in the OA+NS group, but administration of PD effectively alleviated the destruction of lung structures in a dose-dependent manner (Figure 1A). Similar results were obtained regarding lung injury scores and W/D ratios (Figure 1B, 1C). Compared to the control group, lung injury scores and W/D ratios in the OA+NS group were significantly higher (p<0.05). Notably, medium and large doses of PD significantly reduced lung injury scores and W/D ratios induced by OA (p<0.05). Thus, PD ameliorates lung injuries induced by OA in rats.

PD suppresses inflammatory cytokines in OA-induced ARDS rats

To investigate the anti-inflammatory effects of PD, concentrations of TNF-α and IL-1β were examined by ELISA (Figure 2C, 2D). Levels of TNF-α and IL-1β were significantly elevated after OA administration (p<0.01). As expected, medium and large doses of PD significantly inhibited OA-induced inflammatory cytokine production (p<0.05). Therefore, PD suppresses the inflammatory response in OA-induced ARDS rats.
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**PD rescues expression of NKA in rat lung tissues**

It has been established that NKA plays a key role in alveolar fluid transport and edema clearance. Expression of NKA α1 ([Figure 3A]) and β1 subunit ([Figure 3B]) in the OA+NS group was markedly decreased, compared to the control group \( (p<0.01) \). They were increased in OA+PD groups, compared with the OA+NS group. Especially, OA+PD_{M} and OA+PD_{L} groups significantly rescued the suppression of NKA proteins by OA \( (p<0.05) \). Therefore, NKA expression can be increased by PD administration in ARDS rats.

**PD suppresses OA-induced apoptosis**

Apoptosis-related proteins, such as anti-apoptotic Bcl-2, pro-apoptotic Bax, and caspase-3, play pivotal roles in the ARDS process [17]. Hence, Western blot analysis was performed to determine the effects of PD on Bcl-2, Bax, and caspase-3 protein expression in lung tissues of OA-induced rats. As shown in Figure 3C, OA administration obviously increased expression levels of caspase-3 and Bax, whereas it decreased Bcl-2 levels, compared to the control group. However, treatment with PD noticeably downregulated expression of caspase-3 and Bax, while increasing Bcl-2 levels, compared to the OA group. The most significant effects of PD treatment were achieved at a high dose (45 mg/kg). Data indicates that PD may inhibit OA-induced apoptosis.

**PD modulates NF-κB-p65, MAPKs, and PI3K expression in OA-challenged rats**

To investigate the influence of PD on MAPK, PI3K/AKT, and NF-κB signaling pathways in the context of ARDS, expression of NF-κB-p65, MAPKs, and PI3K proteins was analyzed by Western blot. OA prominently promoted expression of NF-κB-p65 and the phosphorylation of MAPKs isoforms ([Figure 4A]). However, treat-
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ment with PD effectively attenuated NF-κB-p65 and p-PI3K expression and the phosphorylation of ERK1/2, JNK1/2/3, and p38. Immunohistochemistry analysis was also used to determine the distribution of NF-κB-p65 in rat lungs after PD intervention. NF-κB-p65 plus staining appeared to be brown. The percentage expressing NF-κB-p65 plus area was increased in the OA+NS group, while it was rescued in PD treatment groups (Figure 4B). Collectively, these data suggest that activation of NF-κB-p65, phosphorylation of PI3K, and phosphorylation of MAPKs in OA-induced rats could be reversed by PD treatment.

Discussion

ARDS is based on alveolar-capillary membrane injuries, followed by the development of pulmonary edema, which may induce both systemic and local inflammation. The recruitment of PMNs into lung tissues is the predominant feature of ALI/ARDS [18]. The present study reported the protective effects of PD against OA induced ARDS, as evidenced by reduced histopathological damage, acute lung injury scores, W/D ratios, and total protein in BALF. Interestingly, PD can increase lung NKA levels in rat lung tissues, suggesting that PD could alleviate
lung edema and enhance the ability of the lungs to clear edema, when used in medium or large doses.

