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Effect of L-Arginine on Pulmonary Artery Smooth Muscle Cell Apoptosis in Rats with Hypoxic Pulmonary Vascular Structural Remodeling

Ingrid Karmane SUMOU[1], Jun-Bao DU[1,3]*, Bing WEI[1], Chun-Yu ZHANG[1], Jian-Guang QI[1], and Chao-Shu TANG[2,3]

[1] *Department of Pediatrics, Peking University First Hospital, Beijing 100034, China;*
[2] *Institute of Cardiovascular Diseases, Peking University First Hospital, Beijing 100034, China*
[3] *Key Laboratory of Molecular Cardiovascular Diseases, Ministry of Education, Beijing 100034, China*

Abstract This study investigated the effect of L-arginine (L-Arg) on the apoptosis of pulmonary artery smooth muscle cells (PASMC) in rats with hypoxic pulmonary vascular structural remodeling, and its mechanisms. Seventeen Wistar rats were randomly divided into a control group (n=5), a hypoxia group (n=7), and a hypoxia+L-Arg group (n=5). The morphologic changes of lung tissues were observed under optical microscope. Using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling assay, the apoptosis of PASMC was examined. Fas expression in PASMC was examined using immunohistochemistry. The results showed that the percentage of muscularized artery in small pulmonary vessels, and the relative medial thickness and relative medial area of the small and median pulmonary muscularized arteries in the hypoxic group were all significantly increased. Pulmonary vascular structural remodeling developed after hypoxia. Apoptotic smooth muscle cells of the small and median pulmonary arteries in the hypoxia group were significantly less than those in the control group. After 14 d of hypoxia, Fas expression by smooth muscle cells of median and small pulmonary arteries was significantly inhibited. L-Arg significantly inhibited hypoxic pulmonary vascular structural remodeling in association with an augmentation of apoptosis of smooth muscle cells as well as Fas expression in PASMC. These results showed that L-Arg could play an important role in attenuating hypoxic pulmonary vascular structural remodeling by upregulating Fas expression in PASMC, thus promoting the apoptosis of PASMC.

Key words hypoxia; pulmonary artery; arginine; apoptosis

Hypoxic pulmonary vascular structural remodeling is an important pathologic basis of hypoxic pulmonary hypertension. The proliferation and hypertrophy of pulmonary vascular smooth muscle cells, the thickening of pulmonary artery media, the muscularization of small peripheral vessels and the increase in extracellular matrix are the main pathologic features of pulmonary vascular structural remodeling [1]. Previous studies showed that L-arginine (L-Arg)/nitric oxide (NO) could alleviate the development of hypoxic pulmonary vascular structural remodeling and hypoxic pulmonary hypertension. However, the molecular mechanism by which L-Arg attenuates hypoxic pulmonary vascular structural remodeling remains unknown [2,3]. Apoptosis and proliferation are two important factors that determine the biological behavior of vascular smooth muscle cells, hence the balance between them influences the vascular structure. *In vitro* studies showed that NO could induce apoptosis of smooth muscle cells [4]. Recent studies showed that supplemental L-Arg partially inhibited pulmonary vascular structural remodeling that occurred secondarily to increase pulmonary pressure due to cold exposure, and NO-induced apoptosis in artery smooth muscle cells might contribute to its regulatory effect on cold exposure-induced pulmonary vascular structural changes. Therefore, we can assume that the effects of NO on pulmonary smooth muscle cell apoptosis

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* Corresponding author: Tel, 86-10-66171122; Fax, 86-10-66134261; E-mail, junbaodu@ht.rol.cn.net

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might be involved in the mechanisms by which L-Arg modulates hypoxic pulmonary vascular structural remodeling. However, sufficient evidence to demonstrate such effects has been lacking.

The present study was designed to examine the influence of the NO precursor, L-Arg, on the apoptosis of pulmonary artery smooth muscle cells (PASMC) and the expression of Fas protein by pulmonary arteries of rats with hypoxic pulmonary vascular structural remodeling.

