West China Second Hospital, Sichuan University; Department of Sports Medicine, Chengdu Sport University, Chengdu, China

Combined effects of the ATP-sensitive potassium channel opener pinacidil and simvastatin on pulmonary vascular remodeling in rats with monocrotaline-induced pulmonary arterial hypertension

Li Jiang, Tongfu Zhou, Hanming Liu

Received September 16, 2011, accepted October 14, 2011

Tongfu Zhou, West China Second Hospital, Sichuan University, 610041 Chengdu, China
lvoice@21cn.com

Pharmazie 67: 547–552 (2012) *doi: 10.1691/ph.2012.1712*

The drugs that are currently used to treat pulmonary hypertension (PH) lack the ability to inhibit or reverse the pulmonary vascular remodeling that occurs during the course of the disease. We propose a novel method that combines the therapeutic powers of the potassium channel opener pinacidil and the statin drug simvastatin. These two drugs do not share similar mechanisms of treating PH. We used rats with monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH) as a model and examined the combined effects of pinacidil and simvastatin on pulmonary vascular remodeling. A series of indicators, including those for pulmonary vascular obstruction, proliferation, and cell phenotype, pulmonary vascular matrix and pulmonary vascular smooth muscle cell phenotype were used to monitor changes in pulmonary structure over the course of disease and treatment in normal controls, untreated PAH rats, pinacidil-treated subjects, simvastatin-treated subjects, and combination-treated subjects. We found that levels of mPAP, right ventricle Fulton index, pulmonary arteriolar wall thickness and muscularization, cell growth rate, transforming growth factor β (TGF-β), lung tissue matrix metalloproteinase-2 (MMP-2), MMP-9 and lung tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), vascular smooth muscle cell (VSMC) contractile protein SM-α-actin, and SM-α-actin mRNA of these different groups were all significantly lower in the combination-treated group than in the untreated group. Subjects in the combination-treated group also showed lower levels than those in either the pinacidil-treated or simvastatin-treated group. These results support our hypothesis and provide basis for a new, more effective therapeutic methods of treating PAH in human patients.

1. Introduction

Pulmonary hypertension (PH) features increased pulmonary vascular resistance and pulmonary arterial pressure. It also involves pulmonary vascular remodeling, the pathologic basis for which is abnormal proliferation of pulmonary vascular endothelial and smooth muscle cells. Pulmonary vascular remodeling can lead to pulmonary arteriolar obstruction (Heath and Edwards 1958; Rabinovitch et al. 1978; Simonneau et al. 2004). Since the 1990 s, prostacyclin, endothelin receptor antagonists, and phosphodiesterase-5 inhibitor have been used clinically to treat PH. While these drugs have improved patients' quality of life and expanded their lifespans to a certain extent, they mostly target expanding pulmonary vessels and work to reduce pulmonary vascular resistance, with very little effect on pulmonary vascular remodeling (Okada et al. 1997; Jeffery et al. 2002; Lam et al. 2005; Liu et al. 2008). In addition, some of these medicines are expensive and inconvenient to administer (Rabinovitch et al. 1987; Zhou et al. 1997; Gong et al. 2004; Li et al. 2006; Stenmark et al. 2006; Badesch et al. 2007). Currently studies on developing new drugs to intervene and treat PH have focused more on anti-remodeling effect rather than vascular expansion effect. KATPCOs and statins are two drugs that have been studied extensively in recent years for their effects on anti-vascular remodeling.

It has also been reported that in treating PAH, drug combinations have higher therapeutic power and less side effect than single drug treatment (Waddell et al. 2002; Galiè et al. 2004; Hoeper et al. 2004; Newman et al. 2004; Humbert et al. 2008). These findings show a new direction for drug development. Treating PH with a single drug may not have the greatest or sustained therapeutic power. As the molecular mechanisms underlying vascular remodeling caused by PH are complicated, drug combination that synergistically target different sites and pathways may substantially increase the therapeutic power (Waddell et al. 2002; Galiè et al. 2004; Hoeper et al. 2004).

However, some researchers argued that combined use of NO and prostacyclin after congenital heart disease surgery showed no synergistic therapeutic effects, which may be related to the competition between the two drugs (Hallioglu et al. 2003). We reason that if two drugs share similar pharmacological action mechanisms, combination treatment may not offer further advantage due to competition; on the other hand, if two drugs target different sites and cell signaling pathways, combination treatment with both should be more potent in treating the disease, without the adverse effect caused by using one drug alone in elevated dose.

