Original Article

Fasudil, an inhibitor of Rho-associated coiled-coil kinase, attenuates hyperoxia-induced pulmonary fibrosis in neonatal rats

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Abstract: Background: Oxygen therapy is important during the management of high-risk neonatal infants, such as those with preterm birth, low birth weight, and asphyxia. However, prolonged exposure to high oxygen concentrations can readily lead to diffuse nonspecific inflammation, which promotes airway remodeling and pulmonary fibrosis. The Rho/Rho-associated coiled-coil kinase (Rho/ROCK) signaling pathway plays an important role in numerous developmental and proliferative diseases. This study was performed to determine the efficacy of ROCK inhibitor fasudil in blocking the development of hyperoxia-induced lung injury and fibrosis in neonatal rats. Methods: Neonatal rats were randomly divided into four groups: air + saline group, air + fasudil group, hyperoxia + saline group, and hyperoxia + fasudil group. The hyperoxia + saline and Hyp + fasudil groups were exposed to 95% oxygen for 21 days and administered intraperitoneal saline or fasudil once daily. The air + saline and air + fasudil group were exposed to 21% oxygen (room air) and administered the same volume of intraperitoneal saline or fasudil. Results: Fasudil-treated rats exhibited improved histopathological changes and decreased lung hydroxyproline content. Fasudil attenuated the protein level of alpha-smooth muscle actin, transforming growth factor-β1, and connective tissue growth factor. Additionally, fasudil reduced the activation of ROCK1 and myosin phosphatase targeting subunit 1 protein in the Rho/ROCK signaling pathway. Conclusions: Fasudil may be a potentially effective therapeutic drug for hyperoxia-induced pulmonary fibrosis.

Keywords: Hyperoxia, neonatal rats, pulmonary fibrosis, fasudil, Rho/ROCK signaling pathway

Introduction

Oxygen therapy is important during the management of high-risk neonatal infants, such as those with preterm birth, low birth weight, and asphyxia. However, prolonged exposure to high oxygen concentrations can readily lead to diffuse nonspecific inflammation in the lung parenchyma and interstitium, which promotes airway remodeling and pulmonary fibrosis [1, 2]. Because of their pulmonary surfactant deficiency and premature antioxidant system, neonatal infants (especially preterm infants) can also develop the additional complication of bronchopulmonary dysplasia (BPD), which is characterized by lung inflammation and fibrosis [3]. Lung immaturity, acute and chronic lung injury, and pulmonary fibrosis are all known to contribute to the development of BPD [4]; however, the pathogenesis of hyperoxia-induced BPD remains unclear.

The Rho/ROCK signaling pathway plays important roles in the regulation of various biological activities involving in cell proliferation, differentiation, contraction, chemotaxis, adhesion, invasion, and other behaviors [5-7]. Rho GTPase, ROCKs (ROCK1, ROCK2) and myosin phosphatase are key signaling molecules in this pathway [5]. ROCK1 is mainly expressed in the lung, liver, kidney, and testis, while ROCK2 is mainly distributed in the brain and heart [8]. Infla-
Inflammatory cytokines induce the conversion of inactive GDP-bound Rho into active GTP-bound Rho. Active Rho then activates its downstream effector ROCK, which stimulates the phosphorylation of myosin phosphatase-targeting subunit 1 (MYPT1) at multiple amino acid loci. Phosphorylation of MYPT1 inactivates myosin light chain phosphatase (MLCP), thus affecting the level of cytoplasmic myosin light chain (MLC).

The Rho/ROCK signaling pathway has gained widespread attention in recent years. Specific ROCK inhibitors have become powerful tools in exploring the molecular role of Rho/ROCK signaling in various diseases. Fasudil, the only clinically approved ROCK inhibitor, was first used in Japan in 1995 as a novel and efficient vasodilator in patients with subarachnoid hemorrhage-induced cerebral vasospasm. Since then, a large number of clinical trials have confirmed its safety [9].