Materials and Methods

Animals and methods of hypoxia

Male Wistar rats weighing between 210 g and 300 g were randomly divided into a control group (n=7), a hypoxia group (n=5), and a hypoxia with L-Arg group (n=5). For hypoxic challenge, the rats of the hypoxia group and the hypoxia+L-Arg group were put into a normobaric hypoxic chamber (Chinese Academy of Medical Sciences, Beijing, China) with an oxygen concentration of 10.0%±0.5%. The animals underwent continuous hypoxic challenge of 6 h per day for 14 d. For rats in the hypoxia+L-Arg group, L-Arg was given intraperitoneally at a dose of 500 mg/kg each day before hypoxic challenge. An equal volume of normal saline was injected intraperitoneally in rats of the control and hypoxia groups. The rats in the control group breathed room air. In these three groups, the routine breeding, feeding and drinking conditions were the same.

Morphologic observation of the pulmonary vessels

After 14 d of treatment, the rats were anesthetized with an intraperitoneal injection of 10% (W/V) urethane and fixed on the operating table. The thoracic cavity was exposed. One side of a lung lobe was removed, fixed in 10% (W/V) formalin and dehydrated in an ascending gradient of alcohol. After the lung tissues were made transparent with dimethylbenzene, they were embedded in paraffin and routinely processed into sections of 5 μm for elastic straining, and weighed and counterstained with Van Gieson stain.

Determination of the percentage of three types of small pulmonary vessels

Observed under 40× optical microscope CHC-212 (Olympus, Tokyo, Japan), the number of small pulmonary vessels including muscularized arteries, partially muscularized arteries and non-muscularized vessels with external diameters between 15 μm and 50 μm were counted and the percentage of each type of vessel was determined.

Determination of relative medial thickness (RMT) and relative medial area (RMA) of small and median pulmonary arteries

An Image Processing & Analyzing System Q550 LW (Leica, Wetzlar, Germany) was used for the analysis of small and median muscularized pulmonary arteries with clearly defined internal elastic laminae and regular shapes. The following parameters were measured: the external diameter of external elastic lamina along the longest and shortest axes (D_1 and D_2), the length of the internal elastic lamina (L_{IEL}) and the area enclosed by the internal and external elastic lamina (A_{IEL} and A_{EEL}). The RMT and RMA of pulmonary arteries with different cut angles α and the conditions of either contraction or relaxation were computed from those measurements using Barth's method [5]. For each rat, 5–10 pulmonary median muscularized arteries measuring 50–150 μm in external diameter, and pulmonary small vessels measuring 15–50 μm in external diameter were assessed. RMT and RMA of the median and small pulmonary muscularized arteries were determined and the average values were computed.

Analysis of apoptosis

Using the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling assay, the apoptotic cells were detected. The paraffin-embedded sections of the lung tissues were used for analysis. The sections were routinely dewaxed and hydrated, then blocked in H_2O_2 and digested for 10–20 min in 100 μg/ml proteinase K at room temperature, and dehydrated with 90% ethanol. Then 4 μl of 5× TdT buffer (Promega, Madison, USA), 1 μl of 2 mM dUTP (Sigma, St. Louis, USA), 15 μl of water and 0.4 μl of TdT was added. The total reaction system remained 20 μl. The slides were put into a humidified chamber for 2 h at 37 ºC and the reaction fluid was thoroughly washed out. The sample was blocked in horse serum (1:100) and kept at room temperature for 20 min, then horseradish peroxidase labeled with avidin and biotin was added. We incubated the sample in a humidified chamber at 37 ºC for 1 h, rinsed it three times, for 5 min each, and developed the color for 2–3 min using 3,3′-diaminobenzidine-tetrachloride (DAB)-H_2O_2, then stained it with hematoxylin. The cells with brown granules in the nucleus were defined as apoptotic cells. For each rat, 15–20 pulmonary arteries were detected, and the numbers of apoptotic smooth muscle cells and total smooth muscle cells were counted. The ratio of numbers of apoptotic cells to smooth muscle cells

in median and small pulmonary arteries was calculated.

Immunohistochemical analysis

After they were dewaxed by dimethylbenzene, the sections of lung tissue were put into water and the antigens were heat-processed (92 ºC–98 ºC) for 10 min. Then the slides were washed twice (5 min each cycle) in phosphate-buffered saline (PBS), blocked in sheep (goat) serum working solution for 10 min, then incubated at 37 ºC for 1 h with anti-Fas antibodies (1:100) (Santa Cruz Biotechnology, Santa Cruz, USA). After being washed twice in PBS (5 min each cycle), the slides were incubated with biotinylated secondary antibodies and then with avidin-biotin complex at 37 ºC for 30 min. Subsequently the sample was washed in PBS, incubated in DAB for 20–30 min and stained in hematoxylin. Sections were dehydrated through an ascending gradient of ethanol, made transparent with dimethylbenzene and observed with a microscope. The brown granules in pulmonary smooth muscle cells were defined as the positive signals.

