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Effects of fasudil on hypoxic pulmonary hypertension and pulmonary vascular remodeling in rats

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Abstract. – AIM: The aim of this study was to investigate the effects of Rho kinase inhibitor-Fasudil on hypoxic pulmonary hypertension (HPH) and pulmonary vascular remodeling in rats.

MATERIALS AND METHODS: A total of 24 Sprague Dawley rats were evenly randomized into control, model and Fasudil intervention groups. Light and transmission electron microscopy were utilized to observe pulmonary vascular remodeling as well as ultrastructural changes in pulmonary arteriole endothelial cells.

RESULTS: The model group showed apparent pulmonary vascular remodeling, pulmonary arteriole endothelial cell injury, the proliferation and swelling of smooth muscle cells around, and the proliferation of collagen fibers. Fasudil intervention improved pulmonary vascular remodeling as well as relieved pulmonary arteriole endothelial cell injury and the proliferation of smooth muscle cells and collagen fibers.

CONCLUSIONS: Fasudil has preventive and reverse effects on HPH, pulmonary vascular remodeling, and pulmonary arteriole endothelial cell injury.

Key Words:
Fasudil, Pulmonary arterial hypertension (PAH), Pulmonary vascular remodeling.

Introduction

Hypoxic pulmonary hypertension (HPH) has been recognized as the most common complication of some cardiopulmonary vascular diseases, which is characterized by increased pulmonary artery pressure, pulmonary vascular remodeling and right ventricular hypertrophy. Without any intervention treatment, HPH will proceed to lead to right heart failure or even death[1,2]. However, its pathogenesis is partially known and an effective pharmacotherapy has not been found. Therefore, to explore the mechanism underlying HPH and, accordingly, to find effective prevention drugs become urgent. HPH is a complicated disease in which various molecules and target cells interact with each other. This feature of HPH hinders the understanding of what factor plays a key role in its initiation and development. Recent years have witnessed more and more attention attracted to the relation of HPH with cardiopulmonary vascular diseases as well as with the small G protein Rho/Rho kinase signaling pathway. Studies[3,4] have demonstrated that selectively blocking of G protein Rho/Rho kinase signaling pathway improves the cardiopulmonary injury as well as its prognosis. However, the exact underlying mechanism remains unknown.

Although the pathogenesis of HPH remains incompletely known, numerous studies have confirmed that the development of HPH is manifested by hypoxic pulmonary vasoconstriction and hypoxic pulmonary vascular remodeling[5]. Nowadays pulmonary vascular remodeling is widely assumed to be a key factor for the development of HPH, as well as a main cause for resistance to antihypertensive drugs for vasodilation. Based on this assumption, the role by pulmonary vascular remodeling in the development of HPH has drawn more and more attention and become a research focus. How to prevent and reverse pulmonary vascular remodeling seems to be a key point for the treatment of HPH. In addition, recent *in vitro* and *in vivo* animal experiments have found that the small G protein Rho kinase inhibitor-Fasudil has noticeable effect on vasodilation and pulmonary artery pressure reduction[6]. However, no further study has been conducted to clarify whether this substance also plays a role in preventing and improving pulmonary vascular remodeling.

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In this study, we established HPH rat models to explore the pathogenesis of HPH and the relation between the hemodynamic and pathomorphism of pulmonary vascular remodeling. Further, we investigated the effects of Fasudil on pulmonary vascular remodeling and HPH.

Materials and Methods

Animal Grouping and of HPH Model Establishment

A total of 24 healthy male Sprague Dawley rats weighing between 250 g and 300 g were supplied by the animal experiment center of Military General Hospital of Nanjing. The HPH rat models were established using the method described in a published paper[7], The experimental animals were kept in hypoxia with a low O_2 concentration of 10 ± 0.5% for 3 weeks, 8 h per day and 6 d per week, whereas the control group was kept in normoxia at the corresponding time. The animals were randomized into model, Fasudil intervention, and control groups with 8 in each. The model group was injected intraperitoneally with 2 ml/kg physiological saline every day. The Fasudil intervention group was injected intraperitoneally with 2 ml/kg Fasudil (Asahi Kasei Co., Nagoya Pharmaceuticals Plant, Japan; 2 mL/vial containing 30 mg Fasudil; batch number: ERSIIKM) (15 ml/kg Fasudil was diluted with physiological saline to get a solution at a final concentration of 2 ml/kg). The control group was injected intraperitoneally with 2 ml/kg physiological saline at corresponding time every day. After 3 weeks, all the animals received anesthesia with an intraperitoneal injection of 40 mg/kg 2% sodium pentobarbital. Right heart catheterization[8] was performed for mean pulmonary artery pressure (mPAP) measurement using a Hewlett-Packard multifunctional physiological recorder. Left carotid artery catheterization[9] was performed for mean carotid artery pressure (mCAP). After pressure measurement, the heart and lungs were collected immediately. The right ventricle (RV) and the left ventricle plus septum (LV+S) were separated and weighed. [RV/(LV+S)] (RVHI) ratio was calculated for right heart hypertrophy evaluation[10].

