Fasudil alleviated hypoxia-induced pulmonary hypertension by stabilizing the expression of angiotensin-(1-7) in rats

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Abstract. – OBJECTIVE: The aim of this study was to investigate the mechanism of fasudil alleviating hypoxic pulmonary hypertension (HPH).

MATERIALS AND METHODS: A total of 50 Sprague-Dawley rats were randomized into control, model and fasudil intervention groups. Hemodynamic and pulmonary pathomorphology data were collected using Powerlab system and hematoxylin and eosin staining. The protein expression of Ang-(1-7) was detected by immunohistochemical staining and ELISA assay in vivo or in vitro. Western blot was utilized to observe the protein expression of ACE2 and HIF-1α in vitro.

RESULTS: Fasudil treatment repressed the elevation of RVSP, RV/(LV+S), attenuated the pulmonary vascular structure remodeling (PVSR) of pulmonary arterioles induced by chronic hypoxia, and stabilized the expression of Ang-(1-7) in vivo and in vitro. In addition, experiments consistently indicated an escalation of ACE2 and a reduction of HIF-1α.

CONCLUSIONS: Our results suggest that fasudil can effectively attenuate PVSR and PH. The underlying mechanism may partially up-regulated Ang-(1-7) and ACE2, and lessened HIF-1α.

Key Words: Hypoxic pulmonary hypertension, Fasudil, Ang-(1–7), ACE2, HIF-1α.

Introduction

Pulmonary hypertension is a chronic hypoxic lung disease, characterized by sustained increase of pulmonary artery pressure and vascular remodeling. Pulmonary vascular structure remodeling (PVSR) serves as an indication of advanced pulmonary hypertension, accompanied with several histological changes such as intima thickening, media hyperplasia, adventitia widening and peripheral vessels muscularization, which ultimately leads to severe pulmonary hypertension, right ventricular hypertrophy and right heart failure with high mortality. Rho-kinase, have significant roles in a variety of cellular functions, including smooth muscle cell (SMC) contraction, cytoskeletal rearrangement, cell proliferation and migration. Studies also suggested that Rho-kinase affected development of PH through its effects on vasoconstriction and cell proliferation. Recent studies demonstrated fasudil, as a ROCK inhibitor, exerts a direct inhibitory effect on pulmonary artery smooth muscle cells (PASMCs) proliferation, whereas the mechanisms of its effects on PASMCs remains unknown.

In addition, pulmonary hypertension (PH) is closely linked with renin-angiotensin system (RAS). Components of the RAS, such as Angiotensin (AGT), angiotensin II (AngII), angiotensin-converting enzyme (ACE) and angiotensin type 1 receptor (AT1R), are associated to the development of PH. Local production of Ang II catalyzed by ACE stimulates the growth of smooth muscle cells, which induces changes of the pulmonary vasculature and subsequently leads to the development of PH. Nevertheless, the recent study stated that ACE2, a homology of ACE, could exert beneficial effect on the development of PH. The local production of Ang II catalyzed by ACE2 and has the antiproliferative and vasodilatating function, opposite to that of Ang II. Hypoxia inducible factor 1α (HIF-1α) is one of the major transcription factors in hypoxia, which translocates into the nucleus, binds to hypoxia-responsive elements, and activates the
expression of genes that promote vascular development. HIF-1α is a key hypoxia-driven signaling pathway in the lung vasculature. Previous reports showed that HIF-1α upregulated ACE protein expression through binding and transactivating the ACE promoter directly during hypoxia, whereas ACE2 protein expression increased during the early stages of hypoxia and decreased to near-baseline levels at the later stages after HIF-1α accumulation. Evidently, ACE2 is regulated in a bidirectional way during hypoxia with a protective effect on the development of HPH. In our work, we found that fasudil significantly enhanced ACE2/Ang-(1-7) expression and reduced HIF-1α expression, thus improving the development of HPH. Considering the above mentioned, we speculated that fasudil may ameliorate chronic hypoxia-induced PVSR and pulmonary hypertension through Ang-(1-7) catalyzed by ACE2. Moreover, our study also suggested that ACE2 expression may be related to the amount of HIF-1α.

