Original Article

Protection of ripasudil, a Rho kinase inhibitor, in lipopolysaccharides-induced acute pneumonia in mice

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Abstract: Pneumonia is a major cause of morbidity and mortality of infectious diseases, especially in children. Ripasudil (K-115), a selective ROCK inhibitor, is a promising emerging drug against glaucoma, and reported to have anti-inflammatory activity. However, the anti-inflammatory effect of ripasudil still remains unclear in pneumonia. The goal of this study is to investigate the role and the underlying mechanism of ripasudil in pneumonia. BALB/c mice were used to establish an acute pneumonia model of mice by injection of lipopolysaccharide (LPS) intraperitoneally. Ripasudil (0.5 mg, 1 mg, 2 mg) was administrated 1 h before the induction of LPS. The histological change of lung tissue was evaluated by hematoxylin-eosin staining and lung wet/dry ratio. Inflammatory cytokines secretion, oxidant-antioxidant factors levels were measured. Cell apoptosis was examined using TNUEL assay. Western blot and qRT-PCR was used to determine gene expressions. Results showed that ripasudil significantly attenuated LPS-induced histological changes, reduced the production of pro-inflammatory cytokines, and alleviated LPS-induced oxidative stress in mice. LPS-induced cell apoptosis and associated protein expression changes were attenuated by ripasudil. Besides, ripasudil reduced the expression of RhoA, and decreased the activity of RhoA/ROCK signaling. Finally, the level of RhoA and eNOS from pneumonia patients exhibited negatively correlated, whereas the level of RhoA was higher while eNOS level was lower than that in the healthy control. The results of the present study indicate that ripasudil attenuate LPS-induced pneumonia in BALB/c mice by ameliorating inflammation, oxidative stress and apoptosis through inhibiting RhoA/ROCK signaling pathway. Ripasudil might be a novel and effective drug for the treatment of pneumonia.

Keywords: Pneumonia, ripasudil, RhoA/ROCK signaling, inflammation, oxidative stress, apoptosis

Introduction

Pneumonia, a lower respiratory tract infection disease, is a leading infectious cause of children morbidity and mortality [1]. On current trends, three children will die from pneumonia in every two minutes [2]. Approximately 88 thousand children died from pneumonia in 2016, and most of them were younger than 2-year-old [3]. In particular, the incidence of pediatric pneumonia in Southeast Asia was ranking first all over the world, seriously threatening the health and life of children patients [4, 5]. Therefore, a clearer understanding of pneumonia and novel effective treatment advances are in urgent need.

Rho-associated protein kinase (ROCK) is a serine/threonine protein kinase, and exists in two isoforms, ROCK1 and ROCK2, which are downstream effectors activated by the small GTP binding protein-RhoA [6, 7]. RhoA/ROCK signaling has recently received a considerable amount of attention due to its involvement in various diseases. Rao J et al showed that blocking RhoA/ROCK signaling ameliorated adhesion and inflammatory infiltration induced by advanced glycation end products (AGEs), indicating a protective role of RhoA/ROCK signaling inhibition in the progression of diabetic nephropathy [8]. Liang J et al demonstrated the protection of blocking RhoA/ROCK signaling in pemphigus vulgaris, suggesting that RhoA/ROCK signaling is a promising useful therapeutic target in skin diseases [9]. RhoA/ROCK signaling aberrant activation was also involved in autoimmune disorders, and blocking of this signaling exhibited a protective role in various dis-
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Eases [10, 11]. Thus, inhibition of RhoA/ROCK signaling plays a protective role in human diseases.

Ripasudil (K-115), a novel, potent, and selective ROCK inhibitor, is a promising emerging drug against glaucoma which has been approved by the Japanese administrative authority [12, 13]. Anti-inflammatory effect of ripasudil has been reported in previous studies, suggesting that ripasudil might be effective in inflammation-related diseases [6, 14]. It is reported that ripasudil attenuated lipopolysaccharide (LPS)-induced inflammation and apoptosis in pulmonary microvascular endothelial cells, indicating an effect of ripasudil in pulmonary diseases [15]. However, whether ripasudil is protective in pneumonia has not been discovered and remains unclear. Thus, the present study aims to investigate the role of ripasudil in the progress of pneumonia in mice to ensure its therapeutic effect.

Materials and methods

Patients and blood collection

The study protocol was approved by the Ethics Committee of The Second Affiliated Hospital of Shaanxi University of Chinese Medicine according to the principles of the Declaration of Helsinki. A total of 30 child patients with pneumonia and 30 aged-matched healthy controls were recruited. Exclude patients with other complications or patients who had received anti-inflammatory treatment. A total of 3 ml fasting peripheral venous blood was obtained from patients and healthy controls. The blood samples were centrifuged at 4000 g at 4°C, and then the supernatant was collected and stored at -80°C for the further research.

Animals and experimental protocols

BALB/c mice were purchased from Slac Laboratory Animal Co. Ltd. (Shanghai, China), and kept in a specific pathogen free (SPF) environment with free access to water and food. This study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Shaanxi University of Chinese Medicine. Mice were randomized into groups (6 mice in each group) consisting of control group, LPS group, and LPS + ripasudil (0.5 mg, 1 mg and 2 mg) groups. BALB/c mice were used to establish an acute pneumonia model of mice by injection of lipopolysaccharide (LPS; Sigma, USA) intraperitoneally. The mice of the control group were administrated the same amount of normal saline. An aliquot of 0.1 ml of ripasudil (Kowa Company, Japan) and vehicle (PBS; Wako Pure Chemicals, Japan) was intraperitoneally injected 1 h before LPS treatment. Lungs and blood were collected for the further research.

Histological analysis

For histological analysis, lung tissues were fixed in 4% formaldehyde, dehydrated and embedded in paraffin, followed by cutting into sections with a thickness of 4 μm. Then the sections were stained with hematoxylin-eosin (H&E) and observed under a light microscope.

Lung wet/dry ratio

The right lung tissues were weighed and then dehydrated at 80°C for 48 h to obtain its dry weight. The severity of pulmonary edema was determined by the ratio of lung wet weight/dry weight.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the Trizol reagent (Life Technologies, UK). The complementary DNA (cDNA) was synthesized using the Prime Script RT Master Mix Kit (Takara, Japan) according to the instructions of manufacturer. Then mRNA level of RHOA was determined using the SYBR Select Master Mix Kit (Thermo Fisher Scientific, USA) according to the instructions of manufacturer. GAPDH expression was applied as the internal control.

Western blot

Proteins were extracted by adding RIPA lysis buffer (Beyotime Tech, Shanghai, China) containing PMSF. The suspension was centrifuged at 12000 g for 10 min at 4°C. The protein concentration was detected using the BCA protein assay.

For western blot, samples were subjected to 10% SDS/PAGE and transferred onto PVDF.
membranes. The membranes were blocked with 5% non-fat milk, followed by incubation of primary antibodies at 4°C overnight. Then the appropriate secondary antibody was applied at room temperature for 2 h. The relative density of protein bands was determined using an enhanced chemiluminescence (ECL) solution.

**Enzyme-linked immunosorbent assay (ELISA)**

The levels of endothelial nitric oxide synthase (eNOS), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and monocyte chemo-attractant protein-1 (MCP-1) in blood were determined by corresponding ELISA kit (Thermo Fisher Scientific, USA) according to the instructions of manufacturer, respectively.

**Reactive oxygen species (ROS) and oxidative stress assay**

Production of ROS was measured using the ROS test protocol (Beyotime Biotechnology, CHN). The activity of lactate dehydrogenase (LDH), superoxide dismutase (SOD) in bronchoalveolar lavage fluid (BALF) were spectrophotometrically assessed according to the test kits following the manufacturer's instructions (Nanjing Jiancheng Biotechnology Institute, China).

**Cell apoptosis assay**

Cell apoptosis of lung tissues were determined by terminal dUTP transferase nick-end labeling (TNUEL) assay. After deparaffinization and hydration, lung tissues were washed with PBS and incubated with proteinase K working solution (Abcam, UK). Then tissues were treated with the TUNEL reaction mixture (Roche, Germany), and maintained in a 37°C incubator for 60 min. TUNEL-positive cells were observed with a confocal microscopy.

**Immunohistochemistry**

After tissues were cut into sections at a thickness of 4 μm, the sections were dewaxed and rehydrated by decreasing gradient of ethanol. Sections were incubated with anti-Rhoa antibody, followed by incubation with secondary antibody. Then the sections continued to be incubated in DAB staining (ZSGB-BIO, Beijing, China) for 5 min, followed by counterstain with hematoxylin for 30 s. All images were viewed under a light microscope.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 6.0 software (La Jolla, CA, USA) and SPSS 22.0 (SPSS, Inc., USA). Differences were analyzed using student's t-test between groups and using one-way ANOVA followed by Tukey's post hoc test among groups. Data were considered statistically significant at P<0.05.

**Results**

**Ripasudil ameliorated LPS-induced histological injury**

The mouse pneumonia model was established by injection of LPS intraperitoneally. Compared with the control group, LPS induced obvious structural destruction, and inflammatory infiltration in pulmonary areas. With the increased concentration (0.5 mg, 1.0 mg, 2.0 mg) of ripasudil, the pulmonary injury was ameliorated (Figure 1A). Lung wet/dry ratio was conducted to assess pulmonary edema. Compared to the control, pulmonary edema was severe after induction of LPS (Figure 1B). While ripasudil significantly declined the level of pulmonary edema. These results suggested that ripasudil ameliorated LPS-induced histological injury.

**Ripasudil suppressed LPS-induced inflammation**

An obvious inflammatory infiltration was observed after LPS induction in mice, indicating a severe inflammatory injury occurred in pulmonary tissue, usually along with production and release of inflammatory cytokines. Thus, the levels of inflammatory cytokines in blood and lung tissues were detected by ELISA and western blot, respectively. As observed in Figure 2A-D, the levels of IL-1β, IL-6, TNF-α and MCP-1 in blood were significantly increased after LPS induction compared to the control. Treatment of ripasudil obviously decreased the cytokines levels at a concentration-dependent way. Western blot assay in Figure 2E-I also showed an increased expression of inflammatory cytokines in LPS group, which was then obviously decreased by ripasudil treatment (1 mg, 2 mg). These results suggested that ripasudil could
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**Figure 1.** Lipopolysaccharide (LPS) was used to induce acute pneumonia in mice. Mice were treated with different concentrations of ripasudil (0.5 mg, 1 mg, 2 mg). A. Hematoxylin-eosin (H&E) staining of lung tissues was performed to evaluate histological changes. B. The degree of pulmonary edema was evaluated by the ratio of lung wet/dry weight. Data were presented as mean ± SD from at least three experiments. Compared to the control group, ***P<0.001; compared to the LPS group, #P<0.05, ###P<0.001.

decrease the production of inflammatory cytokines caused by LPS.

*Ripasudil suppressed LPS-induced oxidative stress*

Compared with the control group, the levels of ROS and LDH were significantly increased after LPS induction, while the levels of SOD and eNOS were significantly decreased after LPS induction (Figure 3A-D). These alterations were reversed by the treatment of ripasudil. Besides, protein expression of zonula occludens-1 (ZO-1) was decreased after LPS induction, which was also increased with the treatment of ripasudil with a concentration-dependent way (Figure 3E).

*Ripasudil decreased LPS-induced cell apoptosis*

Effect of ripasudil on the cell apoptosis in LPS induced pneumonia was shown in Figure 4. Tunnel staining in Figure 4A showed that apoptosis number of cells was increased after LPS induction, and ripasudil significantly deceased cell apoptosis. Apoptosis-related proteins were detected using western blot in Figure 4B. LPS induction significantly decreased the expression of Bcl-2, and increased the expression of
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Bax and cleaved caspase-3. While these alternations were reversed when mice were treated with ripasudil concentration-dependently.

Ripasudil inhibited RhoA-ROCK signaling pathway

To investigate the further potential mechanism of ripasudil in LPS-induced pneumonia, the influence of ripasudil on RhoA-ROCK signaling pathway was observed. As shown in Figure 5A, immunohistochemistry analysis showed that RhoA expression was increased after LPS induction, and was decreased with the treatment of ripasudil. Then western blot assay was performed to detect RhoA-ROCK signaling pathway. As shown in Figure 5B, compared to the control, induction of LPS significantly increased