Various studies have suggested that fasudil is capable of inhibiting inflammation, blocking epithelial-mesenchymal transition (EMT), suppressing cell proliferation and migration, repressing organ reconstruction, and alleviating tissue fibrosis [10-12]. There are numerous reports on the efficacy of fasudil on pulmonary fibrosis in adult mice, nevertheless there are few in neonates. Activation of the Rho/ROCK signaling pathway in bleomycin-induced pulmonary fibrosis has suggested that this pathway is an important therapeutic target for fibrotic diseases [13]. We hypothesized that fasudil effectively blocks the development of hyperoxia-induced pulmonary fibrosis in neonatal rats. For this purpose, we exposed neonatal rats to 95% oxygen for 21 days to establish a hyperoxia-induced lung fibrosis model [14]. Using this model, we observed the effect of fasudil on hyperoxia-induced lung fibrosis. We evaluated the histopathological changes and hydroxyproline content of the lungs in hyperoxia-exposed rats. We also measured the protein level of alpha-smooth muscle actin (α-SMA), transforming growth factor-β1 (TGF-β1), and connective tissue growth factor (CTGF) in lung tissue to elucidate the molecular mechanisms involved in the blocking of hyperoxia-induced lung fibrosis by fasudil in neonatal rats. Finally, we measured the ROCK1 and MYPT1 phosphorylation levels in the Rho/ROCK signaling pathway.

Materials and methods

Animals and treatments

This animal study was approved by our institutional animal research ethics committee. Twenty-four full-term neonatal Sprague-Dawley rats (male and female; body weight, 5.0 ± 0.5 g) were purchased from the experimental animal center of Daping Hospital of the Third Military Medical University, Chongqing, China. All rats were randomly divided into four groups of six rats each: those administered air + saline (Air + NS group), air + fasudil (Air + FAS group), hyperoxia + saline (Hyp + NS group), and hyperoxia + fasudil (Hyp + FAS group). Rats in the Hyp + NS group and Hyp + FAS groups were placed in animal oxygen cage, into which 100% oxygen was constantly flowed. The oxygen concentration was monitored by a digital oxygen concentration meter (CY12C; Xin’an Jiang Analytical Instrument Factory, Jiande, Zhejiang, China) to ensure that it remained at > 95%; the concentration of carbon dioxide remained at < 5%. Rats in the Air + NS and Air + FS groups were exposed to room air (21% oxygen).

Fasudil (Tianjin Chase Sun Pharmaceutical Co., Ltd., Tianjin, China) was intraperitoneally injected at 20 mg/kg once daily for 21 days; the same volume of saline was injected daily as a vehicle. The mother rats were exchanged between the air and hyperoxia groups at 10:00 each morning to prevent the oxygen exposure from affecting their lactation ability. Breeding pads, food, and water were replaced daily. Animal survival rates and body weights were recorded every other day. The oxygen cage was open for less than 1 hour each day. The ambient temperature was 22°C to 26°C, and the humidity was 60% to 70%.

All rats were sacrificed under anesthesia by intraperitoneal injection of 3% chloral hydrate on day 21 following the treatments. The lungs were harvested from each rat. The left lungs were soaked in 4% paraformaldehyde overnight and embedded in paraffin, and the right lungs were frozen in liquid nitrogen for western blot analysis.

Lung histology

The lung sections were stained with hematoxylin-eosin and Masson’s trichrome. The slides were examined for inflammation and fibrosis under a light microscope. The radial alveolar count (RAC), an important index of alveolar...
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development [15], was measured using a vertical line drawn from the center of a respiratory bronchiole to the nearest pleura. Ten different fields were randomly selected from each slide under light microscopy (100× magnification).

Hydroxyproline assay by alkali hydrolysis method

The total collagen content of the lung tissue was determined by analysis of hydroxyproline on day 21 after hyperoxia exposure as previously described [13]. The hydroxyproline assay was conducted using a hydroxyproline test kit (A030-2; Jiancheng Biological Engineering Institute, Nanjing, China) according to the manufacturer’s instructions. Briefly, 1 ml of hydrolysate was added to 30 to 100 mg of lung tissue. The tissue was boiled for 20 minutes and then cooled using flowing tap water. Reaction reagents were added to the lung tissue following adjustment of the pH value, and the absorbance at 550 nm was measured. The content of hydroxyproline in the lung tissue of rats from each group was then calculated according to the manufacturer’s instructions.

Immunohistochemistry

Immunohistochemical analysis was performed using a two-step immunohistochemical detection kit (Catalog No. PV-9001; Zhongshan Jinqiao Biotechnology Co., Beijing, China). Briefly, lung sections were incubated with hydrogen peroxide to block endogenous peroxidase following deparaffinization. After antigen retrieval and blocking, the slides were incubated overnight with polyclonal antibody to α-SMA (1:200 dilution; Abcam, Cambridge, MA, USA). Phosphate-buffered saline was used as a negative control. The next day, the slides were incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody for 1 hour at room temperature. The slides were then stained with Dolichos biflorus agglutinin (DAB) (Zhongshan Jinqiao Biotechnology Co.) and hematoxylin-eosin, dehydrated, clarified, and mounted. Cells with brown gran-
Figure 2. Representative histopathological changes in lung tissue at 21 days. Rats were exposed to hyperoxia and treated with fasudil for 21 days. All rats were sacrificed on day 21, and their lungs were harvested. A-D. Hematoxylin-eosin (200× magnification). E-H. Masson’s trichrome (200× magnification). A, E. Air + NS group. B, F. Air + FAS group. C, G. Hyp + NS group. D, H. Hyp + FAS group.
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The radial alveolar count was calculated as described in the Materials and methods. NS, saline; FAS, fasudil; Hyp, hyperoxia. #P < 0.05 compared with Air + NS group.

