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Relationship between elastolytic activity and pulmonary remodeling in rats with pulmonary hypertension induced by monocrotaline

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Abstract: **Objective** Pulmonary hypertension is a proliferative vascular disease characterized by pulmonary vascular structural remodeling. Until now, the pathogenesis of pulmonary hypertension has not been fully understood. Recent many studies have suggested that endogenous vascular elastase plays a pivotal role in the vascular changes associated with pulmonary hypertension. The study aimed to explore the relationship between elastolytic activity and pulmonary remodeling in rats with pulmonary hypertension induced by monocrotaline. **Methods** One hundred mature Sprague-Dawley rats were randomly assigned into Normal and Model groups (both $n = 50$). Rats in the Model group were induced with pulmonary hypertension by subcutaneous injection of monocrotaline (60 mg/kg). The elastolytic activity was measured by a fluorescence microplate reader and the pulmonary artery pressure was detected by catheterization at 2, 8, 14 and 21 days post-injection. Light microscopic structural analysis of the peripheral pulmonary vasculature was performed at the same time points. **Results** The elastolytic activity increased significantly to $(3.87 \pm 0.19) \times 10^{-8}$ U/mg at 2 days, and then decreased to $(0.18 \pm 0.2) \times 10^{-8}$ U/mg at 8 days after injection, but on the 14th day it increased again to $(1.45 \pm 0.18) \times 10^{-8}$ U/mg and remained high at $(1.91 \pm 0.18) \times 10^{-8}$ U/mg on the 21st day. The extension of muscle into distal arteries was observed on the 8th day post-injection. Increased pulmonary artery pressure and medial wall thickness were present on the 14th day post-injection. **Conclusions** The early increase in the elastolytic activity may be related to the initiation of pulmonary hypertension. The second increase may be involved in the development of this disease.

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Key words: Endogenous vascular elastase; Hypertension, pulmonary; Monocrotaline; Pulmonary remodeling; Rats

肺动脉高压时内源性血管弹性蛋白酶的动态变化与肺血管重建关系的实验研究

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[**摘　要**] 目的　肺动脉高压是一种以肺血管重建为主要病理改变的肺血管增生性疾病,其机制十分复杂,至今尚未完全阐明,近年来的研究表明,内源性血管弹性蛋白酶(EVE)与肺动脉高压时的肺血管病变密切相关,该研究旨在探讨肺动脉高压时 EVE 的动态变化及其与肺血管重建之间的关系。**方法**　100 只成年 SD 大鼠随机分为正常组和模型组(均 $n = 50$)。模型组大鼠皮下注射 60 mg/kg 野百合碱诱导肺动脉高压。于实验第 2,8,14,21 天,每组各处死 12 只大鼠,取 6 只荧光分光光度计检测肺动脉的弹性蛋白离解活性,并通过右心导管法测定肺动脉压力;余下 6 只对周围肺动脉进行组织病理学分析。**结果**　肺动脉的弹性蛋白离解活性在注射野百合碱后的第 2 天即明显升高至$(3.87 \pm 0.19) \times 10^{-8}$ U/mg,第 8 天降为$(0.18 \pm 0.2) \times 10^{-8}$ U/mg,第 14 天再次升高至$(1.45 \pm 0.18) \times 10^{-8}$ U/mg,第 21 天维持在$(1.91 \pm 0.18) \times 10^{-8}$ U/mg。而肌性肺动脉的比例增加在注射野百合碱后第 8 天出现,肺动脉中膜的增厚及肺动脉压力的增高在第 14 天后出现。**结论**　EVE 活性的早期升高可能是肺动脉高压的一种重要触发因素;EVE 活性的第 2 次升高可能与肺高压的进展有关。　[**中国当代儿科杂志,2005, 7(6):479 – 482**]

[**关　键　词**] 内源性血管弹性蛋白酶;高血压,肺性;野百合碱;肺血管重建; 大鼠

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Pulmonary hypertension is a proliferative vascular disease characterized by pulmonary vascular structural remodeling. Until now, the pathogenesis of pulmonary hypertension has not been fully understood. Recently, many studies have suggested that alterations in the composition of extracellular matrix, especially endogenous vascular elastase (EVE), may be involved in the pathogenesis of pulmonary hypertension[1], but the exact mechanism is unclear. In this study, a rat pulmonary hypertension model induced by monocrotaline was used to determine the changes of serine elastase activity in hypertensive pulmonary arteries and its relationship with pulmonary remodeling.