KATPCOs and statins are two drugs that have been studied extensively in recent years for their effects on anti-vascular remodeling. We reason that because KATPCOs and statins

are both anti-vascular remodeling drugs that have been studied relatively extensively but nevertheless do not share similar mechanisms, combined treatment with these two drugs should show synergistic effects. In addition, these two drugs are affordable and easy to administer. This makes them suitable for long-term use in PH patients in developing countries. To our knowledge, this study is the first to examine the combined effects of KATPCOs and statins on pulmonary vascular remodeling.

We used rats with monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH) as a model and examined the combined effects of pinacidil and simvastatin on pulmonary vascular remodeling. Our study will provide insight that may be useful for the development of new, effective therapeutic methods of vascular remodeling in PAH patients. It may also provide a basis for combined clinical PAH treatments.

2. Investigation and results

2.1. Comparison of mPAP and degree of right ventricle hypertrophy

As shown in the Table, the mPAP of group M was significantly higher than that of group C ($P < 0.01$), showing that MCT had successfully induced PAH. The mPAP of all intervention groups (groups MP, MS, and MPS) were all significantly lower than that of group M ($P < 0.01$), showing that all these treatments were effective. The mPAP of group MPS was significantly lower than that of either group MP or group MS ($P < 0.05$), indicating that combined treatment with KATPCO pinacidil and statins provided additional therapeutic value over pinacidil or statins alone. For the Fulton index (RV/(LV + S)), which reflects the degree of right ventricle hypertrophy, results were similar. As shown in the Table, the Fulton index of group M was significantly higher than that of group C ($P < 0.01$), showing that MCT had effectively induced PAH. The Fulton indexes of all intervention groups (groups MP, MS, and MPS) were significantly lower than that of group M ($P < 0.01$), showing that all these treatments were effective. The Fulton index of group MPS was significantly lower than that of either group MP or group MS ($P < 0.05$), indicating that combined treatment with KATPCO pinacidil and statins provided additional therapeutic value over pinacidil or statins alone.

2.2. Comparison of pulmonary arteriolar wall thickness and muscularization

As shown in the Table, pulmonary arteriolar wall thickness (WT%) of group M was significantly higher than that of group C ($P < 0.01$), showing that MCT had induced PAH effectively. The WT%s of all intervention groups (groups MP, MS, and MPS) were significantly lower than that of group M ($P < 0.01$), providing evidence that all these treatments were effective. Importantly, the WT% of group MPS was significantly lower than that of either group MP or group MS ($P < 0.05$), indicating that combined treatment with KATPCO pinacidil and statin provided additional therapeutic value over pinacidil or statins alone. Also as shown in the Table, the muscularization of non-muscular pulmonary arterioli of group M was significantly more pronounced than that of group C ($P < 0.01$), showing that MCT had induced PAH effectively. The muscularization of all intervention groups (groups MP, MS, and MPS) were significantly less pronounced than that of group M ($P < 0.01$), showing that all these treatments were effective. Importantly, the muscularization of group MPS was significantly less pronounced than that of either group MP or group MS ($P < 0.05$), indicating that combined treatment with KATPCO pinacidil and statins provided additional therapeutic value over pinacidil or statins alone.

Table: Comparison of indices between rats in different experimental groups ($\chi \pm s$)

Group	n	mPAP(mmHg)	RV/(LV+S) (%)	Arteriolar wall thickness percentage (WT%)	Muscularization of non-muscular pulmonary arterioli (%)	Arteriolar wall proliferative rate (%)	TGF-β (IOD) × 10^3	MMP-2 (IOD) × 10^3	MMP-9 (IOD) × 10^3	TIMP-1 (IOD) × 10^3	SM-α-actin mRNA	SM-α-actin
Group C	8	18.56 ± 1.20	26.89 ± 2.10	10.87 ± 3.12	16.43 ± 2.12	6.08 ± 1.89	2.23 ± 0.77	1.88 ± 0.25	2.33 ± 0.43	3.19 ± 0.45	0.808 ± 0.121	1.446 ± 0.135
Group M	8	37.20 ± 4.67*	46.81 ± 4.34*	36.47 ± 7.35*	50.09 ± 5.89*	49.78 ± 8.09*	52.34 ± 8.6*	18.13 ± 2.21*	14.86 ± 2.03*	29.08 ± 2.98*	0.213 ± 0.066*	0.513 ± 0.120*
Group MP	8	32.30 ± 2.16△	36.87 ± 3.56△	24.65 ± 4.29△	33.30 ± 4.56△	34.76 ± 6.89△	36.06 ± 9.78△	4.39 ± 1.32△	3.89 ± 0.67△	4.54 ± 0.96△	0.334 ± 0.058△	1.396 ± 0.118△
Group MS	8	31.22 ± 2.43△	37.66 ± 3.35△	25.78 ± 3.24△	34.59 ± 3.90△:	33.34 ± 5.78△	35.67 ± 8.56△	4.01 ± 0.98△	3.99 ± 1.01△	4.78 ± 1.04△	0.327 ± 0.011△:	1.402 ± 0.011△
Group MPS	8	28.43 ± 3.86△#☆	34.89 ± 4.78△#☆	22.90 ± 4.65△#☆	31.56 ± 4.76△#☆	31.89 ± 5.67△#☆	26.78 ± 7.45△#☆	3.86 ± 0.84△#☆	2.90 ± 0.45△#☆	3.90 ± 0.56△#☆	0.719 ± 0.116△#☆	1.426 ± 0.201△#☆