Figure 2. Ripasudil prevents LPS-induced inflammatory response. A-D. The production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and monocyte chemo-attractant protein-1 (MCP-1) was detected using ELISA. E-I. The production of TNF-α, IL-1β, IL-6 and MCP-1 was detected using western blot. Data were presented as mean ± SD from at least three experiments. Compared to the control group, ***P<0.001; compared to the LPS group, *P<0.05, **P<0.01, ***P<0.001.
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the protein expressions of LIM Kinase 1 (LIM-K1), LIMK2, RhoA, ROCK1, ROCK2 and p-cofilin (p-CFL), indicating an activation of RhoA-ROCK signaling, while these protein expressions were decreased with the treatment of ripasudil, indicating that ripasudil decreased the activity of RhoA-ROCK signaling. These results suggested that ripasudil might exert its role through inhibiting the activity of RhoA-ROCK signaling.

The level of RhoA was negatively correlated with eNOS in pneumonia patients

QRT-PCR and ELISA analysis showed that the expression level of RhoA was significantly higher, whereas the level of eNOS in peripheral blood from pneumonia patients was lower than that in the healthy control (Figure 6A, 6B). Statistical analysis exhibited a negatively-correlated relation between RhoA and eNOS in pneumonia patients (Figure 6C). Thus inhibiting RhoA or improving eNOS might be effectively approaches for the treatment of pneumonia, suggesting that ripasudil might be a promising drug for clinical application based on its biological function.

Discussion

Even though the morbidity and mortality rate of pneumonia in children has declined in recent years, it is still the main cause of children death, and nearly 20% children under 5-year-old died of pneumonia [16]. Thus, an action is needed to accelerate prevention and treatment of pneumonia. Here, we introduced LPS to simulate pulmonary infection of Gram-negative bacterial, which usually leads to pneumonia. This study focused on the role and potential mechanism of ripasudil in pneumonia. The experimental results revealed that ripasudil play a protective role in LPS-stimulated acute pneumonia in

Figure 3. Ripasudil prevents LPS-induced oxidative stress. A-C. The production of reactive oxygen species (ROS), lactate dehydrogenase (LDH) and superoxide dismutase (SOD) was detected using the corresponding test protocol following the manufacturer’s instructions, respectively. D. The production of eNOS was detected using ELISA. E. The expression of zonula occludens-1 (ZO-1) was detected using western blot. Data were presented as mean ± SD from at least three experiments. Compared to the control group, ***P<0.001; compared to the LPS group, *P<0.05, **P<0.01, ***P<0.001.
Figure 4. Ripasudil prevents LPS-induced cell apoptosis. A. Apoptosis of lung tissue cells was determined by TNUEL assay. B. Apoptosis-related proteins: Bax, Bcl-2, Cleaved caspase-3 and caspase-3 were detected by western blot and quantified. Data were presented as mean ± SD from at least three experiments. Compared to the control group, ***P<0.001; compared to the LPS group, *P<0.05, **P<0.01, ###P<0.001.
Figure 5. Ripasudil inhibited RhoA-ROCK signaling pathway. A. The expression of RhoA in lung tissue was determined using immunohistochemistry. B. RhoA-ROCK signaling-related proteins: LIM Kinase 1 (LIMK1), LIMK2, RhoA, ROCK1, ROCK2, p-cofilin (p-CFL) and CFL was detected using western blot and quantified. Data were presented as mean ± SD from at least three experiments. Compared to the control group, ***P<0.001; compared to the LPS group, *P<0.05, **P<0.01, ***P<0.001.
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First, we explored the anti-inflammatory effects of ripasudil on LPS-induced acute pneumonia in mice. LPS has been reported to induce inflammation by entering nucleus to stimulate pro-inflammatory cytokines expressions such as TNF-α, IL-1β and IL-6 [17]. The concentrations of TNF-α, IL-1β and IL-6 are detectable and arrived at peak at early phase of inflammation, meanwhile, the lung inflammatory response is a hallmark of pneumonia [18]. In this study, LPS significantly increased the expression levels of TNF-α, IL-1β and IL-6, leading an inflammatory response and pneumonia. Ripasudil could significantly decrease these elevated inflammatory cytokines, effectively inhibited inflammatory response. MCP-1 is a member of chemotactic cytokines (CC) sub-family chemokines, which plays a prominent role in the development of inflammatory response [19]. MCP-1 is usually produced by monocyte, airway epithelial cells, lymphocytes, and macrophages in response to inflammation. Besides, it is regarded as a biochemical marker for an early diagnosis of pneumonia [20]. Thus, inhibition of MCP-1 is an effective way to alleviate pneumonia. In this study, ripasudil made an obvious decrease of MCP-1 in a concentration-dependent way. These alternations that include decreased TNF-α, IL-1β and IL-6, and decreased MCP-1 after ripasudil treatment showed that ripasudil had a well anti-inflammatory activity in LPS-induced acute pneumonia in mice, indicating that ripasudil might serve as a therapeutic strategy of pneumonia through inhibiting inflammation.