The Fas expression in PASMC was examined using a semiquantitative method. We defined the level of expression as negative (–) if there were no positive Fas signals in PASMC, as (+) if 1%–50% cells showed Fas expression, and as (++) if 51%–100% cells showed Fas expression. At least 10 median and small pulmonary arteries were examined for each sample. For the convenience of understanding and statistical procedures, the content of Fas protein expressed in pulmonary arteries was calculated as follows: the percentage of pulmonary arteries with a certain extent of reaction intensity was multiplied by the weighed values of their reaction intensity. The weighed value of reaction intensity was 0, 0.5 and 1.0, if the reaction intensity was –, + and ++, respectively.

Statistical analysis

Data were presented as the mean±SD. The percentage of these three types of pulmonary small vessels, RMT and RMA of the pulmonary muscular arteries, and the integral values of Fas expression in the PASMC were compared using ANOVA. The Q test was used for intergroup comparison. The ratio of apoptotic cells to total PASMCs was analyzed using the Kruskal-Wallis test.

Results

Changes in the percentage of three types of pulmonary small vessels

Under an optical microscope, the muscularized artery has continuous external and internal elastic lamina, the partially muscularized artery has a continuous external elastic lamina and a discontinuous internal elastic lamina, and the non-muscularized vessel has only one single elastic lamina. The percentage of the three types of pulmonary small vessels was significantly different between any two groups (P<0.01; **Table 1**). In the hypoxia group, the percentage of muscularized arteries in pulmonary small vessels was significantly higher than that in the control group (P<0.01). In the hypoxia+L-Arg group, the percentage of muscularized arteries in pulmonary small vessels was significantly lower than that in the hypoxia group (P<0.01). The percentage of non-muscularized vessels in pulmonary small vessels of rats in the hypoxia group was significantly lower than that of the control group (P<0.01). The percentage of non-muscularized vessels in small pulmonary vessels in the hypoxia+L-Arg group was significantly higher compared with that of the hypoxia group (P<0.01).

Changes in the RMT and RMA of the median and small muscularized pulmonary arteries

Fig. 1 shows significant differences in RMT and RMA

Table 1 Changes in three types of pulmonary small vessels and the relative medial thickness (RMT) and relative medial area (RMA) of pulmonary muscular arteries in rats

Group	Type of small pulmonary vessels (%)			RMT (%)		RMA (%)		*n*
	MA	PMA	NMV	Median MA	Small MA	Median MA	Small MA	
Control	9.43±0.77	7.32±0.30	83.25±0.88	7.44±0.39	9.78±0.43	14.30±0.72	18.58±0.78	7
Hypoxia	15.85±0.82[b]	10.58±0.31[b]	73.56±0.69[b]	9.76±0.30[b]	14.87±0.16[b]	18.54±0.54[b]	27.50±0.28[b]	5
Hypoxia+L-Arg	11.79±0.28[ad]	9.64±0.25[b]	78.57±0.28[bd]	8.26±0.14[c]	11.57±0.70[ad]	15.82±0.25[c]	21.77±1.25[ad]	5

[a]P<0.05 versus control; [b]P<0.01 versus control; [c]P<0.05 versus hypoxia group; [d]P<0.01 versus hypoxia group. Data are expressed in mean±SD. L-Arg, L-arginine; MA, muscularized artery; NMV, non-muscularized vessel; PMA, partially muscularized artery.