The tissue was homogenized with radioimmunoprecipitation assay lysis buffer containing phenylmethylsulfonyl fluoride. The total protein was extracted, and the protein concentration was determined using a bicinchoninic acid assay. Fifty micrograms of protein from each sample was loaded into 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking in 5% bovine serum albumin at room temperature for 1 hour, the membrane was then incubated with specific primary antibodies overnight at 4°C. The next day, the membrane was incubated with a secondary antibody followed by development using an enhanced chemiluminescence kit (Catalog No. KGP1123; Key Gen Biotech, Nanjing, China). The primary antibodies included anti-α-SMA antibody (1:200), anti-TGF-β1 antibody (1:1000) (GeneTex, Inc., Irvine, CA, USA), anti-CTGF antibody (1:1000) (GeneTex), anti-ROCK1 antibody (1:500), anti-p-MYPT1 antibody (1:1000), anti-MYPT1 antibody (1:1000) (Cell Signaling Technology, Inc., Beverly, MA, USA), and rabbit anti-β-actin polyclonal antibody (1:2000) (Si Zhen Bai Biological Technology, Beijing, China).

Statistical analyses were performed using SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, NY, USA). Data are expressed as mean ± standard deviation. Comparison of differences among groups was performed using one-way analysis of variance followed by LSD test. The cumulative survival rate was calculated using Kaplan-Meier survival analysis. Statistically significant differences were indicated by P values of < 0.05.

Results

Effects of fasudil on body weight and mortality

Compared to air groups, the body weights of hyperoxia groups were significantly reduced following 21 days of hyperoxia treatments (P < 0.05). The body weight of Hyp + FAS group was even lower than that of Hyp + NS group; however, there was no significant difference between the two groups (P > 0.05) (Figure 1A).

The animal survival rates were 100.0% in the Air + NS and Air + FAS groups, 66.7% in the Hyp + NS group, and 58.3% in the Hyp + FAS group. The survival rates were significantly lower in the hyperoxia than air groups (P < 0.05). The survival rate in the Hyp + FAS group was lower than that in the Hyp + NS group; however, there was no significant difference between the two groups (P > 0.05) (Figure 1B).

Effects of fasudil on histopathological changes

The effect of fasudil on the development of hyperoxia-induced inflammation and fibrosis was examined on day 21 (Figure 2A-H).
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Mal, well-alveolarized lung structure was observed in the Air + NS and Air + FAS groups. In contrast, hyperoxia treatment induced the development of a disordered lung tissue structure, alveolar collapse, obvious alveolar wall thickening, and numerous blue-stained stripes and flakes indicating collagen deposition. Although fibrotic lesions were observed in the Hyp + FAS group, both the extent and intensity of the lesions were less severe than those of the Hyp + NS group.

Effects of fasudil on RAC

We measured the RAC value on day 21 to observe the effect of fasudil on the development of neonatal rat lungs (Figure 3). RAC values were similar between the Air + NS and Air + FAS groups. However, hyperoxia treatment significantly lowered the RAC values in the Hyp + NS group ($P < 0.05$). The RAC value in the Hyp + FAS group was slightly higher than that in the Hyp + NS group, but there was no statistically significant difference.

Effects of fasudil on hydroxyproline level

We measured the content of hydroxyproline on day 21 to quantitatively observe the difference in the degree of pulmonary fibrosis in hyperoxic rats with and without fasudil treatment (Figure 4). The hydroxyproline content in the Air + NS, Air + FAS, Hyp + NS, and Hyp + FAS groups were 271.66 ± 36.25, 283.76 ± 41.82, 588.78 ± 51.74, and 588.78 ± 51.74 µg/g, respectively. The content of hydroxyproline was similar in the air groups. In contrast, hyperoxia treatment resulted in significantly elevated lung tissue hydroxyproline levels ($P < 0.05$). The content of hydroxyproline was significantly lower in rats treated with fasudil than in those exposed to hyperoxia alone ($P < 0.05$).
Effects of fasudil on α-SMA

