

# Resveratrol Attenuates Monocrotaline-Induced Pulmonary Hypertension in Rats

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## ABSTRACT

**Background and Objectives :** Resveratrol (RVT) is a polyphenolic phytoalexin, and it has been demonstrated to be capable of protecting against cardiovascular disease. The aim of this study was to identify whether RVT might protect against monocrotaline (MCT)-induced pulmonary hypertension and whether vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) are involved in the beneficial effects. **Materials and Methods :** Thirty Sprague Dawley rats were divided into three groups: the control (n=6), the MCT (n=12) and the MCT with RVT (5 mg/kg/day, n=12) groups. After 28 days, the tissue samples were obtained for morphometric analysis and Western blotting. **Results :** In the MCT group, the right ventricle/(left ventricle+septum) weight ratio was significantly increased compared with that of the control group ( $0.51 \pm 0.07$  vs.  $0.20 \pm 0.03$ ,  $p < 0.01$ ), which was markedly suppressed in the RVT treated group ( $0.35 \pm 0.08$ ,  $p < 0.01$ ). Histological analysis also showed that MCT treatment increased the medial wall thickness of the pulmonary arterioles compared with that of the control group ( $36 \pm 8\%$  vs.  $17 \pm 5\%$ ,  $p < 0.01$ ), which also was significantly suppressed in the RVT treated group ( $27 \pm 5\%$ ,  $p < 0.01$ ). In addition, Western blot demonstrated the decreased expression of VEGF in the MCT group ( $p < 0.01$ ), which was upregulated after long term RVT treatment ( $p < 0.01$ ). The expression of eNOS was increased after MCT treatment ( $p < 0.01$ ), but upregulation of eNOS could not be reversed by the RVT treatment. The expression of iNOS was not significantly modulated. **Conclusion :** These results suggest that RVT attenuates MCT-induced pulmonary hypertension and it may represent a new strategy for the treatment of pulmonary hypertension. (Korean Circulation J 2006;36:683-687)

**KEY WORDS :** Resveratrol ; