Int J Clin Exp Pathol 2015;8(10):12169-12176
www.ijcep.com /ISSN:1936-2625/IJCEP0010708

Original Article

Ruscogenin exerts beneficial effects on monocrotaline-induced pulmonary hypertension by inhibiting NF-κB expression

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Received August 9, 2015; Accepted August 10, 2015; Epub October 1, 2015; Published October 15, 2015

Abstract: This study aims to examine the effect of ruscogenin on pulmonary arterial hypertension (PAH) and to determine the mechanism underlying this effect. We isolated pulmonary vascular smooth muscle cells (PVSMCs) from the pulmonary artery of the rats; the PVSMCs were cultured *in vitro* and then were treated with platelet-derived growth factor (PDGF), PDGF + ruscogenin, or PDGF + ruscogenin + parthenolide. We randomized Sprague-Dawley rats into five groups as follows: control group, PAH group, low-dose group, medium-dose group, and high-dose group; the rats in the low-, medium-, and high-dose groups received the vehicle and ruscogenin 0.1, 0.4, and 0.7 mg/kg, respectively, from day 1 to day 21 after injection of monocrotaline (MCT). We measured the mean pulmonary arterial pressure (mPAP), right ventricular systolic pressure (RVSP), and medial wall thickness of the pulmonary artery (PAWT). We examined the levels of the nuclear factor kappa B (NF-κB) protein by using immunohistochemistry and western blot analysis, and the mRNA levels of NF-κB in PVSMCs were evaluated using real-time polymerase chain reaction (PCR). The mPAP, RVSP, and PAWT and the protein and mRNA levels of NF-κB were significantly higher in the PAH model group than in the control group ($P < 0.05$). Ruscogenin induced a significant dose-dependent decrease in the mPAP, RVSP, and PAWT and in the NF-κB expression in the PAH group ($P < 0.05$), which suggests that ruscogenin will also exert dose-dependent effects on MCT-induced PAH through the inhibition of NF-κB.

Keywords: Ruscogenin, pulmonary hypertension, expression, nuclear factor-kappa B, monocrotaline

Introduction

Pulmonary arterial hypertension (PAH) is a serious and progressive disease that can be triggered by many cardiopulmonary or other diseases [1]. This disease is characterized by vascular remodeling, which leads to an increase in pulmonary vascular resistance and eventually to right heart failure [2]. The key mechanism underlying vascular remodeling is the proliferation and migration of vascular smooth muscle cells (VSMCs) and with some cytokines [3]. Nuclear factor kappa B (NF-κB) is associated with inflammatory reaction, immunological response, and proliferation and apoptosis of eukaryotic cells [4]. The NF-κB inhibitor pyrrolidinedithiocarbamate decreased the expression of vascular cell adhesion molecule-1 (VCAM-1) and the infiltration of macrophages and ameliorated pulmonary hypertension in a rat model of monocrotaline-induced PAH [5], which suggests that the activation of NF-κB may be associated with the development of PAH. A previous study has shown that the expression of NF-κB is increased in the lung tissue of rats with chronic hypoxia [6], which indicates a potential role of NF-κB in the pathogenesis of hypoxic pulmonary hypertension. Although the morbidity and mortality in patients with PAH has decreased with effective pharmacological treatment, to date, a complete cure has not been developed for this devastating disease.

Ruscogenin (RUS) (**Figure 1**), first isolated from *Ruscus aculeatus*, is a major steroidal sapogenin of the Chinese herb Radix *Ophiopogon japonicus*. Ruscogenin exerts significant antithrombotic activity and anti-inflammatory activi-

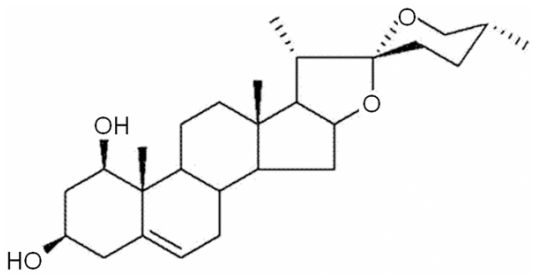

Figure 1. Chemical structure of ruscogenin.