* denotes when compared to group C, $P < 0.01$; △: denotes when compared to group M, $P < 0.05$; #: denotes when compared to group MP, $P < 0.05$; ☆: denotes when compared to group MS, $P < 0.05$

2.3. Comparison of pulmonary arteriolar wall proliferative rate and TGF-β expression

As shown in the Table, the pulmonary arteriolar wall proliferative rate of group M was significantly higher than that of group C ($P < 0.01$), showing that MCT had induced PAH effectively. The proliferative rates of all intervention groups (groups MP, MS, and MPS) were significantly lower than that of group M ($P < 0.01$), showing that all these treatments were effective. Importantly, the pulmonary arteriolar wall proliferative rate of group MPS was significantly lower than that of either group MP or group MS ($P < 0.05$), indicating that combined treatment with KATPCO pinacidil and statins provided additional therapeutic value over pinacidil or statins alone.

Also as shown in the Table, the TGF-β expression level (IOD) of group M was significantly higher than that of group C ($P < 0.01$), showing that MCT had induced PAH effectively. The TGF-β expression levels (IOD) of all intervention groups (groups MP, MS, and MPS) were significantly lower than that of group M ($P < 0.01$), showing that all these treatments were effective. Importantly, the TGF-β expression level (IOD) of group MPS was significantly lower than that of either group MP or group MS ($P < 0.05$), indicating that combined treatment with KATPCO pinacidil and statin provided additional therapeutic value over pinacidil or statins alone.

2.4. Comparison of MMP-2, MMP-9, and TIMP-1 levels via semi-quantitative immunochemical testing

We used MMP-2, MMP-9, and TIMP-1 levels as indicators of changes in the pulmonary vascular matrix. As shown in the Table, the MMP-2, MMP-9, and TIMP-1 levels (IOD) of group M were significantly higher than those of group C ($P < 0.01$), showing that MCT had induced PAH effectively. The MMP-2, MMP-9, and TIMP-1 levels (IOD) of all intervention groups (groups MP, MS, and MPS) were significantly lower than those of group M ($P < 0.01$), showing that all these treatments were effective. The MMP-2, MMP-9, and TIMP-1 levels (IOD) of group MPS were significantly lower than those of either group MP or group MS ($P < 0.05$), indicating that combined treatment with KATPCO pinacidil and statin provided additional therapeutic value over pinacidil or statins alone.

2.5. Comparison of VSMC contractile protein SM-α-actin mRNA levels via real-time fluorescence-based quantitative PCR

We used levels of VSMC contractile protein SM-α-actin mRNA as an indicator of changes in pulmonary vascular smooth muscle cell phenotypes. As shown in the Table, the SM-α-actin mRNA level of group M was significantly lower than that of group C ($P < 0.01$), showing that MCT had induced PAH effectively. The SM-α-actin mRNA levels of all intervention groups (groups MP, MS, and MPS) were significantly higher than that of group M ($P < 0.01$), showing that all these treatments were effective. The SM-α-actin mRNA level of group MPS was significantly higher than that of either group MP or group MS ($P < 0.05$), indicating that combined treatment with KATPCO pinacidil and statin provided additional therapeutic value over pinacidil or statins alone.

2.6. Comparison of VSMC contractile protein SM-α-actin expression level via Western blotting

We used the expression level of VSMC contractile protein SM-α-actin a complementary indicator of changes in pulmonary vascular smooth muscle cell phenotypes. As shown in the Table, the SM-α-actin expression level of group M was significantly lower than that of group C ($P < 0.01$), showing that MCT had induced PAH effectively. The SM-α-actin expression levels of all intervention groups (groups MP, MS, and MPS) were significantly higher than that of group M ($P < 0.01$), showing that all these treatments were effective. The SM-α-actin protein expression level of group MPS was significantly higher than that of either group MP or group MS ($P < 0.05$), indicating that combined treatment with KATPCO pinacidil and statins provided additional therapeutic value over pinacidil or statins alone.