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol had been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Huaiían First Hospital affiliated to Nanjing Medical University.

Pulmonary Arteriole Sample Preparation

The right upper lung peripheral tissue was taken and fixed in 4% paraformaldehyde phosphate buffer overnight. These were followed by paraffin embedding and section cutting (RM2135 type paraffin microtome; Leica, Solms, Germany). Hematoxylin-eosin (HE) staining and elastic tissue staining were performed to observe the morphological changes in pulmonary arterioles under an optical microscope (Olympus Corporation, Shinluku, Japan). Cardiopulmonary vessel associated parameters were analyzed by the MPIAS-500 multimedia color pathological image analysis system (Shanghai Tongji Medical University, China). These parameters included the outer diameter, wall thickness, wall area, lumen area and total wall area of pulmonary arterioles with a diameter less than 200°m. Then, wall thickness/outer diameter (WT%), wall area/total wall area (WA%) and lumen area/ total wall area (LA%) ratios were calculated as the indices of pulmonary vascular morphology for further statistical analysis. The right lower lung peripheral tissue was taken and cut into 1 mm × 1 mm × 1 mm pieces. glutaraldehyde (2.5%) and osmic acid (1%) were used for pre-fixation and post-fixation, respectively. These were followed by acetone dehydration, EPON 812 embedding and section cutting by LKB-V-type ultramicrotome. After uranyl acetate and lead citrate staining, ultrastructural changes of pulmonary arteriole endothelial cells were observed using transmission electron microscopy (JEM-1010, Namiki, Japan).

Statistical Analysis

Data were presented as (x ± s and analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Analysis of variance (F test) was performed for comparisons among groups, and the least significant difference method was utilized for comparisons between groups. $p < 0.05$ was considered statistically significant.

Results

The Effects of Fasudil on the mPAP, mCAP and RVHI

Compared with the control group, the model group exhibited noticeably increased mPAP and

Table I. The results of mPAP, mCAP, RVHI, WT%, WA%, LA% in all groups (x ± s).

Group	n	mPAP (mmHg)	mCAP (mmHg)	RVHI	WT%	WA%	LA%
Test group	8	15.25 ± 0.91	121.13 ± 9.80	0.25 ± 0.02	13.24 ± 2.03	31.81 ± 3.62	68.20 ± 3.62
Model group	8	31.38 ± 1.98[1]	113.54 ± 14.84	0.47 ± 0.03[1]	31.13 ± 5.74[1]	54.93 ± 3.34[1]	45.07 ± 3.34[1]
Fasudil group	8	16.63 ± 1.53[2]	110.04 ± 12.24	0.27 ± 0.02[2]	17.08 ± 2.24[2]	37.30 ± 3.69[2]	62.70 ± 3.69[2]
F value		202.95	1.239	220.183	37.962	67.376	67.376
p		0.000	0.318	0.000	0.000	0.000	0.000

Note: [1]Compared with control group, $p < 0.01$; [2]Compared with model group, $p < 0.01$.

RVHI (both $p < 0.01$). Compared with the model group, the Fasudil intervention group showed significantly decreased mPAP and RVHI (both $p < 0.01$). No significant differences in mCAP were observed among the groups. The results are summarized in Table I.

The Effect of Fasudil on Vascular Morphology

Vascular morphology was observed using light microscopy. HE staining showed thin and well-continuous pulmonary arteriole walls and evenly-distributed and thickness-consistent endothelial cells in the control group. The model group displayed discontinuous pulmonary arteriole endothelial cells, proliferated and swelling smooth muscle cells, thickened vessel walls, and decreased lumen sizes. Compared with the model group, the Fasudil intervention group exhibited lessened vessel wall thickening and lumen size decreasing (Figure 1). Elastic fiber staining demonstrated a noticeable-widened distance between the inner and outer elastic fibers, as well as thickened pulmonary arteriole walls on the sections of the model group. In contrast, the Fasudil intervention group showed a reduced widened distance between the inner and outer elastic fibers of pulmonary arterioles (Figure 2). The WT%, WA% and LA% results of the pulmonary arteriole image analysis are summarized in Table I. In addition, the ultrastructural changes in pulmonary arteriole endothelial cells were observed under transmission electron microscopy and the results are shown in Figure 3.