ties such as inhibition of leukocyte adhesion and migration, improvement of liver injury, and inhibition of lipopolysaccharide (LPS)-induced acute lung injury [7-10]. Previous studies have shown that the endothelial-protective effects and the possible anti-inflammatory mechanism of ruscogenin is associated with the suppression of intercellular adhesion molecule-1 (ICAM-1) expression in endothelial cells mainly through the inhibition of the NF-κB signaling pathway [11-12]. A recent study showed that ruscogenin significantly attenuates LPS-induced acute lung injury by inhibiting the expression of tissue factor (TF) and inducible nitric oxide synthase (iNOS) and activating of NF-κB p65, which indicates that ruscogenin may be used as a potential therapeutic agent for acute lung injury (ALI) or sepsis [7].

The effects of ruscogenin on PAH remain to be clarified. Therefore, in the present study, we established a rat model of MCT-induced PAH, and we administered ruscogenin and determined the mRNA and protein levels of NF-κB to investigate whether ruscogenin exerts its effects via the NF-κB signaling pathway.

Materials and methods

Cell culture

The pulmonary artery of the rat was identified and pulmonary vascular smooth muscle cells (PVSMCs) were isolated, which were cultured in M199 media supplemented with 10% fetal bovine serum for 48 h. Then, the rat PVSMCs were divided into four groups as follows: Group 1, the control group that received no treatment; Group 2, stimuli group that received only platelet-derived growth factor (PDGF, 10 ng/mL) for 12 h; Group 3, intervention group that received PDGF (10 ng/mL) and ruscogenin (0.1, 1, or 10 μg/mL) for 12 h; and Group 4, double intervention group that received PDGF (10 ng/mL) and ruscogenin (0.1, 1, or 10 μg/mL), but received the third disposal preparation, an NF-κB inhibitor parthenolide (20 μmol/L) for 12 h.

Animal experiments

All experiments were performed using male Sprague-Dawley rats weighing between 180 and 220 g and fed freely with a standard rat diet and water. The Experimental Animal Center of Nanjing Medical University (Nanjing China) provided 50 rats, which were randomly divided into three groups: control group (n = 10), model group (n = 10), and treatment group (n = 30). Ruscogenin was isolated from the tubers of Ophiopogon japonicus by successive chromatographic steps, and the purity of the sample obtained analyzed using high-performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD) was 98.6% [9]. The control group received normal saline (NS) at a volume equivalent to that of ruscogenin and the PAH model group received a single intraperitoneal injection of MCT (dissolved in 1 N HCL buffered to pH 7.0 with 1 N NaOH) at a dose of 60 mg/kg body weight [13] followed by the solvent from day 1 to day 21; the low-dose, medium-dose, and high-dose groups received oral administration of ruscogenin 0.1, 0.4, and 0.7 mg/kg (dissolved in distilled water with 5% ethanol), respectively, from day 1 to day 21 after MCT injection. All the doses of the drugs were selected on the basis of the results of previous studies [10]. This study was performed 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 was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of Nanjing Medical University.

Hemodynamic studies

On day 22, the rats were weighed and were anesthetized with an intraperitoneal injection of urethane (1.0 g/kg). According to our previous method [14], a polyethylene catheter (PE 10; 427400, Becton Dickinson, USA) and heparin-saline (125 U/ml) were inserted into the pulmonary artery through the right ventricle from the right jugular vein. Then, the catheter was connected to the MPA Acquisition and Analysis