Materials and methods

Study design

One hundred mature Sprague-Dawley rats weighing 250-300 g were randomly assigned into Control and Model groups (both $n = 50$). Model rats received a single subcutaneous injection of monocrotaline (60 mg/kg) (Aldrich, chemical, USA) in the hind flank[2]. Control rats received an equivalent volume of 0.9% normal saline.

Measurement of mean pulmonary pressure

At 2, 8, 14 and 21 days after injection, 6 rats in each group were selected randomly to measure the mean pulmonary pressure. Briefly, the rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg). The right external jugular vein was isolated via a right neck incision and ligated distally with 3-0 silk. A performed polyethylene catheter was inserted into the vein through a small longitudinal incision. With the aid of a pressure transducer and a physiological monitor, the catheter was advanced slowly and repeatedly until a pulmonary pressure tracing was obtained. The catheter was then secured and the mean pulmonary pressure was recorded.

Measurement of pulmonary artery elastolytic activity

After the hemodynamic measurements, a midline sternotomy provided access to the pulmonary artery. The central pulmonary arteries (main trunk from above the valve, right and left branches to the hilum) were dissected. The vessels were further cleaned of all adhering fat, blood, and loose connective tissues, and then weighed. The extraction of elastase was done as previously described[3]. The protein samples were analyzed for their abilities to degrade fluorescein-conjugated bovine neck ligament elastin (EnChek Elastase Assay Kit; Molecular Probes, USA). Analyses followed the assay kit protocol, with reaction buffer as a negative control and porcine pancreatic elastase as a positive control. Elastase activity was expressed as equivalent units of activity of porcine pancreatic elastase.

Morphometric assessment

At 2, 8, 14 and 21 days after injection, an additional six rats in each group were selected to perform morphometric study. The method for morphometric assessment was followed the description of Ilkiw[4] and was modified. Briefly, the rats were mechanically ventilated (RSP1002, Kent Scintific, USA) through a tracheostomy (peak inspiration pressure 10 mmHg, 75 breaths/min) under pentobarbital sodium anesthesia (50 mg/kg). A midline sternotomy was performed to expose the heart and lungs. Heparin (500 U) was injected into the right ventricle and allowed to circulate for 2 minutes. The central pulmonary artery was cannulated through a small right ventriculotomy incision with a polyethylene tubing. The lung was then perfused with 37℃ heparinized phosphate-buffered saline at 20 cm H_2O pressure for 5 minutes to clear the lungs of blood, then the heart and lungs were removed while maintaining ventilation. The lung was subsequently perfused with a hot (60℃) radiopaque barium-gelatin mixture at 100 cm H_2O pressure for 5 minutes. The pulmonary artery cannula was clamped and the lung was inflated and fixed by perfusion through the trachea tube with 10% formalin at 36 cm H_2O pressure for 72 hours. A block of tissue including the entire cross section of the midregion of the left lung was taken, processed, and embedded in paraffin wax. Sections were stained for elastin with Gomori's aldehyde fuchsin and counterstained by the Van Gieson method. All barium-filled arteries with external diameters in the range of 15-50 μm were assessed at ×400 magnification. Extension of muscle into normal nonmuscular peripheral arteries was evaluated by the percentage of muscular arteries. The thickness of tunica media of normally muscular arteries with external diameters in the range of 100-200 μm were measured with KS400 image analysis system. The thickness percentage of tunica media, an index of medial hypertrophy, was calculated according to the following formula: percent wall thickness = (2 × tunica media thickness)/external diameter × 100%.