Oxidative stress is closely associated with toxic inflammation reactions, along with excessive production of reactive oxygen and nitrogen species, which is connected to damage biomembranes, compromise cell function and lead to pulmonary injury [21]. Antioxidant enzymes and free radicals are classical oxidative stress markers, and high-exposure of LPS usually trigger aberrant expression of antioxidant enzymes and free radicals in the lung homogenates of mice, thus the balance between these radicals should be maintained to protect lung tissues against oxidative damage [21-23]. Here, an overproduction of ROS and MDA, and decreased expression of SOD and eNOS, was measured after LPS induction in mice. However, these alternations were reversed by ripasudil, suggesting that ripasudil could play a protective role against LPS-induced oxidative damage.

Aberrant activation of RhoA/ROCK signaling has been reported to be associated in various diseases [9, 24]. Cofilin (CFL) is an actin-binding protein that is essential for the depolymerization of actin filaments, and phosphorylation of CFL decreases its actin binding affinity, resulting in an accumulation of filamentous actin, and depolymerization of actin [25]. CFL is a downstream effector of LIMK1, thus LIMK1 regulate the balance of phosphorylation and dephosphorylation of CFL to influence cellular movement, promoting cell proliferation and migration [26, 27]. LIMK has two main isoforms, LIMK1 and LIMK2. Both of LIMK1 and LIMK2 are phosphorylated by upstream kinases ROCK1. Therefore, the function of RhoA/ROCK signaling was exerted mainly through their downstream effectors, LIMK and CFL, and

Figure 6. The level of RhoA was negatively correlated with eNOS in pneumonia patients. A. The mRNA level of RhoA in peripheral blood from pneumonia patients was detected using qRT-PCR. B. The level of eNOS in peripheral blood from pneumonia patients was detected using ELISA. C. The level of RhoA and eNOS in pneumonia patients was negatively correlated.
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This signaling transduction pathway could regulate actin, which is important to determining cell shape and cellular movement, in response to different extracellular stimuli [28]. A previous study has reported that leptin stimulation to human nucleus pulposus cells induced cytoskeleton reorganization, while the RhoA inhibitor and ROCK inhibitor could prevent leptin-induced phosphorylation of LIMK1 and CFL and attenuate the effects that leptin caused [29]. In the present study, the stimulaton of LPS activated the RhoA/ROCK/LIMK/CFL signaling activity by evidenced of elevated expressions of LIMK, RhoA, ROCK, and phosphorylation of CFL, which further caused lung inflammatory injury [30]. It has been reported that Fasudil, ROCK inhibitor, retarded high glucose-induced inflammatory injury in human umbilical vein endothelial cells, indicating that inhibition of RhoA/ROCK pathway has the potential ability against inflammatory response [31]. Here, the ROCK inhibitor, ripasudil, significantly attenuated LPS-induced inflammatory response, oxidative stress and cell apoptosis, and reduced the activity of RhoA/ROCK/LIMK/CFL signaling at a concentration-dependent manner, indicating that ripasudil has potential therapetic ability in pulmonary injury by inhibiting the activity of RhoA/ROCK/LIMK/CFL signaling pathway.

Conclusion

In the present study, the anti-inflammatory and anti-apoptotic effects of ripasudil were explored in vivo. The results suggested that ripasudil play a protective role in LPS-induced pneumonia, and effectively attenuated inflammatory response, oxidative stress and cell apoptosis partly through inhibiting the activity of RhoA/ROCK signaling pathway. The data here provided a potential therapeutic strategy for the treatment of pneumonia, and more pre-clinical explorations are in need in the future.

Disclosure of conflict of interest

None.

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