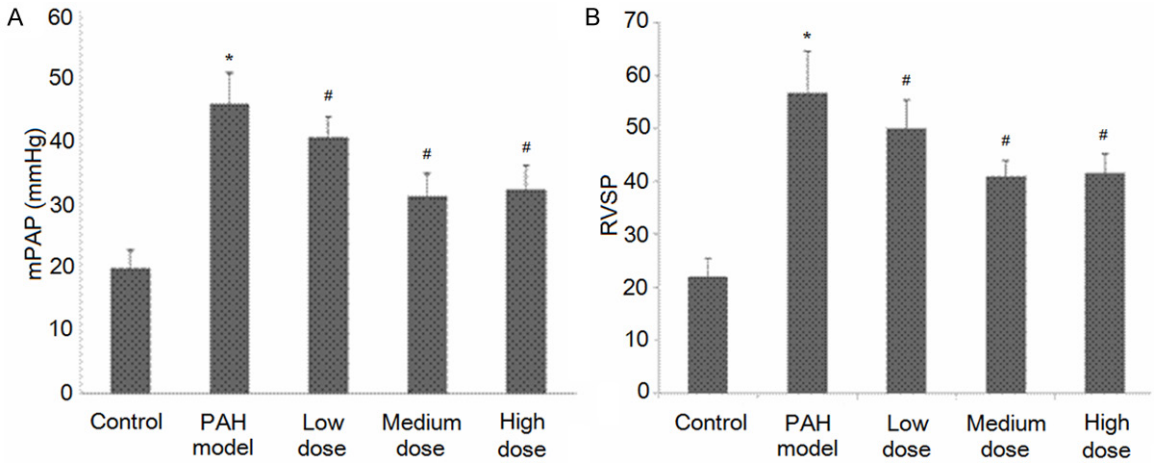

Figure 2. Effect of ruscogenin on hemodynamics in MCT treated rats. (A) mPAP (B) RVSP Results are given as mean ± SD (n = 10). $^{\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MCT group (× 400).

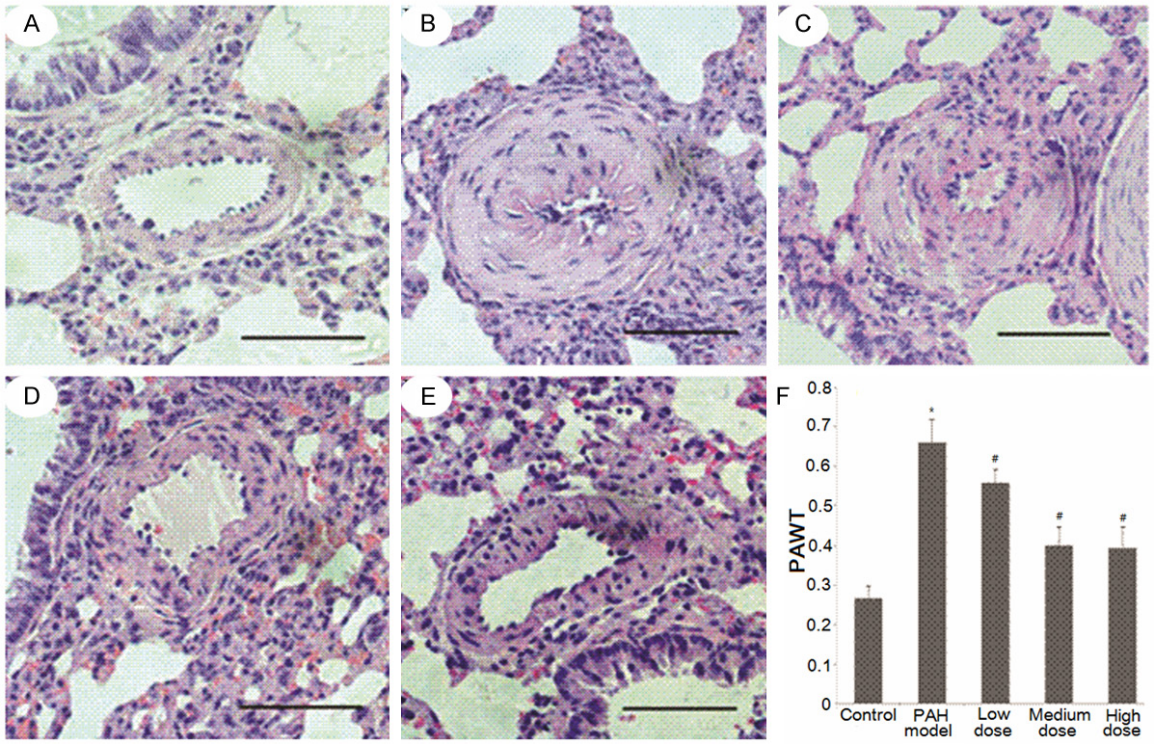

Figure 3. Medial wall thickness in muscular pulmonary arteries in each group. (Scale bar: 50 μm, H&E staining). A. Control group. B. MCT group. C. MCT + ruscogenin (0.1 mg/kg) group. D. MCT + ruscogenin (0.4 mg/kg) group. E. MCT + ruscogenin (0.7mg/kg) group. F. Morphology analyses were performed on pulmonary arteries with outer diameters of 25-200 μm. Results are given as mean ± SD (n = 10). $^{\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MCT group (× 200).