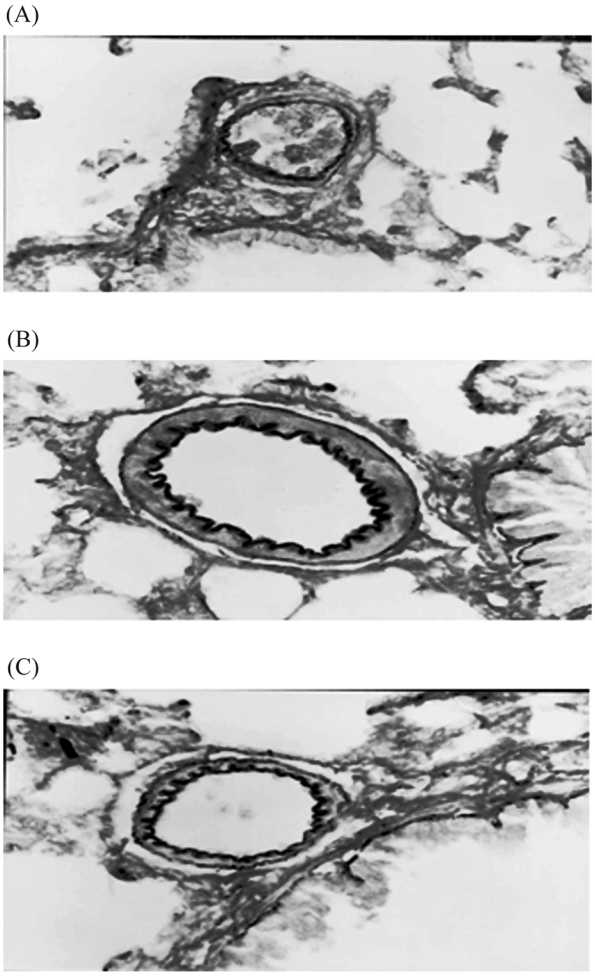


Fig. 1 Changes in relative medial thickness and relative medial area of median muscularized pulmonary arteries in rats

(A) The normal morphologic structure of media of pulmonary artery in the control group. (B) A thickened media of pulmonary artery is shown in the hypoxia group. (C) A thickened media of pulmonary artery was reversed in the hypoxia+L-arginine group. Magnification, 400×.

of median and small muscularized pulmonary arteries in rats in each of the three groups. The quantitative results are shown in **Table 1**. The RMT and RMA of median muscularized pulmonary arteries in the hypoxia group were both significantly increased compared with those of the control group (P<0.01), and also higher than those of small muscular pulmonary arteries (P<0.01). In the hypoxia+L-Arg group, the RMT and RMA of median muscularized pulmonary arteries were both significantly lower than those in the hypoxia group (P<0.05). But there was no significant variation in these parameters between the hypoxia+L-Arg group and the control group (P>0.05). The RMT and RMA of small muscularized pulmonary arteries in the hypoxia+L-Arg group were significantly lower than those in the hypoxia group (P<0.01).

Apoptosis of PASMC

The apoptosis of PASMC is shown in **Fig. 2**. There was a difference in the ratio of apoptotic smooth muscle cells to smooth muscle cells in the small and median pulmonary arteries between any two groups (P<0.05) (**Table 2**). The ratio of apoptotic smooth muscle cells to smooth muscle cells of the median and small pulmonary arteries in the hypoxia group was lower than that in the control group (P<0.05). In the hypoxia+L-Arg group, the ratio of

