Abstract. – Objectives: To investigate whether Angiotensin-(1-7) [Ang-(1-7)] could prevent the development of monocrotaline (MCT) induced pulmonary arterial hypertension and vascular remodeling.

Material and Methods: 30 Sprague-Dawely rats were randomly assigned into three groups: control group, pulmonary arterial hypertension (PAH) group, and PAH + Ang-(1-7) group. Rats in the PAH group and PAH + Ang-(1-7) group received 60 mg/kg monocrotaline (MCT) injection subcutaneously and after 24 hours received either saline or 24 µg/kg/h of Ang-(1-7) injection via osmotic minipumps for 4 weeks. Those rats in the control group were firstly injected saline subcutaneously and then received saline injection via osmotic minipumps.

Results: After four weeks, in the PAH group, right ventricular systolic pressure (RVSP), right ventricular hypertrophy index (RVHI), percentage of wall thickness (WT%) and percentage of wall area (WA%) were significantly increased, and the level of endothelial nitric oxide synthase (eNOS) protein, eNOS ser 1177-phosphorylation, Akt-phosphorylation were significantly decreased compared with control group. However, RVSP, RVHI, WT%, WA% were dramatically decreased in PAH + Ang-(1-7) group and the level of eNOS protein, eNOS ser 1177-phosphorylation, Akt-phosphorylation were significantly increased compared with PAH group.

Conclusion: Those results suggest that Ang-(1-7) could prevent the development of monocrotaline induced pulmonary arterial hypertension and vascular remodeling, which appears to be associated with up-regulation of eNOS activation via Akt pathway.

Key Words: Angiotensin-(1-7), Monocrotaline, Pulmonary arterial hypertension, Endothelial nitric oxide synthase.

Introduction

Monocrotaline (MCT) is an inactive alkaloid obtained from plant origin and is biotransformed to toxic metabolites such as MCT pyrrole in the liver. The metabolite first encountered the pulmonary arterial bed, which results in several pathological changes including endothelial cell injury, smooth muscle cell (SMC) migration and proliferation, vascular remodeling, right heart failure and death.

The heptapeptide Ang-(1-7) is considered to be one of the biologically active products of renin-angiotensin system. It is formed from Ang I and II by several endopetidases including recently identified angiotensin converting enzyme-2 (ACE2). The biological functions of Ang-(1-7) include vasodilatation and inhibition of cell proliferation. The functions of Ang-(1-7) has been recognized to oppose the effects of Ang II. Long-term administration of angiotensin-converting enzyme inhibitors (ACEI) and AT1 receptor blocker might increase the plasma levels of Ang-(1-7) suggesting that Ang-(1-7) might contribute to the pharmacological effects of both ACEI and ARB. Some results had shown that ACEI and ARB might prevent the development of pulmonary arterial hypertension (PAH) and vascular remodeling of pulmonary hypertension induced by monocrotaline. The mechanism might involve the up-regulation of eNOS expression.

Endothelial dysfunction caused by endothelial injury is believed to play a key role in the initiation of pulmonary artery hypertension and was observed in any kind of human pulmonary artery hypertension. Because eNOS is
the major source of nitric oxide (NO) production in vascular endothelial cell, it is regarded as an important regulator of cardiovascular homeostasis. eNOS plays a major role in vasodilation and blood pressure regulation\textsuperscript{10}. eNOS is also believed to be involved in the regulation of the pulmonary vascular tone. Akt protein kinase is an upstream signaling effector for eNOS activation. eNOS is phosphorylated in endothelial cell at Ser1177 by Akt\textsuperscript{16}. Lack of eNOS is associated with accelerated vascular remodeling with high flow stress. Impaired eNOS might also contribute to the pathogenesis of pulmonary arterial hypertension\textsuperscript{10,12}.

Heart and blood vessels are important targets for the formation and actions of Ang-(1-7). Ang-(1-7) might release NO and prostaglandins causing vasodilation, inhibition of cell growth\textsuperscript{1,8,11}. However, effects of Ang-(1-7) on the model of pulmonary artery hypertension induced by monocrotamine were unknown. In this study, we investigated whether long-term infusion Ang-(1-7) could prevent the development of monocrotamine induced pulmonary arterial hypertension and vascular remodeling in rats, and whether Akt/eNOS pathway played a role in this process.

**Materials and Methods**

**Animal**

All procedures were carried out in accordance with guidelines for the humane use of laboratory animal at our Institute and were approved by local authorities. 30 adult male Sprague-Dawley rats (280-320 g, provided by the experimental animal center of Sun Yat-sen University, Guangdong, China) were housed at 25°C with a 12:12-hr light: dark cycle, and allowed to free access to standard food and water.