Discussion

HPH has been recognized as an important pathophysiological factor for the initiation and development of numerous cardiopulmonary diseases in clinic practice. However, the pathological mechanism of HPH remains unclear. Although early studies suggested that HPH resulted from enhanced pulmonary vasoconstriction, more and more studies have indicated that HPH of varied causes takes pulmonary vascular remodeling as its pathological basis[2,10,11]. Pathological changes in pulmonary vascular remodeling include the proliferation and hypertrophy of pulmonary arteriole endothelial cells and smooth muscle cells, the muscularization of pulmonary arterioles in alveoli, as well as increases in extracellular matrixes (EMs) such as collagen and elastic fibers. All these changes are jointly contributed to thickened vessel walls and reduced lu-

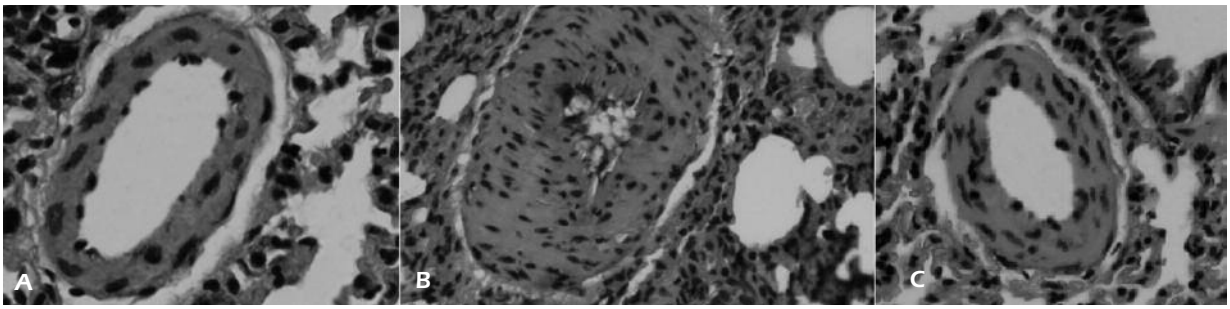

Figure 1. HE staining of pulmonary arterioles from rats (SP, ×400). ***A,*** Control group. ***B,*** Model group. ***A,*** Fasudil group.

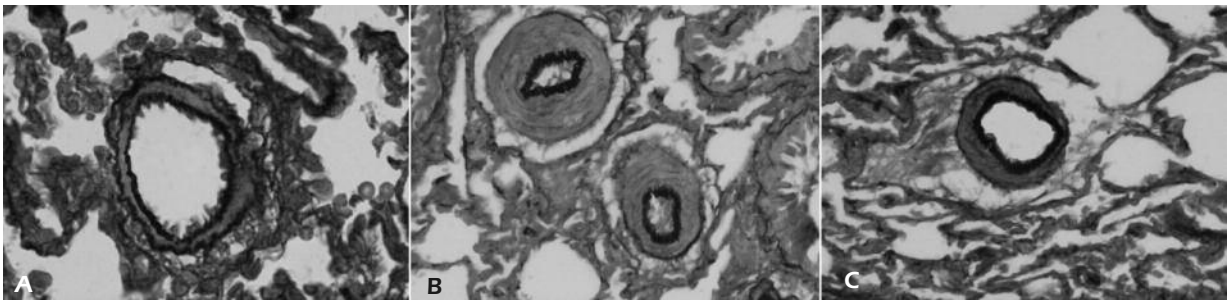

Figure 2. Elastic fiber staining of pulmonary arterioles from rats (SP, ×400). ***A,*** Control group. ***B,*** Model group. ***C,*** Fasudil group.

men sizes[11-13]. Numerous experiments have demonstrated that hypoxia-induced vasoconstrictors secreted by pulmonary endothelial cells (typically, endothelin-1 (ET-1)) have effects on both increased pulmonary vascular tension and enhanced vascular cell proliferation; in contrast, vasodilators [typically, nitrous monoxide (NO)] function in an opposite way[12,14]. Nowadays pulmonary vascular remodeling is widely assumed to be a key factor for the development of HPH, as well as a main cause for resistance to antihypertensive drugs for vasodilation[15-17]. Based on this assumption, how to prevent and reverse pulmonary vascular remodeling seems to be a key point for the treatment of HPH.