Statistical analysis

The data were presented as $\bar{x} \pm s$. Student's t test was used to assess the differences between groups at the same time point. Comparisons among different time points in the same group were based on one-way analysis of variance (ANOVA). Differences were consid-

ered statistically significantly at $P<0.05$. All analyses were conducted with the SPSS10.0 software.

Results

Mean pulmonary artery pressure

The mean pulmonary artery pressure in the Model group was not significantly different from that of the Control group 2 and 8 days after monocrotaline injection. At 14 days, the mean pulmonary artery pressure in the Model group increased significantly when compared with that in the Control group ($P<0.05$). The increase in the mean pulmonary artery pressure was more significant at 21 days ($P<0.01$). The Control group kept a stable mean pulmonary artery pressure during the experiment. See Figure 1.

Pulmonary artery elastolytic activity

Two days after monocrotaline injection, the pulmonary artery elastolystic activity of the Model group increased significantly compared with that of the Control group ($P<0.01$), but restored to normal on the 8th day ($P>0.05$). The pulmonary artery elastolytic activity increased significantly again on the 14th day ($P<0.01$), and another significant increase occurred on the 21st day ($P<0.01$). The Control group kept a stable pulmonary artery elastolytic activity during the experiment. See Figure 2.

Extension of muscle into distal arteries

The extension of muscle into distal arteries was evaluated by the percentage of muscular arteries. At 8 days after monocrotaline injection, muscular arteries significantly increased as compared with those of control rats ($P<0.01$). The abnormality continued and increased at 14 and 21 days (both $P<0.01$). In control rats, only a slight increase in muscular arteries was observed at 21 days ($P<0.05$). See Table 1.

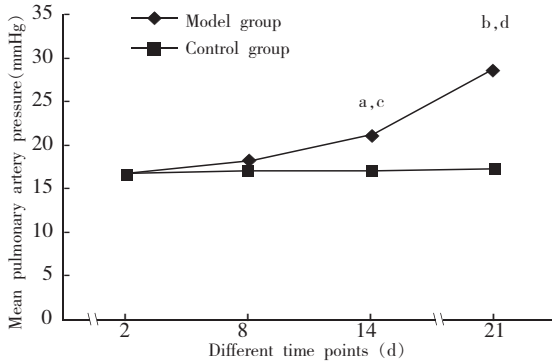

Figure 1 The mean pulmonary artery pressure in the two groups at different time points. Compared with the Control group at the same time point a $P<0.05$, b $P<0.01$; Compared with the same group at different time points c $P<0.05$, d $P<0.01$

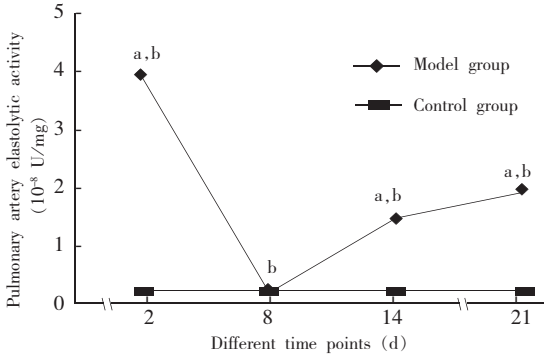

Figure 2 The pulmonary elastolytic activity of the two groups at different time points. a Compared with the Control group at the same time point $P<0.01$; b Compared with the same group at different time points $P<0.01$

Table 1 Percentage of muscular arteries and wall thicknes in two groups at different time points

($\bar{x}\pm s$, $n=12$)