Materials and Methods

Animals and Reagents

Male Sprague-Dawley (SD) rats (160 ±15g) were purchased from the Animal Center of the Fourth Military Medical University (Xi’an, China). All procedures were approved by the Animal Care and Use Committee of the Fourth Military Medical University and abided by the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996). Rats were randomly separated into three groups including control group (n=10), hypoxia group (n=10) and hypoxia+fasudil group (n=30, three different doses of fasudil). SD rats were exposed to 8 h/d in a hypobaric hypoxia chamber (10% oxygen) for 6 weeks. After exposure to hypoxia for 4 weeks, the chronic hypoxia-treated rats randomly received intragastric administration with three different doses of fasudil (10 mg/kg, 30 mg/kg and 75 mg/kg per day) for an additional 2 weeks. Age-matched male rats were housed in room air (21% oxygen) as normal control.

Fasudil was obtained from Chase Sun Pharmaceutical Co., Ltd. (Tianjin, China). Ang-(1-7) polyclonal antibody were obtained from Santa Cruz (Santa Cruz, CA, USA). ACE2, β-actin and HIF-1α monoclonal antibodies were purchased from Millipore (Millipore, Billerica, MA, USA). The ELISA kit to determine Ang-(1-7) was obtained from R&D (R&D Systems Inc., Minneapolis, MN, USA).

Hemodynamic Experiments and Tissue Preparation

After 6 weeks of hypoxia, the animals were anesthetized with 20% ethylurethan (5 ml/kg i.p.) and a soft silicagel catheter linked to a Powerlab system (AD Instruments, Colorado Springs, CO, Australia) was inserted into the right jugular vein. The right ventricle peak systolic pressure (RVSP) waveforms were displayed on the monitor when the catheter arrived in the right ventricle chamber. Meanwhile, the mean carotid artery pressure (mCAP) was recorded via a special catheter inserted into the carotid artery. After the hemodynamic data were collected, lung and heart were harvested. The weight of right ventricle (RV) and left ventricle plus septum (LV+S) were obtained, and the ratio of RV/(LV+S) was calculated as an index of RV hypertrophy. The lower lobe of the right lung were placed in neutral buffer (pH 7.4) containing 10% formalin.

Morphological Investigation

The lung tissue were embedded in paraffin and sectioned into 5µm thick sections after soaked in 10% formalin for 72 hours, then processed hematoxylin and eosin staining. Microscopic evaluation showed structure remodeling of the pulmonary arterioles. Total 45 pulmonary arterioles (external diameters of 50-180 µm) in approximate round shape were obtained from each group. The average size of arterioles was 78µm. The outside diameter and inside diameter of pulmonary arterioles were measured by an image-processing program (Image-Pro Plus, Version 5.1, Media Cybernetics, Rockville, MD, USA). The medial wall thickness, the cross-sectional area of medial wall, and the total cross-sectional vessel area were obtained. Pulmonary vascular structure remodeling was assessed by percent medial wall thickness (WT%) and percent medial wall area (WA%) two indices: WT% = 100 × (medial wall thickness) / (vessel semi-diameter); WA% = 100 × (crosssectional medial wall area) / (total cross-sectional vessel area). All the morphological analysis was conducted via a double-blind method.
**Immunohistochemical Staining Assay**

5 µm lung sections were deparaffinized, rehydrated, and blocked by being incubated in 0.3% H2O2 for 30 mins. Once the antigen retrieval was performed, the slides were incubated for 1 h with normal goat serum, and then incubated with Ang-(1-7) at 4°C overnight. After sections were washed in phosphate buffered saline (PBS), then incubated with corresponding secondary antibody for 1 h at 37°C. Diaminobenzidine (DAB) signal detection method was performed for 8 mins at room temperature. Negative controls were performed using mouse serum as the primary antibody. Positive staining was colored in deep brown. All sections were evaluated using an Olympus BX50 optical microscope equipped with an image analysis program (Image ProPlus version 6.0, Immagini e Computer, Milan, Italy) and analyzed quantitatively. The integrated optical density (IOD) was calculated for arbitrary areas, measuring in 10 fields for each sample using a 40×objective.