**Experimental Protocol**

MCT (Sigma, St Louis, MO USA) 600 mg was dissolved in 3.6 ml 1M HCl, and 6-8 ml distilled water was added. This solution neutralized with 1M NaOH was adjusted to PH 7.4. 30 experimental rats were randomly assigned into three groups: control group, PAH group and PAH +Ang-(1-7) group. The rats in the PAH group and PAH +Ang-(1-7) group received 60 mg/kg of MCT injection subcutaneously and after 24 hours received either saline or 24 µg/kg/h of Ang-(1-7) (Bachem, Torrance, CA, USA) via osmotic minipumps (model 2ML4, ALZET Osmotic Pumps: Durect Corporation, Cupertino, CA, USA) for 4 weeks. The rats in the control group were firstly received saline injection subcutaneously and after 24 hours received saline via osmotic minipumps for 4 weeks.

**Measurements of Hemodynamic Parameters and Right Ventricular Hypertrophy**

Hemodynamic parameters were used to evaluate the effects of Ang-(1-7) on the development of pulmonary arterial hypertension. Four weeks after MCT injection, each rat was anesthetized with sodium 10% chloral hydrate (0.3 ml/100 mg, i.p.), polyethylene catheter was inserted into the right ventricular through the jugular vein to record the right ventricular systolic pressure. Then, the heart and lung were quickly removed, and part of them were frozen at –80°C for molecular study and the rest were fixed in 4% formaldehyde for structural study. The right ventricle was dissected from the left ventricle (LV) and the septum (S) and was weighted to determine right ventricular hypertrophy index (RVHI= RV/ LV+S).

**Morphometric Evaluation**

After the infusion of 10% buffered formalin and paraffin-embedding, lungs were cut at 4-µm sections, and the sections were stained with hematoxylin and eosin (HE). Photo image was captured of each lung section (6 fields each animal), and measurements of arterial diameter and area were attained by the Zeiss-KONTRON IBAS 2.5 Automatic Image Analysis System (Zeiss, Munich, Germany). Arteries with an external diameter from 50 to 100 µm were evaluated for the occlusion of pulmonary microvessels. The median thickness was expressed as follows: percentage wall thickness =[(external vessel diameter – luminal diameter)/external vessel diameter]×100; percentage wall area =[(external vessel area – luminal area)/external vessel area]×100

**Western Blot Analysis**

Lung was snap-frozen and homogenized in tissue protein extraction reagent (Pierce Chemical Co, Rockford, IL, USA). The protein concentration was determined with a protein assay system according to the manufacturer (Bio-Rad DC; BioRad Laboratories, Hercules, Ca USA). Equal amounts of total protein, 30 µg each, were sub-
Ang-(1-7) might prevent the development of monocrotaline induced PAH in rats

Morphometric Evaluation
The degree of arterial remodeling (percentage of arterial medial thickness and area) of arteries with an external diameter from 50 to 100 um was significantly elevated in PAH rats. Ang-(1-7) treatment resulted in significantly lower medial thickness and area of the pulmonary arteries compared with those in the PAH groups (Figure 2).

Effect of Ang-(1-7) on Phospho-eNOS Activity
eNOS activation was evaluated by measuring eNOS phosphorylation at Ser1177. In PAH group, eNOS phosphorylation at Ser 1177 in lung tissue was significantly less than those in control group. In PAH +Ang-(1-7) group, eNOS phosphorylation at Ser 1177 in lung tissue was significantly greater than those in PAH group (Figure 3A).

Effect of ang-(1-7) on level of eNOS protein expression
As shown in Figure 3B, in PAH group, the amount of eNOS protein expression in lung was significantly less than those in control groups. In PAH +Ang-(1-7) group, the amount of eNOS protein expression in lung was significantly greater than those in PAH group.

Effect of Ang-(1-7) on Phospho-Akt activity
As shown in Figure 3C, the degree of Akt Ser 473 phosphorylation was significantly decreased

Results
Hemodynamic Parameters and Right Ventricular Hypertrophy
Right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index(RVHI) was significantly higher in PAH group than control group, suggested the occurrence of pulmonary arterial hypertension. The elevation of RVSP and RVHI was significantly lower by long-term Ang-(1-7) administration (Figure 1).

Figure 1. A, Ang-(1-7) improved RVSP. B, RVHI in rats with MCT-induced PAH. Compared with the control group, RVSP and RVHI was increased in the PAH group, RVSP and RVHI was significantly lower in Ang-(1-7) group: *p<0.01 vs.control; /p<0.01 vs. PAH group.
in PAH group. The lower of Akt Ser 473 phosphorylation was significantly increased by long-term Ang-(1-7) administration.

**Discussion**

Our study shows that Ang-(1-7) might prevent the development of monocrotaline induced pulmonary arterial hypertension and vascular remodeling of pulmonary hypertension, and its mechanism appears to be associated with upregulation of eNOS activation via Akt pathway.