Myofibroblasts play important roles in promoting inflammation, tissue repair, and pulmonary fibrosis. Therefore, we examined the expression and location of the myofibroblast-specific marker α-SMA in lung tissue by immunohistochemistry and measured the protein content by western blot analysis (Figure 5A-E). Immunohistochemical staining of lung sections showed that α-SMA-positive cells were mainly expressed in the bronchial smooth muscle cells and vascular smooth muscle cells of the rats in the air groups. Only small numbers of α-SMA-positive cells were observed in the alveolar septa, alveolar surfaces, and bronchiolar epithelium. Conversely, after exposure to hyperoxia, large numbers of α-SMA-positive cells were noted in the alveolar septa and alveolar surfaces. Substantially fewer α-SMA-positive cells were present in the hyperoxia-exposed lung tissue after fasudil treatment.

Semiquantitative western blotting revealed that the α-SMA protein levels were similar between the Air + NS and Air + FAS groups. In contrast, the α-SMA protein levels were significantly higher in the hyperoxia groups than in the air groups (P < 0.05). However, the levels were significantly lower after fasudil treatment (P < 0.05) (Figure 5E).

Effects of fasudil on TGF-β1 and CTGF

The profibrotic cytokines TGF-β1 and CTGF play key roles in the occurrence and development of pulmonary fibrosis. We examined the protein levels of TGF-β1 and CTGF in lung tissue on day 21 by western blotting (Figure 6A and 6B). The protein levels of both TGF-β1 and CTGF were similar between the Air + NS and Air + FAS groups. In contrast, hyperoxia treatment was associated with significantly higher TGF-β1 and CTGF protein levels in both the Hyp + NS and Hyp + FAS groups (P < 0.05). However, the levels were significantly lower in the Hyp + FAS group than in the Hyp + NS group (P < 0.05).

Effects of fasudil on p-MYPT1 and ROCK1 protein levels

The expression of ROCK1 and p-MYPT1 in lung tissue after hyperoxia exposure was measured by western blotting (Figure 7A and 7B). The levels of ROCK1 and phosphorylated MYPT1 exhibited no differences between the two air groups. Hyperoxia exposure stimulated a rising trend in the ROCK1 protein level (P > 0.05), but a significantly increased MYPT1 phosphorylation level. Fasudil treatment reduced the expression of ROCK1 protein (P > 0.05) and significantly decreased the MYPT1 phosphorylation level (P < 0.05).
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Discussion

To the best of our knowledge, this is the first report of the attenuation of hyperoxia-induced pulmonary fibrosis by fasudil, a ROCK inhibitor, in neonatal rats. In this study, fasudil significantly (1) improved hyperoxia-induced pathological changes and reduced the content of hydroxyproline in the lungs of neonatal rats, (2) inhibited both expression of the myofibroblast-specific marker α-SMA and production of the profibrotic cytokines TGF-β1 and CTGF, and (3) inhibited the activation of ROCK1 and MYPT1 in the Rho/ROCK signaling pathway.

Transformation of fibroblasts into myofibroblasts plays a pivotal role in the pathogenesis of fibrosis [16]. Myofibroblasts are a special type of fibroblasts that possess the functions of both fibroblasts and smooth muscle cells. α-SMA is considered to be a characteristic marker of myofibroblasts [17]. Known sources of myofibroblasts include local fibroblasts, bone marrow-derived fibrocytes, and resident epithelia cells (through epithelial mesenchymal transition) [18, 19].

Myofibroblasts secrete cytokines, chemokines, growth factors, extracellular matrix (ECM), and protease and play important roles in inflammation, remodeling, and fibrosis in various tissues [20, 21]. The Rho/ROCK signaling pathway is capable of inducing EMT, regulating the production of myofibroblasts, and participating in the development of fibrosis [22]. Studies have shown that fasudil blocks bleomycin-induced pulmonary fibrosis by inhibition of EMT and downregulation of α-SMA mRNA and protein levels [13]. Y-27632, another ROCK inhibitor, inhibits renal fibrosis through similar mechanisms [23]. Our findings are consistent with these reports [16], in which hyperoxia significantly upregulated and fasudil significantly reduced the expression of α-SMA on the alveolar surface. These results imply that hyperoxia may promote the transition of alveolar epithelial cells to myofibroblasts. We speculate that the antifibrotic effect of fasudil in neonatal rats may be mediated by inhibition of EMT.