3. Discussion

In recent years, clinical trials have shown that PAH patients treated with prostacyclin, bosentan, or sildenafil alone usually continue to deteriorate. On the contrary, drug combinations, which have more therapeutic power than single drug treatments, may stall or even reverse the pulmonary vascular remodeling that occurs in PAH patients (Waddell et al. 2002; Galiè et al. 2004; Hoeper et al. 2004; Newman et al. 2004; Humbert et al. 2008). Because combination treatment can enhance the therapeutic power while avoiding the adverse effects caused by high doses of single drugs, researchers have paid increasing attention to the subject. KATPCOs and statins are two drugs that have been studied extensively for their effects on preventing vascular remodeling. They act through different pharmacological mechanisms, so when used in combination, they undergo no competition. Instead, these two drugs target different sites and cell signaling pathways in a synergistic fashion, therefore providing even more therapeutic power than the sum of both drugs acting alone.

In our study, we used a MCT-induced PAH rat model to investigate the effect of combined use of KATPCO pinacidil and simvastatin on pulmonary vascular remodeling. We found that for indicators of vascular thickening and proliferation such as MMP-2, MMP-9, TIMP-1, PCNA, TGF-β, in MCT-induced PAH group (group M) their levels were significantly higher than those in the control group ($P < 0.05$), which demonstrated typical symptoms of PAH; in pinacidil-treated and simvastatin-treated PAH group (group MP and group MS), their levels were significantly lower than those in the untreated group M, demonstrating that both pinacidil and simvastatin both contributed to reversing vascular remodeling caused by PAH; importantly, in group MPS that was treated with pinacidil and simvastatin combined, the levels of these indicators were significantly lower than those in group MP and group MS that was treated by a single drug ($P < 0.05$). On the contrary, for VSMC contractile protein SM-α-actin and its mRNA that mark the non-proliferative state of pulmonary blood vessels, in group M their levels were significantly lower than those in the control group ($P < 0.05$); in group MP and group MS, their levels were significantly higher than those in the untreated group M; and in group MPS the levels of these indicators were significantly higher than those in group MP and group MS ($P < 0.05$). These results strongly support our hypothesis that combined treatment with pinacidil and simvastatin offers improved therapeutic power in treating PAH compared to either drug alone.

Matrix metalloproteinases (MMPs) are a type of proteases in the extracellular matrix that are involved in collagen degradation. They play important roles in ECM remodeling, cell migration and proliferation. It has been shown that in animal models with induced PH, MMPs expression is up-regulated, suggesting that MMPs may contribute to the formation of pulmonary arterial hypertension and the accompanying pulmonary vascular remodeling. Tissue inhibitors of metalloproteinases are specific

MMPs inhibitors and dynamically balanced with MMPs. In our study we found the changes in TIMP level paralleled that in MMP levels. This could possibly be because as MMPs activity decreased, TIMP level decreased correspondingly to keep the MMPs/TIMPs balance. Our results show that simvastatin and KATPCO pinacidil can both inhibit MMPs hence the progress of MCT-induced PH; in addition, combined use of the two drugs had synergistic effects in decreasing MMPs activity, suggesting that the combination treatment could inhibit ECM collagen accumulation in PH development and reverse vascular remodeling.

PCNA is closely related to DNA synthesis and plays an important role in cell proliferation. PCNA expression starts to increase substantially at the G_2 phase and peaks at the S phase, consistent with DNA synthesis activity. Therefore the expression level of PCNA in the cells is a good indicator of cell proliferation. Our results show that simvastatin and KATPCO pinacidil can both inhibit PCNA expression hence the proliferation of pulmonary arteriolar wall cells in the PAH development; in addition, combined use of the two drugs have synergistic effects in decreasing the PCNA expression level, suggesting that the combined treatment could inhibit pulmonary arteriolar wall cell proliferation via decreasing the expression level of PCNA, hence the anti-vascular remodeling effect.