Group	Percentage of muscular arteries (%)				F	Percentage of wall thickness (%)				F
	2 d	8 d	14 d	21 d		2 d	8 d	14 d	21 d	
Control	15.5 ± 2.9	17.2 ± 2.6	18. 7 ± 2.5	20.8 ± 3.1	3.99	2.38 ± 0.26	2.52 ± 0.38	2.57 ± 0.35	2.63 ± 0.29	0.64
Model	16. 7 ± 3.1	32.7 ± 4.2	40.7 ± 5.0	60.7 ± 6.6	82.75	2.67 ± 0.37	2.88 ± 0.36	4.20 ± 0.37	28.35 ± 4.68	169.89
t	1.94	6.68	9.59	10.82		1.45	1.62	10.74	13.98	
P	>0.05	<0.01	<0.01	<0.01		>0.05	>0.05	<0.01	<0.01	

Wall thickness of muscular arteries

There was no significant difference in the thickness percentage of tunica media at either 2 or 8 days after injection between the Model and the Control groups. At 14 days post-injection, the thickness percentage of tunica media increased significantly in the Model group as compared with that of controls ($P<0.01$), and a further increase occurred at 21 days ($P<0.01$). The control rats had a stable thickness percentage of tunica media.

Discussion

EVE is an enzyme of approximately 20 kD in molecular weight, which is related to the serine proteinase adipsin and localizes largely to vascular smooth muscle

cells. EVE probably has an important nurturing function in the embryonic vascular development, however, once reactivated in response to injury postnatally, it can stimulate a zealous and misdirected remodeling that thickens the vessel wall and occludes the lumen[5].

The role of EVE in the pathogenesis of pulmonary hypertension was first suggested by morphological study. Early in l986, Rabinovitch et al[6] reported endothelial injury associated with increased fragmentation of the internal elastic lamina in pulmonary arteries from patients with pulmonary hypertension. This suggested that a proteolytic enzyme of degrading elastin may be related to the mechanism of pulmonary remodeling. Moreover, several studies demonstrated that increases in elastase activity preceded other morphological and biochemical responses[6,7]. A causal relationship between the increased EVE activity and pulmonary hypertension was suggested by a further study in which over-expression of the serine elastase inhibitor elafin protected transgenic mice from hypoxic pulmonary hypertension[8].

In this study, the changes of monocrotaline-induced elastase activity in pulmonary arteries were determined. The data showed that the elastolytic activity increased significantly 2 days after the monocrotaline injection, then returned to normal at 8 days, but increased again at 14 days and remained high till 21 days. According to previous studies[9,10], the mechanism of the production of elastase can be explained as follows: the monocrotaline, an alkaloid toxin, can induce endothelial injury in pulmonary arteries. The structural and functional alterations in the endothelium may result in loss of barrier function. As a result, a serum factor may penetrate the subendothelium and gain access to underlying SMC. The serum factor can facilitate the adhesion of elastin to a 67 kD elastin binding protein, which is on VSMCs surface, and thus increases the activity of EVE in VSMCs by a mitogen-activated protein kinase signaling mechanisms involving increased nuclear expression of the transcription factor acute myelogenous leukemia-1 (AML-1).

In this study, the development of pulmonary hypertension was assessed in rats by cardiovascular catheter. The evolution of structural changes in peripheral arteries was studied by morphometric techniques. The results demonstrated that the extension of muscle into distal arteries appeared 8 days after monocrotaline injection and the increased medial wall thickness and pulmonary artery pressure were present at 14 days. Two increases in the EVE activity were found during the experiment. The first increase was observed 2 days after monocrotaline injection and preceded the structural remodeling and hemodynamic changes, suggesting that the increase of the EVE activity might be related to the initiation of pulmonary hypertension. The second increase in the activity was noted at 14 days when medial hypertrophy occurred, suggesting that the second increase of the EVE activity might be related to the progression of pulmonary hypertension.

On the basis of the study, it is speculated that the inhibition of the early increase of elastolytic activity may prevent the development of pulmonary hypertension, whereas the inhibition of the second increase of elastolytic activity may retard the progression of the disease.

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