**Cell Culture and Treatment**

PASMCs were obtained by tissue explant culturing method. Pulmonary arteries were isolated from SD rats. After the adventitial layers together with surrounding tissue were removed, the pulmonary arteries were dissected into small pieces and then placed in a overturned culture flask. DMEM (Dulbecco’s Modified Eagle Medium, HyClone, Logan, UT, USA) with 10% FBS was added in the flask. After 4 hours, the flask was carefully turned over, and the medium immerced the tissue pieces cultured at 37°C in 21% oxygen condition. The PASMCs grew out in about a week, and cell passage was performed when the cells grew to 70% confluence. Cells were used between passages 3 to 6. Smooth muscle cell identity was verified by positive staining for smooth muscle α-actin (mouse monoclonal antibody; Sigma-Aldrich, St. Louis, MO, USA) at each passage. The cells were seeded at 1 × 10⁶ cells in culture dishes (Jet Biofil, L Inc, Canada) and allowed to grow for 3 days. Then cells underwent serum-starvation for 24 hours. The media was then changed to containing 5% FBS phenol-red-free DMEM with fasudil in various dosages. There were altogether 5 groups, including one normoxia group, one hypoxia exposure group alone, and three hypoxia exposure groups treated with three different dosages of fasudil (10 µmol/L, 30 µmol/L, 90 µmol/L). Cells were cultured under 21% or 3% oxygen condition for another 48 hours. After treatment, the cell lysates were obtained for the Western blot and ELISA assays.

**Western Blot Assay**

Total lysates were obtained from harvested cultured PASMCs. PASMCs homogenates were conducted in RIPA lysis buffer (Beyotime Inc., Jiangsu, China). The protease inhibitor of phenylmethylsulfonyl fluorid (PMSF, 1 Mm) was added to the RIPA buffer in advance. Equivalent amounts of protein (50 µg) from each sample were separated on 12% SDS-polyacrylamide gels, and then transferred onto 0.22 µM nitrocellulose filter membranes (Millipore, Billerica, MA, USA). The bands were then incubated with ACE2, β-actin and HIF-1α at 4°C overnight and then incubated with corresponding secondary antibody for 1 h at 37°C. The signals were detected by ECL kit (BestBio Inc, Shanghai, China).

**ELISA assay**

The concentrations of Ang-(1-7) in PASMCs was measured using ELISA kits according to the manufacturer’s instructions.

**Statistical Analysis**

The statistical significance between groups was analyzed using GraphPad Prism v 4.0 Software (San Diego, CA, USA). And statistical analysis were assessed by analysis of variance (one-way ANOVA) or LSD-t test for multiple comparisons. The data were shown as means ± SD. A significant difference was accepted as significant if p<0.05.

**Results**

**Fasudil Normalized Chronic Hypoxia-Induced PH in Rats**

As shown in the Figure 1, chronic hypoxia did not influence mCAP considerably, the groups treated with three dosages of fasudil showed a reduction in mCAP, but these decreases were not statistically significant (Figure 1A). The RVSP was measured by catheterization via jugular vein to right ventricle, which substitutes for the pulmonary artery pressure. After exposure to hypoxia for 6 weeks, the RVSP was significantly elevated in hypoxia group compared with the control group (Figure 1B, **p<0.01 vs. control group). However, the RVSP was significantly lower in the hypoxia+fasudil group than in the hypoxia group. There was significant difference of RVSP between the hypoxia+ fasudil and hypoxia group (Figure 1B, *p<0.05, **p<0.01 vs. hypoxia group). RV/(LV+S)%e, known as an index of RV hypertrophy, was significantly in-
Fasudil alleviated HPH by stabilizing Ang-(1-7) in rats creased by hypoxia (Figure 2A, **p<0.01 vs. control group), but the hypoxia-induced RV hypertrophy were markedly inhibited in the hypoxia + fasudil group (Figure 2A, *p<0.05, **p<0.01 vs. hypoxia group). The WT% of the arterioles, recognized as the index of pulmonary artery remodeling, increased significantly after hypoxia exposure compared with the control group (Figure 2B and Figure 3, **p<0.01 vs. control group). In the hypoxia + fasudil group, the WT% was notably smaller than in the hypoxia group. There was significant difference of the WT% between hypoxia+fasudil and hypoxia group. Likewise, the WA% in the hypoxia group was also significantly higher than in the control group (Figure 2C and Figure 3, **p<0.01 vs. control group) and there was a significant difference of WA% between hypoxia + fasudil and hypoxia group (Figure 2C and Figure 3, *p<0.05, **p<0.01 vs. hypoxia group).