Our studies showed that the experimental rats in hypoxia for 3 weeks presented elevated pulmonary artery pressure, right heart hypertrophy, thickened pulmonary arteriole walls, and narrowed lumens. These findings suggested the successful establishment of HPH rat models. In them, discontinuous pulmonary arteriole endothelial cells, proliferated and swollen smooth muscle cells, thickened vessel walls, and decreased lumen sizes were observed after HE staining. Other pathological changes included an increased muscularized artery proportion, a decreased non-muscularized artery proportion, and the muscularization of non-muscular vessels. Furthermore, these models showed increases in WT% and WA% and a decrease in LA%. Their apparently-widened distance between the inner and outer elastic fibers and thickened pulmonary arteriole walls indicated that pulmonary vascular remodeling, characterized by enhanced pulmonary arteriole muscularization, increased middle layer thickness and decreased lumen sizes, is a major manifestation of the development of HPH. In addition, transmission electron microscopy showed proliferated and swollen smooth muscle cells and proliferated collagen fibers in the models. All the aforementioned findings suggest that preventing and reversing pulmonary vascular remodeling is very likely to be a key point in the treatment of HPH.

Recent years have witnessed more and more attention to the relation between HPH and the Rho/Rho kinase signaling pathway. Studies[3,6,18] have discovered that the activation of the

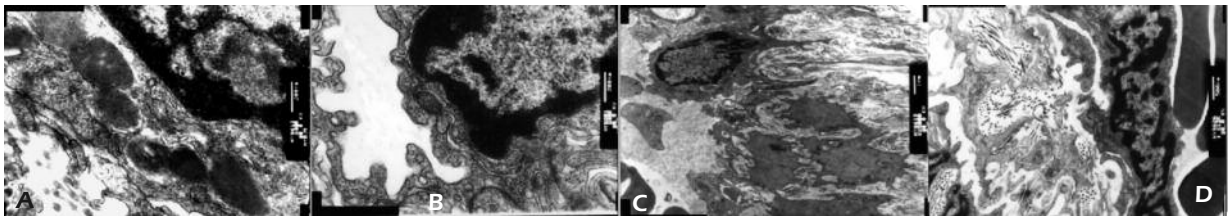

Figure 3. Ultrastructural changes in rat pulmonary arteriole endothelial cells using transmission electron microscopy. ***A,*** The model group showed swelling of pulmonary arteriole endothelia cells and disappearance of mitochondrial outer membrane with cristal disorder, breaks and vanishment (indicated by arrows) (SP, ×4000). ***B,*** The Fasudil group JlS/J showed normal pulmonary arteriole endothelia cells and clear mitochondrial crista (indicated by arrows) (SP, ×4000). ***C,*** The model group showed proliferation and swelling of smooth muscle cells and an increase in the number of elastic fibers (SP, ×5000). ***D,*** The Fasudil group showed normal pulmonary arteriole endothelia cells, normal elastic tissues, a thinner smooth muscle layer, and a smaller number of collagen fibers compared with the model group (SP, ×12000).

Rho/Rho kinase signaling pathway by hypoxia is a critical process of the initiation of HPH. Rho kinase inhibitors as a new type of antihypertensive drugs have a remarkable effect on HPH[3,5,6,19]. However, no further studies on whether these drugs also play a role in preventing and improving pulmonary vascular remodeling have been found in literature. Fasudil as a typical Rho kinase inhibitor[20] is a new isoquinoline sulfanilamide derivative. It inactivates Rho kinase by competing with ATP for ATP binding sites in the Rho kinase catalytic domain. Furthermore, it reduces the phosphorylation of the myosin light chain (MLC) and dilates constricted vessels by regulating the myosin phosphatase binding subunit (MBS) and MLC phosphorylation. Recent studies have demonstrated that[5,6,21,22] Fasudil improves the balance between ET and NO as well as enhances endothelium-mediated diastolic effect by inhibiting the secretion of ET-1 and promoting the secretion of NO by endothelial cells. In this study, the results showed that Fasudil intervened in the development of PHP-reduced pulmonary artery pressure and right heart hypertrophy. It improved pulmonary arteriole endothelial cell injury and meanwhile relieved pulmonary arteriole middle layer thickening and lumen size narrowing. These findings strongly suggest that Fasudil has the effects of reducing pulmonary artery pressure, improving right heart hypertrophy and pulmonary arteriole endothelial cell injury, and ameliorating pulmonary vascular remodeling. These effects are presumably realized through its actions on smooth muscle cell constriction, the balance between endothelium-derived vasodilators (typically, NO) and vasoconstrictors (typically, ET-1), and cell growth gene expression.

Conclusions

Study focus on the pharmaceutical treatment of HPH has changed from the exclusive use of vasodilators to the application of drugs against pulmonary vascular function and remodeling as the understanding of the pathogenesis of HPH deepens. Fasusil effectively inhibits the main pathological change in HPH ñ pulmonary vascular remodeling ñ but without bringing such side effect as hypotension. Its application in preventing HPH and inhibiting pulmonary artery remodeling can be promising.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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