Transforming growth factor-β (TGF-β) belongs to a super family of regulatory factors involved in cell growth and differentiation. It can transform the phenotype of normal mechanocytes; under the coexistence with epidermal growth factor (EGF), TGF-β changes the specially property of growing adherent to substratum of inocytes so that they can grow in the extracellular matrix. In the disease development of PAH, TGF-β increases the growth of mechanocytes, facilitates the expression of extracellular matrix (ECM) proteins such as collagen and fibronectin, and inhibits the degradation of ECM, thus plays an important role in pulmonary vascular remodeling. Our results show that simvastatin and KATPCO pinacidil can both down-regulate TGF-β expression and inhibit the mechanocyte growth as well as expression of ECM collagen and fibronectin, which can alleviate or reverse the pulmonary vascular remodeling caused by PAH; in addition, combination of the two drugs has synergistic effect in decreasing TGF-β expression level, suggesting that the combined treatment could inhibit the mechanocyte growth and ECM collagen and fibronectin expression via decreasing TGF-β, hence providing its therapeutic effect on pulmonary vascular remodeling.

Vascular smooth muscle cells (VSMC) are located in the middle layer of blood vessels. They can determine vasoactivity, vascular structure and vascular tension. Based on structure and function VSMCs are categorized into contractile and synthetic types. The contractile VSMCs are in a differentiated state and cannot proliferate; α-SM-actin is a marker protein for contractile VSMCs. The synthetic VSMCs are in de-defferentiated state; they can respond to external mitogens and start to proliferate. The transformation from contractile VSMCs to synthetic VSMCs is a prerequisite for PAH-caused vascular remodeling. Our results show that simvastatin and KATPCO pinacidil can both increase SM-α-actin and SM-α-actin mRNA levels and inhibit the transformation from contractile to synthetic VSMCs, which can alleviate or reverse the pulmonary vascular remodeling caused by PAH; in addition, combined use of the two drugs have synergistic effect in increasing SM-α-actin and SM-α-actin mRNA levels, suggesting that the combined treatment are more potent in inhibiting the VSMC phenotype transformation caused by PAH, hence have a stronger effect in reversing pulmonary vascular remodeling.

In conclusion, we report here that KATPCO pinacidil and simvastatin can both inhibit the progress of pulmonary arterial hypertension and alleviate the pulmonary remodeling caused by PAH in MCT-induced PAH rat model; they both inhibit the transformation of PASMCs from contractile type to synthetic type, hence inhibit PASMC proliferation. In addition, the combined use of KATPCO pinacidil and simvastatin exerts greates therapeutic power than either drug alone, suggesting that the combination of the two drugs has a synergistic effect on treating pulmonary vascular remodeling caused by PAH.

4. Experimental

4.1. Animal preparation

Forty male Sprague-Dawley rats (180–220 g) were transported to Sichuan University Huaxi Hospital Animal Research Center and raised for 4–5 days on food and water *ad libitum* before the experiments began. The rats were randomly assigned to five groups of eight rats each: a normal control group (group C), an MCT (Sigma, Shanghai) injection group (group M), an MCT injection + oral simvastatin group (Shujiangzhi, 20 mg/tablet, Hangzhou Shamodong Pharmaceutical Corporation) (group MS); an MCT injection + oral pinacidil group (Sigma) (group MP); and an MCT injection + oral simvastatin + oral pinacidil group (group MPS).

All rats were provided with food and water *ad libitum* and euthanized on the 28th day. Rats in group C received no special treatment. For group M, MCT was dissolved in 0.5 mol/L HCl. The pH was adjusted to 7.4 by 0.5 mol/L NaOH. The mixture was injected subcutaneously on the back (60 mg/kg) on the first day, and the same amount of saline was administered intragastrically daily. For group MP, MCT was injected subcutaneously on the back (60 mg/kg) on the first day, and KATPCO pinacidil was administered intragastrically at 2 mg/kg daily. For group MS, MCT was injected subcutaneously on the back (60 mg/kg) on the first day, and simvastatin was administered intragastrically at 2 mg/kg daily. For group MS, MCT was injected subcutaneously on the back (60 mg/kg) on the first day, and pinacidil and simvastatin were administered intragastrically at 2 mg/kg each daily.

4.2. Measurement of hemodynamic parameters and heart weight

Mean pulmonary arterial pressure (mPAP) of all rats was measured on the 28th day: 10% chloral hydrate (400 mg/kg) was injected for intraperitoneal anesthesia. The catheter was placed through the external jugular vein, and mPAP was measured by a multi-parameter monitor (PM-5000). After mPAP measurement, animals were euthanized, and their hearts and right lungs were harvested. Atria and main vessels were removed from the heart, and the right ventricle (RV) and left ventricle plus septum (LV+S) were isolated. After washing and drying with filter paper, the RV and (LV+S) were weighed. The Fulton Index was calculated as RV/(LV+S).