Damage of the capillary endothelium and alveolar epithelium has been correlated to impaired fluid removal from the alveolar space, resulting in accumulation of protein-rich fluid inside the alveoli and the release of pro-inflammatory cytokines, such as TNF, IL-1, and IL-6 [19]. Neutrophils are also recruited to the lungs by cytokines, which are then activated and release toxic mediators, such as reactive oxygen species and proteases [20]. TNF-α is an important mediator in early inflammation of lungs [21]. It can induce the production of IL-1β by vascular endothelium, stimulate macrophages to release chemotactic factors for PMN, and lead to increased pulmonary capillary permeability and interstitial edema [22]. Activated PMNs, which are recruited by TNF-α, release a variety of active enzymes and oxygen free radicals, promoting the production of various inflammatory factors which aggravate lung damage [23]. In addition, TNF-α and IL-1β may also regulate expression and activity of ion channels involved in fluid transport [24]. The present study found that medium and large doses of PD treatment dramatically prevented the increase of TNF-α, IL-1β, and PMNs in the BALF of OA-challenged rats, suggesting that PD treatment ameliorates OA-induced lung neutrophil infiltration and inflammation.

Moreover, apoptosis can amplify inflammatory responses in lung diseases [17]. As expected, this study found increased expression of apoptosis-related proteins, caspase 3 and Bax, in the lungs of OA-induced ARDS rats. In contrast, the anti-apoptotic protein Bcl-2 was decreased. Caspase-3 was analyzed because it has been considered the most important effector caspase and can be activated by any of the initiator caspases [25]. Medium and large doses of PD treatment rescued OA-induced upregulation of Bax and caspase-3 expression and down-regulation of Bcl-2 expression. Present results demonstrate that PD treatment may prevent lung cell apoptosis in OA-induced lung injuries.

The present study also explored other potential mechanisms underlying the protective effects of PD on OA-induced ARDS. MAPKs, including ERK1/2, JNK, and p38, are involved in the inflammatory response. In addition, activation of MAPKs signaling pathways can lead to the activation of NF-κB and induce expression of pro-inflammatory genes [26]. PI3K/Akt signaling pathways have been reported to regulate NF-κB expression [27]. NF-κB can regulate expression of cytokines and cell adhesion molecules during inflammation and the transcriptional activity of NF-κB depends on the post-translational modification of p65 [28]. Therefore, this study analyzed the effects of PD on OA-induced phosphorylation of PI3K, p38, ERK1/2, JNK1/2/3, and NF-κB-p65 in lung tissues of OA-induced ARDS rats. Results demonstrated that OA significantly increased the phosphorylation of MAPKs, PI3K, and NF-κB p65 signaling proteins. However, treatment with PD considerably inhibited the phosphorylation of PI3K, p38, JNK1/2/3, ERK1/2, and NF-κB-p65. MAPK, PI3K/AKT, and NF-κB signaling pathways play important roles in inflammatory lung diseases, such as ARDS. It has been reported that activation of MAPKs can lead to the generation of various cytokines, including IL-1β and TNF-α [29]. In addition, MAPKs and PI3K pathways play important roles in inflammation and edema [30]. The present study found that PD also upregulated expression of NKA. Elevated NKA activity of alveoli is important in removing excessive water in the alveoli and reducing leakage of albumin rich protein solution, thus decreasing lung edema, a relevant factor for ARDS outcomes [31]. Results of the present study do not conclusively show whether MAPKs and PI3K are located upstream or downstream of NKA signaling pathways. However, present findings indicated a possible association between the amelioration of ARDS and activation of MAPKs and PI3K pathways when PD was administrated in OA-induced ARDS.

The present findings suggest that PD administration can impede the progression of ARDS. In addition, PD might inhibit lung inflammation and alleviate lung edema via inhibition of MAPKs/NF-κB or PI3K/NF-κB signaling pathways. Therefore, present data confirms that PD may be used as a promising agent for the prevention and treatment of ARDS in clinical practice.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81560679 and No. 81660687) and the Natural Science Research Foundation of Jilin Province
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for Sciences and Technology (grant No. 2016-0101185JC).

Disclosure of conflict of interest

None.

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