System (Goleta, CA, USA) by using a pressure transducer to record the right ventricular systolic pressure (RVSP) and the mean pulmonary artery pressure (mPAP) by using a multiparameter monitor PM-8000.

Morphometric analysis of the pulmonary arteries

The thorax was opened and the lung tissue was fixed in formalin for histological evaluation or

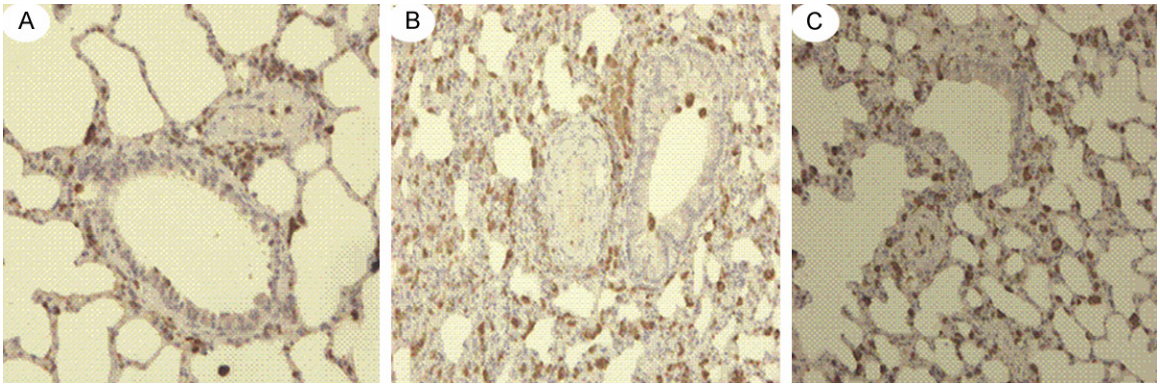

Figure 4. Immunohistochemical analysis of pulmonary artery in each group. Brown indicated positive staining for NF-κB (magnification, × 200). A. Control group. B. MCT group. C. MCT + ruscogenin (0.4 mg/kg) group.

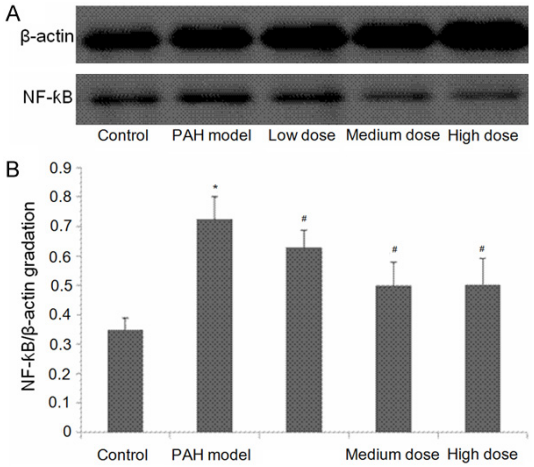

Figure 5. Western blot analysis of NF-κB expression in each group. The band intensities were assessed by scanning densitometry. A. Blots were normalized to α-Tublin or p65 expression. B. All data presented are the mean ± SD of 8 rats. $^{\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. PAH model group (n = 10 for each group).

were transferred to an Eppendorf tube and then frozen in liquid nitrogen for western blot analysis and real-time polymerase chain reaction (PCR) after hemodynamic measurements. We stained a 4-μm section of the fixed lung tissue with hematoxylin and eosin (H&E) and captured the photomicrographs. The external diameter and the medial wall thickness were measured in 20 pulmonary arteries ranging in the size from < 50 μm and 51-200 μm per lung section. We analyzed at least six vessels in each rat. We calculated the medial wall thickness and medial wall areas of each pulmonary artery as follows: % wall thickness = [(external diameter-internal diameter)/external diameter] × 100 and % wall area = [(total area-internal area)/total area] × 100. A blinded observer measured all vessels with perceptible media by using Image-Pro Plus 6.0 [15, 16].