4.3. Lung tissue preparation

Surplus blood was removed from the right lung by irrigating the pulmonary artery using saline followed by slow injection of 4% paraformaldehyde PBS (0.01 mol/L, pH 7.14) at a pressure of 20 cmH_2O. After 5 min, the right lower lung was isolated and placed in 10% buffered neutral formalin for 48 h. Following paraffin embedding, 5 μm serial sections were taken for hematoxylin and eosin (HE) staining, and lung tissue and vessel conditions were observed under light microscope.

Orcein staining was performed on the prepared slices to observe elastic fibers, which appeared red-brown or dark brown after staining. The primary antibodies used for immunohistochemical staining were PCNA (FL-261) rabbit antibody (Santa Cruz Biotechnology, #sc-7907, dilution rate 1:50), rabbit polyclonal to alpha smooth muscle actin (Abcam, #ab5694, dilution rate 1:50), mouse anti-MMP-2 (Santa Cruz Biotechnology, #SC13594, dilution rate 1:50), MMP-9 (Millipore, #AB19016, dilution rate 1:50), TGFβ1 (V) (Santa Cruz Biotechnology, #sc-146, dilution rate 1:50), rabbit TIMP-1 antibody (Beijing Boaosen Biotech Corporation, #bs-0415R, dilution rate 1:50). The secondary antibody was EnvisionTM (K500711) from a rabbit/mouse kit (DAKO).

4.4. Stereology and immunochemical assessment of lung tissue

For each animal, 20 high power fields (X400) were randomly selected and 30–60 intra-acinar arterioli (diameter 50–150 μm) were counted as fully muscularized (mesolamella containing a complete muscular layer), partially muscularized (mesolamella containing an incomplete muscular layer), and not muscularized (no obvious muscular layer) based on degree of positive stain by SM-α-actin. Muscularization was calculated as muscularization% = (fully muscularized vessel number + partially muscularized vessel number)/(total number of observed vessels) × 100%.

Pulmonary arteriolar wall thickness was measured in the following manner: Twenty 50–150 μm pulmonary arterioli were randomly selected from each sample and wall thickness (distance between endangium and adventitia) and outside diameter were measured. Nikon and spot image sampling and processing systems were used to plot internal and external contour lines and measure the distance. Wall thickness percentage was calculated as (pulmonary arteriolar wall thickness percentage%) = (2 × wall thickness)/(outside diameter) × 100.

Under a light microscope, 15–30 15–150 um pulmonary arterioli were randomly selected from good PCNA immunochemical stained slices. A cell was considered PCNA-positive if its nucleus appeared evenly brown. The total number of cells and of PCNA-positive cells on the arteriolar walls were counted and the proliferative rate was calculated as proliferative rate% = PCNA positive cell number/total cell number × 100%.

To examine MMP-2, MMP-9, and TIMP-1 expression levels, well-stained slices were selected. A cell was considered positive if brown particles appeared evenly on the endochylema or cell membrane. Semi-quantitative analysis was performed with Image-Pro Plus imaging analysis software (Media Cybernetics) under X400 light microscope. For each slice, five fields were randomly selected using systematic sampling principles and input into a computer as measuring fields. Integral optical density (IOD) was calculated.

4.5. Measurement of SM-α-actin mRNA levels

Real-time fluorescence-based quantitative PCR was used to measure SM-α-actin and β-actin levels. The rat SM-α-actin and β-actin gene sequences were downloaded from the NCBI database, and Primer Premier 5 software was used to design the amplimer and probe. To avoid genomic DNA contamination, introns were skipped. Syntheses of primers for SM-α-actin and β-actin and Taqman probe modification was performed and quality controlled by the TaKaRa company. The content was centrifuged immediately before opening, and TE (pH 8.0) was added to dilute the concentration to 10 μM. The preparation was then stored at –20 °C for fluorescence-based quantitative PCR testing. The primer and probe sequences were as follows: SM-α-actin: sense primer 5′-GTTCGAAACCTTCAATGTTCTT-3′, antisense primer 5′-CCATTCAAGCTGTGCTCTCGCT-3′, probe FAM-5′-ATCTACGAGGGCTATGCACTG-3-TAMRA′; β-actin: sense primer 5′-GACCCAGATCATGTTTGAGACC-3′, antisense primer 5′-GCAGTAATCTCCTTCTGCATCC-3′.