Endothelial cells are the key site for the formation and metabolism of Ang-(1-7). Endothelial cells possess Mas receptor. The Mas proto-oncogene encodes a seven-transmembrane-domain G-protein-coupled orphan receptor which is identified as an Ang-(1-7) receptor. Ang-(1-7) binds to the Mas receptor and has subsequent function effects. Some reports show that Ang-(1-7) is a vasodilator agent in several vascular beds, including canine and porcine coronary arteries, canine middle cerebral artery, rabbit renal afferent arterioles, rat aorta, and mesenteric normotensive and hypertensive rats. But contradictory results are reported in human vessels. Concerning the vasodilator effects of Ang-(1-7) it has been marked differences in species and vascular beds. However, Ang-(1-7) the prevented development of the monocrotaline induced pulmonary arterial hypertension in rats is unknown. In our study, 4 weeks after MCT injection, right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (RVHI) was significantly reduced by long-term Ang-(1-7) administration, suggesting that Ang-(1-7) might prevent the development of monocrotaline induced pulmonary arterial hypertension in rats. Ang-(1-7) inhibited
the vascular smooth muscle cells (VSMCs) proliferation stimulated by platelet-derived growth factor (PDGF), Ang II and endothelin. Ang-(1-7) might attenuate neointimal formation and smooth muscle cell proliferation after balloon injury of rat arteries. In our study, percentage of wall thickness (WT%) and percentage of wall area (WA%) were used as the index of arterial remodeling. Four weeks after MCT injection, WT% and WA% was significantly higher. The elevation of WT% and WA% was significantly reduced by long-term Ang-(1-7) administration, suggesting that Ang-(1-7) might reverse vascular remodeling progress.

Growing evidences showed that the cardiovascular actions of Ang-(1-7) appeared to be associated with release of nitric oxide (NO). NO plays a central role in endothelial function, maintaining vasodilatory tone, inhibiting platelet aggregation and adhesion, and regulating vascular smooth muscle cells contraction and proliferation. NO is synthesized from L-arginine by NO synthase (NOS). There are 3 subtypes of NOS (iNOS, eNOS, and nNOS). Research showed that eNOS

**Figure 3.** Effect of Ang-(1-7) on Phospho-eNOS Activity (A), t-eNOS (B), p-Akt (C). In PAH group, eNOS phosphorylation at Ser 1177, t-eNOS, and p-Akt in lung tissue was significantly less than those in control group. In PAH + Ang-(1-7) group, p-eNOS, t-eNOS, and p-Akt was significantly greater than those in PAH group. *p<0.01 vs.control; †p<0.01 vs. PAH group.
played the most important role in keeping pulmonary circulation. eNOS should be responsible for NO availability. ACEI and ARB might prevent the development of PAH and vascular remodeling of monocrotaline induced pulmonary hypertension. The mechanism of action might involve the up-regulation of eNOS expression. Ang-(1-7) might contribute to the pharmacological effects of both ACEI and ARB. Whether Ang-(1-7) reverses vascular remodeling of pulmonary arterial hypertension induced by monocrotaline in rats through up-regulation of eNOS is unknown.

eNOS activity is regulated by various kinases, such as eNOS Ser1177-phosphorylation. Reports have shown that eNOS is phosphorylated at Ser-1177 in cultured endothelial cells in response to vascular endothelial growth factor, insulin-like growth factor, fluid shear stress, and bradykinin.

In our study, the amount of eNOS protein expression and eNOS Ser1177-phosphorylation in lung tissue in PAH group was significantly less than those in control group. The decrease of eNOS protein expression and eNOS Ser1177-phosphorylation was significantly increased by long-term Ang-(1-7) administration. These observations suggested that part of the pathogenesis of the chronic changes found in monocrotaline induced PAH are associated with loss of eNOS and that Ang-(1-7) may function, at least in part, by up-regulation eNOS Ser1177-phosphorylation, preserving eNOS expression.

Akt is an upstream signaling effector for eNOS activation. Akt is expressed in various cell types including smooth muscle cell and endothelial cells. Akt activation was monitored by the state of Akt phosphorylation. Some researchers reported that eNOS was phosphorylated in endothelial cell at Ser1177 by the Akt, leading to a 2-fold increase in eNOS catalytic activity and NO production.

Recently Sampaio et al study showed that human endothelial cells possess GPCR Mas and Ang-(1-7) through Mas, stimulates eNOS activation and NO production via Akt-dependent pathway. In our investigation, the amount of Akt Ser 473 phosphorylation in lung tissue in PAH group was significantly decreased than those in control group. The decrease of Akt Ser 473 phosphorylation was significantly increased by long-term Ang-(1-7) administration. In summary, our findings suggested that Ang-(1-7) prevent the development of pulmonary arterial hypertension and vascular remodeling of monocrotaline induced pulmonary hypertension in rats. Its mechanism appeared to be associated with up-regulation eNOS activation via Akt pathway.

In conclusion, pulmonary arterial hypertension is a serious clinical problem because of its high morbidity and mortality. The MCT-induced pulmonary arterial hypertension model is a noninvasive, slowly developing, hemodynamically relevant rat model that mirrors human PAH, leading to right ventricular hypertrophy. Our study suggests that Ang-(1-7) prevent the development of PAH and vascular remodeling of monocrotaline induced pulmonary hypertension. We hope it could bring a novel therapeutic strategy for the treatment of pulmonary arterial hypertension.

References

Ang-(1-7) might prevent the development of monocrotaline induced PAH in rats