Immunohistochemical analysis

Sections were incubated with mouse NF-κB p65 antibody (Beyotime Institute of Biotechnology, Shanghai, China) after the standard processes of immunohistochemical analysis, such as dipping in wax and dewaxing were performed; then, the sections were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Beyotime Institute of Biotechnology, Shanghai, China) at 37°C for 20 min followed by color development with diaminobenzidine (DAB) and counterstaining. Finally, the sections were visualized by incubating with the DAB solution and by weakly counter-staining with hematoxylin. The positive cells were counted in five random high power fields under a light microscope (× 400) and each group randomly selected five sections.

Western blot analysis

Cytoplasmic and nuclear proteins were extracted from the frozen lung tissue using the bicinchoninic acid (BCA) protein kit (Pierce) according to the manufacturer's protocol. Total protein (30 μg/lane) was separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), incubated with specific antibodies against NF-κB/p65 and

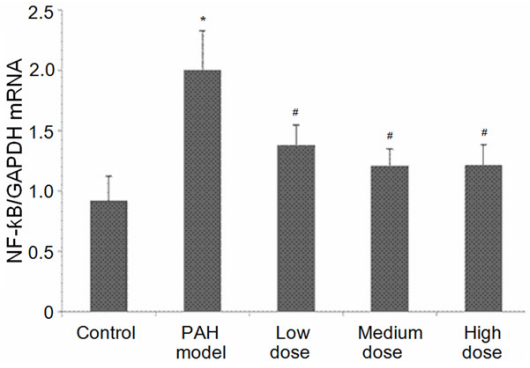

Figure 6. Ruscogenin decreased NF-κB mRNA level. RT-PCR analysis of NF-κB mRNA level in each group. Rusults were means ± SD from five independent experiments. $^{\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. PAH model group (n = 10 for each group).

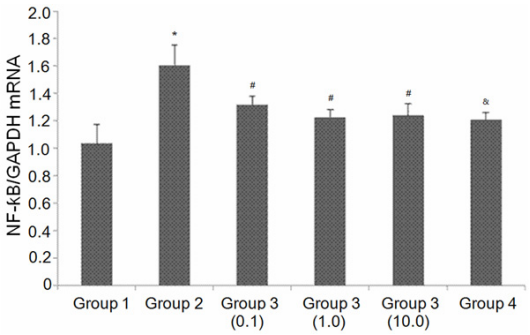

Figure 7. Ruscogenin inhibits NF-κB expression in cultured vascular smooth muscle cells. NF-κB mRNA level in PVSMCs was evaluated by RT-PCR. Group 1 normal cells without any stimuli, group 2 cells stimulated by PDGF (10 ng/ml), group 3 cells preincubated with ruscogenin (0.1 μg/ml or 1 μg/ml or 10 μg/ml) before stimulated by PDGF (10 ng/ml), group 4 cells preincubated with NF-κB inhibitor parthenolide (20 μmol/l) before stimulated by PDGF (10 ng/ml). Results were means ± SD form four independent experiments.

GAPDH at 4°C overnight, and then were incubated with secondary antibody of mouse IgG for 1 h after washing twice with TBST. The blots were detected using Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, USA) according to the manufacturer's protocol. The resulting images were analyzed using a NIH Image software.

RT-PCR

Total RNA was extracted from the frozen pulmonary tissue or cultured cells by using Trizol according to the manufacturer's instructions (Invitrogen, USA). Reverse transcription polymerase chain reaction (RT-RCR) was performed according to the instructions in the Takara RNA PCR Kit 3.0 (Cambridge, MA, USA). Semiquantified beta-actin mRNA was used as an internal standard. cDNA was synthesized, and the primers used for RT-PCR were as follows: 5'-TCTGGCGCAGAAGTTAGGT-3', and 5'-CCAGAGACCTCATAGTTGT-3'. DNA amplification was performed using a thermocycler under the following conditions: for NF-κB p65, 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 60 s, and extension at 72°C for 60 s; for β-actin, 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 60 s. RT-PCR products were measured by photodensitometry using a gel image analysis system after agarose gel electrophoresis and ethidium bromide staining.

Statistical analysis

All the measurement data were described as mean ± standard deviation (SD). The comparison between 2 groups was analyzed using unpaired Student's *t*-test, and two-way analysis of variance (ANOVA) was used to analyze the difference between more than 2 groups. $P < 0.05$ was considered statistically significant, and all the statistical analyses were performed using SPSS11.5 (SPSS Inc. Chicago, USA).