4.6. Measurement of SM-α-actin protein expression level

Western blotting was used to assess SM-α-actin protein expression levels. Lung tissues were ground to fine powder with liquid nitrogen. The powder was collected, added to 300 μl protein lysate and mixed well before being placed on ice for 30 min. The suspension was then centrifuged at 12,000 rpm and 4 °C for 20 min. The supernatant was collected and stored in smaller volumes of 20 μl each at –180 °C. A BCA kit (Biyuntian) was used to measure the protein concentration of all samples, which were then stored at –70 °C. To prepare for electrophoresis, 100 μl 2 × SDS loading buffer was added to 100 μl sample, whirl mixed, heated at 95 °C for 5 min, and centrifuged at 12,000 rpm and 4 °C for 20 min. Supernatant was collected and stored at –20 °C until use. SDS-PAGE was performed. The gray values of SM-α-actin and β-actin bands obtained from the same SDA-PAGE were compared and the grayness value of SM-α-actin was calculated.

4.7. Statistical analysis

Data are presented as mean ± standard deviation (X ± S). One-way ANOVA was performed for statistical processing. The Q-test was performed for paired comparison, and SPSS 13.0 software was used to run the statistical analyses. Test results with $P < 0.05$ were considered statistically significant.

Acknowledgement: Supported by 08zc045, Sichuan Department of Education, China

References

Agapito AF, Sousa L, Oliveira JA, Feliciano J, Cacela D, Quininha J (2005) Eisenmenger syndrome in the adult–experience with new drugs for the treatment of pulmonary hypertension. Rev Port Cardiol 24:421–431.

Apostolopoulou SC, Manginas A, Cokkinos DV, Rammos S (2005) Effect of the oral endothelin antagonist bosentan on the clinical, exercise, and haemodynamic status of patients with pulmonary arterial hypertension related to congenital heart disease. Heart 91:1447–1452.

Badesch DB, Abman SH, Simonneau G, Rubin LJ, McLaughlin VV (2007) Medical therapy for pulmonary arterial hypertension: updated ACCP evidence-based clinical practice guidelines. Chest 131: 1917–1928.

Barst RJ, McGoon M, McLaughlin V, Tapson V, Rich S, Rubin L, Wasserman K, Oudiz R, Shapiro S, Robbins IM, Channick R, Badesch D, Rayburn BK, Flinchbaugh R, Sigman J, Arneson C, Jeffs R, Beraprost Study Group (2003) Beraprost therapy for pulmonary arterial hypertension. J Am Coll Cardiol 41: 2119–2125.

Cowan KN, Jones PL, Rabinovitch M (2000) Elastase and matrix metalloproteinase inhibitors induce regression and tenascin-C antisense prevents progressive vascular disease. J Clin Invest 105: 21–34.

Galiè N, Beghetti M, Gatzoulis MA, Granton J, Berger RM, Lauer A, Chiossi E, Landzberg M, Bosentan Randomized Trial of Endothelin Antagonist Therapy-5 (BREATHE-5) Investigators (2006) Bosentan therapy in patients with Eisenmenger syndrome: a multicenter, double-blind, randomized, placebo-controlled study. Circulation 114: 48–54.

Galiè N, Seeger W, Naeije R, Simonneau G, Rubin LJ (2004) Comparative analysis of clinical trials and evidence-based treatment algorithm in pulmonary arterial hypertension. J Am Coll Cardiol 43: 81–88.

Gong LM, Du JB, Shi L, Shi Y, Tang CS (2004) Effects of endogenous carbon monoxide on collagen synthesis in pulmonary artery in rats under hypoxia. Life Sci. 74: 1225–1241.

Guignabert C, Raffestin B, Benferhat R, Raoul W, Zadigue P, Rideau D, Hamon M, Adnot S, Eddahibi S (2005) Serotonin transporter inhibition prevents and reverses monocrotaline-induced pulmonary hypertension in rats. Circulation 111: 2812–2819.

Hallioglu O, Dilber E, Celiker A (2003) Comparison of acute hemodynamic effect of aerosolized and intravenous iloprost in secondary pulmonary hypertension in children in children with congenital heart disease. Am J Cardiol 92: 1007–1008.

Heath D, Edwards JE (1958) The pathology of hypertensive pulmonary vascular disease. Circulation 18: 533–547.

Herget J, Novotna J, Bibova J,Povýšilová V, Vaňková M, Hampl V (2003) Metalloproteinase inhibition by Batimastat attenuates pulmonary hypertension in chronically hypoxic rats. Am J Physiol Lung Cell Mol Physiol 285: L199–L208.

Hoeper MM, Faulenbach C, Golpon H, Winkler J, Welte T, Niedermeyer J (2004) Combination therapy with bosentan and sildenafil in idiopathic pulmonary arterial hypertension. Eur Respir J 24: 1007–1010.

Humbert M (2008) Update in pulmonary arterial hypertension 2007. Am J Resp Crit Care Med 177: 574–579.