Figure 1. Analysis of hemodynamic data. (A) mean carotid artery pressure (mCAP). (B) right ventricular systolic pressure (RVSP). *p<0.01, significant difference from Control group. *p<0.05, **p<0.01, significant difference from the hypoxia group. Data are means ± SD (n = 10).

Figure 2. Effects Fasudil on right ventricle hypertrophy index in chronic hypoxia-induced pulmonary vascular structure remodeling of rats. (A) Right ventricle hypertrophy index RV/(LV+S)%. (B) Medial wall thickness (MT%) of pulmonary arterioles. (C) Medial wall area (MA%) of pulmonary arterioles. Data are means ± SD. (n = 45). **p<0.01, significant difference from the Control group. *p<0.05, **p<0.01, significant difference from the hypoxia group.
Protein Expression of Ang-(1-7) in Rat Lung Tissue and Cultured PASMCs

In an attempt to investigate whether Ang-(1-7) were involved in hypoxia-induced pulmonary hypertension and artery remodeling, the protein levels of Ang-(1-7) in the experimental groups were measured by immunohistochemical staining, which showed that the relative Ang-(1-7) level in hypoxia group was significantly lower than that in the control group (Figure 4A and 4B, **p<0.01 vs. control group). Meanwhile, the lysates of PASMCs in each group were tested for by ELISA assays, with results indicating significantly lower levels of relative Ang-(1-7) in hypoxia group than in the control group (Figure 5, *p<0.05 vs. control group). In contrast, markedly higher levels of the relative Ang-(1-7) were observed in all three dosages of fasudil treatment groups than in the hypoxia group (Figure 4, #p<0.05, ##p<0.01 vs. hypoxia group).

Figure 3. Hematoxylin and eosin staining pulmonary arterioles (magnification ×20). a) Control group. b) Hypoxia group. c) Hypoxia+Fasudil (10 mg/kg) group. d) Hypoxia+Fasudil (30 mg/kg) group. e) Hypoxia+Fasudil (75 mg/kg) group.

Figure 4. Ang-(1-7) immunohistochemical analysis of lung tissue of rats in different group. A) Expression of Ang(1-7) determined by immunohistochemical staining with Ang(1-7) antibody. B) IOD values of Ang(1-7) staining. Data are means ± S.D. **p<0.01 vs. control group, *p<0.01 vs. hypoxia group.
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Protein Expression of ACE2 and HIF-1α in Cultured PASMCs

Our next step focused on ACE2 and HIF-1α, the relative ACE2 level in hypoxia group was significantly lower than that of the control group in cultured PASMCs (Figure 6A and 6B, *p<0.05 vs. control group). The relative ACE2 level in the three dosages of fasudil treatment groups were all significantly higher than that of the hypoxia group (Figure 6A and 6B, /p<0.05, //p<0.01 vs. hypoxia group), and the relative ACE2 level of hypoxia + fasudil (30 mg/kg) group was the highest. However, the relative HIF-1α level in hypoxia group was significantly higher than that of the control group in cultured PASMCs (Figure 6A and 6C, **p<0.01 vs. control group). The relative HIF-1α level in the three dosages of fasudil treatment groups were all significantly lower compared with the hypoxia group (Figure 6A and 6C, //p<0.01 vs. hypoxia group).