Results

Ruscogenin treatment improved hemodynamics in a rat model of MCT-induced PAH

On day 22, the mPAP and RVSP were significantly higher in the MCT-treated group than in the control group (mPAP: 41.38 ± 4.72 mmHg vs. 19.13 ± 2.75 mmHg, $P < 0.01$; RVSP: 58.88 ± 7.57 mmHg vs. 24.88 ± 4.49 mmHg, $P < 0.01$) (**Figure 2A**, **2B**), which resulted in pulmonary hypertension and RV dysfunction. However, the mPAP decreased in rats treated with ruscogenin at the doses of 0.4 mg/kg (28.88 ± 4.22 mmHg, $P < 0.01$ vs. MCT group) and 0.7 mg/kg (30.13 ± 7.08, $P < 0.01$) (**Figure 2A**). In addition, the RVSP decreased after treatment with ruscogenin at the doses of 0.1 mg/kg (51 ± 6.52 mmHg, $P < 0.05$ vs. MCT group), 0.4 mg/kg (40.13 ± 6.33 mmHg, $P < 0.01$), and 0.7 mg/kg (42.25 ± 7.72, $P < 0.01$) (**Figure 2B**).

Ruscogenin treatment prevented vascular remodeling in a rat model of MCT-induced PAH

Control group (**Figure 3A**), MCT group (**Figure 3B**), MCT + ruscogenin (0.1 mg/kg) group (**Figure 3C**), MCT + ruscogenin (0.4 mg/kg) group (**Figure 3D**), MCT + ruscogenin (0.7 mg/kg) group (**Figure 3E**), and PAWT analysis (**Figure 3F**). We examined the morphology of small pulmonary arteries and measured the relative wall thickness of the pulmonary artery (PAWT). MCT induced severe thickening of the walls of the pulmonary arteries; the PAWT was significantly higher in the PAH model group than in the control group (0.66 ± 0.09 for MCT vs. 0.27 ± 0.05 for control, $P < 0.01$) (**Figure 3F**), and the lumen appeared stenosed or occluded. However, treatment with ruscogenin reversed these pathological changes (0.57 ± 0.08 for RUS 0.1 mg/kg, $P < 0.05$ vs. MCT group; 0.41 ± 0.08 for RUS 0.4 mg/kg, $P < 0.01$; and 0.39 ± 0.07 for RUS 0.7 mg/kg, $P < 0.01$) (**Figure 3F**). Scale bar: 50 μm, H&E staining).

Ruscogenin inhibits NF-κB expression in the pulmonary tissue of a rat model of PAH

The expression of NF-κB in the pulmonary tissue measured using immunohistochemistry in the control groups (**Figure 4A**), model group (**Figure 4B**), and ruscogenin (0.4 mg/kg) group (**Figure 4C**) suggested that the diameter of the pulmonary artery in the model group was greater than that in the control group, but the arterial diameter markedly decreased after ruscogenin treatment.

Western blotting analysis in different groups (**Figure 5A**) showed that the expression level of NF-κB protein in the PAH model group was significantly higher than that in the control group ($P < 0.05$) (**Figure 5B**), but the NF-κB expressed decreased significantly in the medium-dose and high-dose groups ($P < 0.05$) (**Figure 5B**).

The results of expression of mRNA of NF-κB were consistent with those of the protein, which was higher in the PAH model group than in the control group (2.71 ± 0.21 vs. 1.00 ± 0.07, $P < 0.05$), and NF-κB mRNA levels decreased after treatment with medium-dose and high-dose ruscogenin (2.71 ± 0.21 vs. 1.06 ± 0.33, $P < 0.05$) (**Figure 6**), which suggests that the expression of NF-κB mRNA was inhibited with ruscogenin in a rat model of PAH (**Figure 6**).