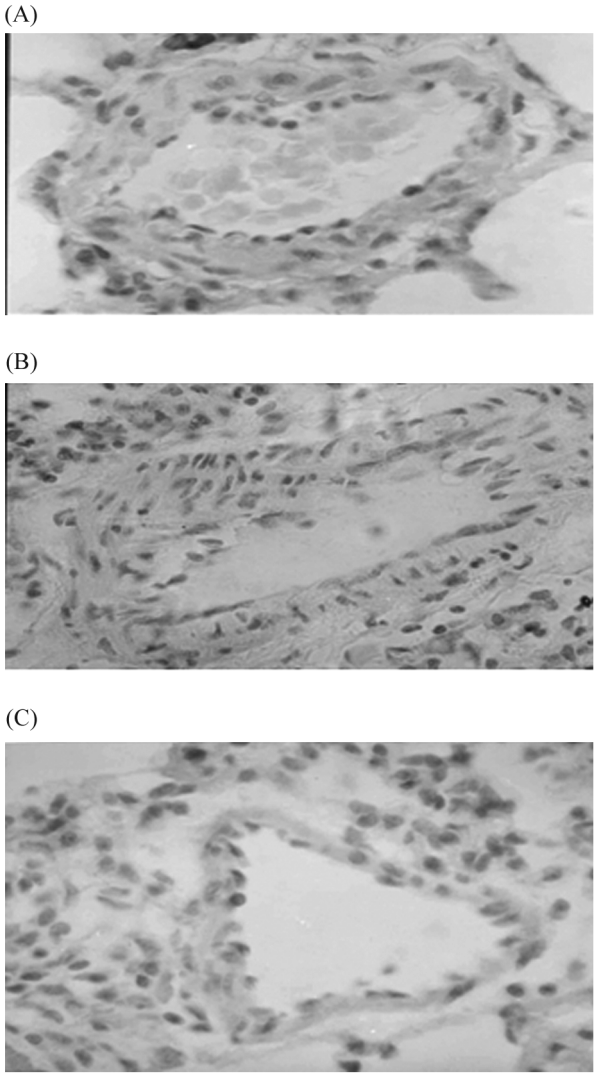


Fig. 2 Smooth muscle cell apoptosis in median pulmonary arteries in rats

(A) Control. (B) Hypoxia group. (C) Hypoxia+L-arginine group. Magnification, 1000×.

Table 2 Ratio of apoptotic cells to total smooth muscle cells in pulmonary arteries in rats

Group	Median pulmonary artery	Small pulmonary artery	*n*
Control	0.092±0.003	0.116±0.014	7
Hypoxia	0.056±0.004[a]	0.050±0.013[a]	5
Hypoxia+L-Arg	0.135±0.034[b]	0.202±0.043[b]	5

[a]$P<0.05$ versus control group; [b]$P<0.05$ versus hypoxia group. L-Arg, L-arginine. Data are expressed in mean±SD.

apoptotic smooth muscle cells to total smooth muscle cells of the median and small pulmonary arteries was higher than that of the hypoxia group ($P<0.05$). However, there was no significant difference in the ratio between the hypoxia+L-Arg group and the control group ($P>0.05$).

Fas expression in smooth muscle cells of pulmonary arteries

As shown in **Table 3**, the level of Fas expression in the hypoxia group was significantly lower than in the control group ($P<0.05$). The level of Fas expression in the hypoxia+L-Arg group was significantly higher than in the hypoxia group ($P<0.05$). **Fig. 3** shows the results of Fas expression in smooth muscle cells of median pulmonary arteries of the different experimental groups.

Table 3 Integral score of Fas expression in pulmonary artery smooth muscle cells of rats

Groups	Median pulmonary artery	Small pulmonary artery	*n*
Control	41.43±1.43	39.29±1.30	7
Hypoxia	13.00±2.00[a]	10.00±3.54[a]	5
Hypoxia+L-Arg	57.00±2.55[ab]	53.00±2.55[ab]	5

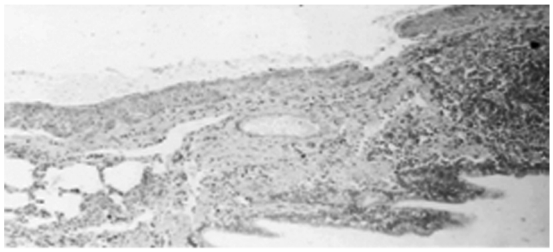
[a]$P<0.05$ versus control; [b]$P<0.05$ versus hypoxia group. L-Arg, L-arginine. Data are expressed in mean±SD.

Discussion

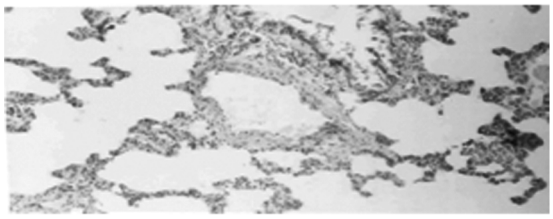
The results of this study showed that pulmonary arteries were remodeled after two weeks of normobaric hypoxia. The degree of muscularization of the pulmonary small vessels was significantly enhanced. The RMT and RMA of the median and small pulmonary arteries were significantly increased. We found that the smooth muscle cell apoptosis in pulmonary arteries and the expression of *Fas*, the protein of an apoptosis-related gene, were significantly suppressed during the process of pulmonary vascular remodeling. Cellular apoptosis and proliferation are two important processes determining the biological behavior of vascular smooth muscle cells, therefore the imbalance between them would influence vascular structure. Our findings suggested that suppression of PASMC apoptosis might be involved in the mechanisms of hypoxic pulmonary vascular remodeling.