Discussion

PH is a lung disease of high mortality. The mechanisms of the pulmonary vasculature remodeling are complex and not yet fully elucidated19. As is widely acknowledged, the RAS system is essential to the regulation of pulmonary vascular and can possibly promote vascular remodeling in the lung20. The RAS system includes vasoconstrictive axis (ACE/AngII/AT1R) and vasodilator axis (ACE2/Ang-(1-7)/Mas)21. ACE2 degrades vasoconstrictive AngII to the vasodilator peptide Ang-(1-7), which balanced the vasoconstrictive axis of the RAS system. Thus, ACE2 and Ang-(1-7) may be considered vasoprotective component of RAS system22. On the other hand, studies testified that overexpression of ACE2 or its activation23,24 could prevent the development of PH. It was also suggested that Ang-(1–7) overexpression was most possibly regulated by ACE2, which in turn stimulated the Mas receptor to trigger a protective effect. ACE2/Ang-(1–7)/Mas axis, as a new therapeutic measure, is effective in treating pulmonary fibrosis and pulmonary hypertension5. In addition, recent research found that HIF-1α downregulated ACE2 in human PASMCs (hPASMCs) and induced the proliferation and migration of hPASMCs during hypoxia18, suggesting a protective role of ACE2 in the development of HPH18,25.

ROCK has an evident effect on pulmonary vascular structure remodeling26. Fasudil, is a selective inhibitor of ROCK clinically applicable27. Evidence supported that fasudil is an attractive agent for treatment of PH. However, to date, the mechanisms of fasudil on PASMCs still remains unclear26,27, which incent our research team to investigate the signal pathway of its effect on HPH.

According to the above mentioned, fasudil could effectively attenuate pulmonary hypertension and ventricular hypertrophy. As is illustrated in this study, Ang-(1–7), ACE2 and HIF-1α played significant roles in relieving pulmonary hypertension by fasudil. In the present study, chronic hypoxia exposure significantly elevated RVSP, increased RV/(LV+S), WT% and WA%, while rats treated with fasudil in three different doses after intermittent hypoxia exposure showed relatively smaller increases in RVSP, less ventricular hypertrophy and less vascular remodeling. Immunohistochemical staining showed fasudil increased the expression of Ang-(1-7) in hypoxia. Moreover, experiments in vitro also demonstrated that fasudil enhanced Ang-(1-7) and ACE2 expression in three different doses, and reduced HIF-1α expression. In a word, the study suggested that the reduction of Ang-(1–7) and ACE2 induced by hypoxia could be effectively reversed by fasudil treatment, probably through inhibiting HIF-1α expression.
Series of researches on fasudil testified that it could effectively alleviate various types of PH through various mechanisms\textsuperscript{28,29}. Our previous work have identified antioxidant effects of fasudil against PH and participation of the modulated expression levels of Trx1 and HIF-1α in MCT-induced PH models of rats\textsuperscript{30}. Our result shows that fasudil significantly inhibited the development of hypoxia-induced PH, as a consequence of the regulated expression levels of Ang-(1–7), ACE2 and HIF-1α. Although studies have demonstrated the effects of fasudil on PH were related with Ang-(1–7), ACE2 and HIF-1α, more explicit mechanism need to be explored.

Conclusions

We demonstrated that suppression of fasudil on Ang-(1–7) and ACE2 may relate with inhibiting the production of HIF-1α, and subsequently alleviated the hypoxia induced pulmonary hy-
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pertension through its inhibitory effects. Therefore, we concluded that stabilized Ang-(1–7) application participated in the attenuation of HPH, and the increase of ACE2 through down-regulating HIF-1α may be responsible for the increased expression Ang-(1–7).

Acknowledgements
This work was supported by National Nature Science Foundation of China (No1270328; No 81471816).

Conflicts of interest
The authors declare no conflicts of interest.

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