Ruscogenin inhibits NF-κB expression in cultured PVSMCs

Previous *in vitro* studies clarified the inhibitory effects of simvastatin on NF-κB expression in PVSMCs. Results of real-time quantitative PCR showed that the mRNA level of NF-κB increased in rat PVSMCs at 12 h after treatment with PDGF (10 μg/mL) (Group 2 vs. Group 1, 1.50 ± 0.003 vs. 1.06 ± 0.003); treatment with ruscogenin (0.1, 1, or 10 μg/mL) (Group 3 vs. Group 2, 0.95 ± 0.002 vs. 1.50 ± 0.003) decreased the NF-κB mRNA levels, which was similar to that observed with preincubation with the positive control parthenolide, an NF-κB inhibitor, at a concentration of 20 μmol/L, which significantly inhibited PDGF-induced NF-κB expression (Group 4 vs. Group 2, 0.76 ± 0.008 vs. 1.50 ± 0.003) (**Figure 7**). Thus, our results indicate the inhibitory effects of ruscogenin on NF-κB expression in cultured PVSMCs.

Discussion

Various animal models have been used to characterize the pathophysiology of PAH and to test novel therapeutic strategies; injection of MCT and exposure to hypoxic conditions are used most frequently to establish these animal models. The MCT model was introduced more than 40 years ago [17]; while MCT is not intrinsically toxic, it is activated to the reactive MCT pyrrole, the initial dehydrogenation product of MCT, by hepatic cytochrome p450 3A [18]. MCT exposure causes endothelial cell injury followed by a massive infiltration of mononuclear cells into the perivascular regions of arterioles and muscular arteries [19], which plays a role in the pathogenesis of PAH in humans [20].

Kou *et al.* showed that ruscogenin induces a significant improvement in the prognosis of MCT-induced infection [10]; treatment with ruscogenin at a of dose 0.7 mg/kg increased the survival rates at 21 days from 67% to 100% and at 42 days from 0% to 50%. To confirm the beneficial effect and determine the possible mechanism underlying this effect, we designed an experiment and showed that preventive ruscogenin administration was effective in attenuating pulmonary vascular remodeling, development of pulmonary hypertension, and improv-

ing right ventricular dysfunction in MCT-treated rats. The results of hemodynamic and histological studies confirmed these effects. The protective effects of ruscogenin appeared from the dose of 0.1 mg/kg and became more apparent at the dose of 0.4 mg/kg; a further increase in the dose of ruscogenin (0.7 mg/kg) did not increase the effect (**Figure 2**). On the basis of these results, we selected the dose of 0.4 mg/kg of ruscogenin for some parts of our experiment.

Under normal conditions, NF-κB is bound to inhibitory factor (IκB) that prevents the transcription of NF-κB. Many factors induce the dissociation of NF-κB from IκB and lead to gene transcription change by the translocation of the active forms of NF-κB p50 and p65 to the nucleus [4]. NF-κB plays an important role in various pathophysiological process, including inflammatory reaction, immunological response, cell proliferation, and apoptosis. NF-κB levels are higher in rat models of PAH, which is related to the pathogenesis of hypoxic pulmonary hypertension [5]. Moreover, the results from our *in vivo* experiments showed that the protein and mRNA levels of NF-κB p65 were higher in the PAH model group than in the control group. The results of our *in vitro* experiments were consistent with the results above, which showed that PDGF stimulation induced an increase in the NF-κB mRNA levels in the cultured vascular smooth muscle cells. Our results indicate that the development of PAH is associated with an upregulation of NF-κB. Ruscogenin suppresses the activation of NF-κB p65 *in vivo* and *in vitro* [7, 11, 12]. The expression of NF-κB p65 (Ser 536) in the lung tissue and in the PVSMCs (**Figure 5B**) significantly decreased by treatment with ruscogenin, which might also be the possible mechanism for downregulation of inflammatory responses in a rat model of MCT-induced PAH. NF-κB activation regulates the proliferation of pulmonary artery smooth muscle cell (PASMCs) *in vitro* [21]. Further studies are required to determine whether ruscogenin directly regulates the NF-κB signaling pathway in PASMCs. Our results indicate that similar to ruscogenin, other therapeutic agents inhibiting the NK-κB pathway may be developed for the treatment of PAH.

In conclusion, our results show that NF-κB plays an important role in the development of PAH, and ruscogenin exerts beneficial effects on PAH by inhibiting the expression of NF-κB.

Acknowledgements

The study was supported by the Funds of the National Natural Science Foundation of China (WPX) under Contract No. 8127357 and (HW) under Contract No. 30971319.

Disclosure of conflict of interest

None.

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