The present study revealed that the administration of L-Arg, a NO precursor, alleviated the degree of muscularization of small pulmonary vessels in hypoxic rats. The RMT and RMA of the pulmonary median and small muscularized arteries were decreased. These facts implied that L-Arg alleviated the development of hypoxic pulmonary vascular remodeling, which was consistent with the

(A)



(B)



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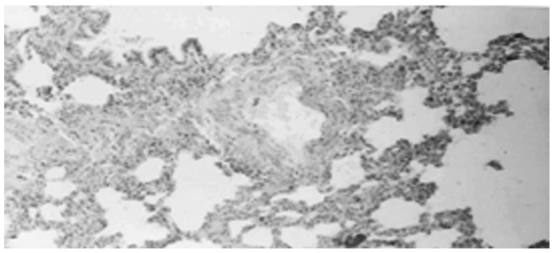

Fig. 3 Fas expression in smooth muscle cells of median pulmonary arteries in rats

(A) Control group. (B) Hypoxia group. (C) Hypoxia+L-arginine group. Magnification, 100×.

previous findings [2].

However, the cellular or molecular mechanism by which L-Arg alleviated hypoxic pulmonary vascular remodeling is still not clear. It was found that NO had direct suppressive effects on vascular smooth muscle cell proliferation [6], which may be one of the mechanisms by which L-Arg modulated hypoxic pulmonary vascular remodeling. In addition, NO could modulate hypoxic pulmonary vascular remodeling by inhibiting the synthesis and secretion of smooth muscle cell proliferation promoting factors, such as endothelins, platelet-derived growth factor and fibroblast growth factor, therefore indirectly suppressing cell proliferation [7–9]. Recent studies showed that, in a pattern contrary to cell mitogenesis (mitosis), apoptosis along with proliferation determined the number of vascular smooth muscle cells, and the balance between apoptosis and proliferation maintained the constancy of the total number of cells. Vascular smooth muscle remodeling is probably the result of cooperative modulation of proliferation and apoptosis. It was noted that an increase in cell apoptosis resulted in a decrease in the number of vascular smooth muscle cells, thereby altering the structure of the vascular wall [10]. An *in vitro* study on the effect of NO on apoptosis of cultured PASMC in rats by Smith and coworkers [4] proved that NO could promote PASMC apoptosis. Recent studies on the effects of NO precursor L-Arg on pulmonary vascular structural remodeling in broilers with pulmonary hypertension induced by cold exposure drew the conclusion that supplemental L-Arg partially inhibited pulmonary vascular structural remodeling that occurred secondary to cold manipulation [11]. Therefore, it could be assumed that the smooth muscle cell apoptosis induced by L-Arg might be involved in the mechanisms responsible for the development of hypoxic pulmonary vascular structural remodeling. It was also found in another recent study that L-Arg could reduce the synthesis of extracellular matrix-collagen and increase its degradation, thus having an important modulating effect on pulmonary vascular matrix remodeling induced by high pulmonary blood flow [12]. However, no experimental data exists on the mechanism by which L-Arg influences the apoptosis of PASMC in rats with hypoxic pulmonary vascular structural remodeling. Our results indicated that the smooth muscle cell apoptosis of pulmonary small and median arteries was suppressed apparently along with pulmonary vascular structural remodeling after chronic hypoxia. Exogenous administration of L-Arg, NO promoted the apoptosis of PASMC and alleviated the hypoxic pulmonary vascular structural remodeling. Hence, the above results suggested that the inducement of smooth muscle cell apoptosis by L-Arg might be involved in the regulation of hypoxic pulmonary vascular structural remodeling and hypoxic pulmonary hypertension, which agreed with the previous findings [13].

The mechanism by which L-Arg modulates pulmonary artery smooth muscle cell apoptosis is still unknown. Apoptosis-related genes might be involved in this process. Fas is a kind of uni-transmembrane glycosylated receptor protein on the surface of cells and belongs to the family of tumor necrosis factor and nerve growth factor receptors. It could induce cell apoptosis when conjugated with Fas ligand [14]. The results of our study showed that smooth muscle cell apoptosis was suppressed along with the downregulation of Fas expression in PASMC after hypoxia. PASMC apoptosis was promoted along with the stimulation of Fas expression when exogenous L-Arg was administered. Therefore, it is suggested that Fas expression induced by PASMC might be one of the mechanisms by which L-Arg modulates smooth muscle cell apoptosis and therefore attenuates hypoxic pulmonary vascular structural remodeling in rats.

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Huang-Tian YANG