Jeffery TK, Morrell NW (2002) Molecular and Cellular Basis of Pulmonary Vascular Remodeling in Pulmonary Hypertension. Progress in Cardiovasc Dis 45: 173–202.

Lam CF, Peterson TE, Croatt AJ, Nath KA, Katusic ZS (2005) Functional Adaptation and Remodeling of Pulmonary Artery in Flow-Induced Pulmonary Hypertension. Am J Physiol Heart Circ Physiol. 289: H2334–2341.

Li XH, Du JB, Bu DF, Tang XY, Tang CS (2006) Sodium hydrosulfide alleviated pulmonary vascular structural remodeling induced by high pulmonary blood flow in rats. Acta Pharmacol Sin 27: 971–980.

Liu B, Zhou TP, Wang XM (2008) A differential study on four pulmonary vascular remodeling modes in four pulmonary arterial hypertesion animal models. Chin J Pathophysiol 24: 289–293.

Newman JH, Fanburg BL, Archer SL, Badesch DB, Barst RJ, Garcia JG, Kao PN, Knowles JA, Loyd JE, McGoon MD, Morse JH, Nichols WC, Rabinovitch M, Rodman DM, Stevens T, Tuder RM, Voelkel NF, Gail DB, National Heart, Lung and Blood Institute/Office of Rare Diseases (2004) Pulmonary arterial hypertension future directions: Report of a national heart, lung and blood institute/office of rare diseases workshop. Circulation 109: 2947–2952.

Nishimura T, Faul JL, Berry GJ, Vaszar LT, Qiu D, Pearl RG, Kao PN (2002) Simvastatin attenuates smooth muscle neointimal proliferation and pulmonary hypertension in rats. Am J Respir Crit Care Med 166: 1403–1408.

Nishimura T, Vaszar LT, Faul JL, Zhao G, Berry GJ, Shi L, Qiu D, Benson G, Pearl RG, Kao PN (2003) Simvastatin rescues rats from fatal pulmonary hypertension by inducing apoptosis of neointimal smooth muscle cells. Circulation 108: 1640–1645.

Okada K, Tanaka Y, Bernstein M, Zhang W, Patterson GA, Botney MD (1997) Pulmonary hemodynamics modify the rat pulmonary artery response to injury. A neointimal model of pulmonary hypertension. Am J Pathol 151: 1019–1025.

Rabinovitch M, Andrew M, Thom H, Trusler GA, Williams WG, Rowe RD, Olley PM (1987) Abnormal endothelial factor VIII associated with pulmonary hypertension and congenital heart defects. Circulation 76: 1043–1052.

Rabinovitch M, Haworth SG, Castaneda AR, Nadas AS, Reid LM (1978) Lung biopsy in congenital heart disease. Circulation 58: 1107–1112.

Rubin LJ (2002) Therapy of pulmonary hypertension: The evolution from vasodilators to antiproliferative agents. Am J Resp Crit Care Med 166: 1308–1309.

Simonneau G, Galiè N, Rubin LJ, Langleben D, Seeger W, Domenighetti G, Gibbs S, Lebrec D, Speich R, Beghetti M, Rich S, Fishman A (2004) Clinical classification of pulmonary hypertension. J Am Coll Cardiol 43: 5S–12S.

Singh TP, Rohit M, Grover A, Malhotra S, Vijayvergiya R (2006) A randomized, placebo-controlled, double-blind, crossover study to evaluate the efficacy of oral sildenafil therapy in severe pulmonary artery hypertension. Am Heart J 151: 851.e1–5.

Stenmark KR, Davie N, Frid M, Gerasimovskaya E, Das M (2006) Role of the adventitia in pulmonary vascular remodeling. Physiology 21: 134–145.

Waddell TK, Bennett L, Kennedy R, Todd TR, Keshavjee SH (2002) Heart-lung or lung transplantation for Eisenmenger syndrome. J Heart Lung Transplant 21: 731–737.

Zhao L, Zhou TP, Liu HM (2007) The effect of simvastatin on high pulmonary blood flow and monocrotaline-induced pulmonary arterial hypertension rat model. Journal of Sichuan University (Medicine) 38: 463-465.

Zhao SS, Zhou TP, Liu B (2007) The effect of simvastatin on pulmonary vascular remodeling in pulmonary aterial hypertension rat model. J Appl Clin Pediatr 22: 1001–1003.

Zhou AQ (1997) Cardiac catheterization-Diagnosis and treatment of congentital cardiac diseases. Shandong Science and Technology Press. pp. 93–202.