TGF-β1 plays important regulatory roles in various pathological processes involving inflammatory responses, tissue repair, and organ fibrosis [24-26]. As a downstream target of TGF-β1, CTGF collaboratively promotes fibroblast proliferation and accumulation, stimulates the synthesis of collagen and fibronectin, and accelerates deposition of the ECM [27]. Yin et al. [28] reported that both TGF-β1 and CTGF were significantly upregulated in rats with bleomycin-induced pulmonary fibrosis. Application of anti-TGF-β1 antibody and anti-CTGF antibody significantly inhibits secretion and synthesis of collagen and other ECM proteins. In the present study...

Figure 7. Protein levels of (A) ROCK1 and (B) phosphorylation of MYPT1 in lung tissue. Rats were exposed to hyperoxia and treated with fasudil for 21 days. All rats were sacrificed on day 21, and their lungs were harvested. a. Air + NS group; b. Air + FAS group; c. Hyp + NS group; d. Hyp + FAS group. #P < 0.05 compared with Air + NS group; &P < 0.05 compared with Hyp + NS group.
study, hyperoxia upregulated the protein levels of both TGF-β1 and CTGF in lung tissue, and fasudil treatment significantly decreased these levels. This finding suggests that both TGF-β1 and CTGF play important roles in the pathogenesis of hyperoxia-induced pulmonary fibrosis. Inhibition of the profibrotic cytokines TGF-β1 and CTGF is likely to be an important molecular mechanism that contributed to the antifibrotic effect of fasudil in this study.

Rho/ROCK signaling is a ubiquitous pathway in various tissues of the body and mediates the process of tissue fibrogenesis through its functional contributions to the inflammatory response, its ability to block EMT, and its regulation of the cytoskeleton and cell migration, as well as other activities [23, 29, 30]. Nagatoya et al. [31] reported that the RhoB mRNA and protein levels were significantly upregulated in unilateral ureteral obstruction-induced fibrotic kidneys. Shimizu et al. [8] showed that the ROCK2 mRNA and protein levels were increased in bleomycin-induced fibrotic lungs. These studies suggest that the Rho/ROCK signaling pathway plays important roles in the pathogenesis of organ fibrosis. In the present study, the expression of ROCK1 protein in lung tissue slightly increased and the expression of its downstream active target p-MYPT1 protein was significantly upregulated following 21 days of hyperoxia exposure. Fasudil treatment slightly reduced the ROCK1 protein levels and significantly reduced the p-MYPT1 expression. These results suggest that the Rho/ROCK pathway was activated at the protein level in this hyperoxia-induced pulmonary fibrosis model. The Rho/ROCK signaling pathway may be involved in hyperoxia-induced pulmonary fibrosis.

In addition to its profibrotic role, ROCK also plays an important role in lung development in neonates; e.g., it is involved in the formation of pulmonary gas exchange units and expansion of the lungs [32, 33]. We examined the RAC values following fasudil treatment to study the role of fasudil in lung development in neonatal rats. Our data suggest that fasudil has no apparent effects on RAC values and that inhibition of the ROCK pathway by fasudil may have little effect on normal lung development in neonatal rats. This finding is in line with that reported by Ziino et al. [34]. One explanation is that fasudil cannot completely inhibit ROCK activity as previously reported [32]. Therefore, the remaining ROCK activity is able to maintain the normal lung development. We also noted that fasudil had no obvious effect on the body weight or mortality of the normoxic neonatal rats in the present study. Under hyperoxic conditions, however, fasudil appeared to have an amplified effect on hyperoxia-induced body weight loss and increased mortality in rats. The underlying mechanism of this phenomenon is unclear. One possible mechanism involves the low tissue perfusion caused by inhibition of the ROCK pathway.

In summary, fasudil blocks the development of hyperoxia-induced pulmonary fibrosis in neonatal rats. The antifibrotic effect is possibly mediated by decreasing the levels of the profibrotic cytokines TGF-β1 and CTGF and inhibiting the development of myofibroblasts in lung tissue, thereby inhibiting ECM synthesis. The regulatory mechanism of fasudil may be related to its inhibition of activation of the Rho/ROCK signaling pathway. Targeting the Rho/ROCK signaling may represent a novel therapy for hyperoxia-induced pulmonary fibrosis. The effect of fasudil on lung development and other organs remains to be further studied. Clinically, we may be able to use local therapy specifically targeting lung, such as inhalation of fasudil to reduce pulmonary fibrosis so as to avoid the systemic effects of fasudil on human body.

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Disclosure of conflict of interest

None.

Authors’ contributions

JL, FX and HXD conceived and designed the experiments. XJQ and WN performed the experiments and analyzed the data. XJQ wrote the paper. FF contributed reagents, materials and laboratory environment.

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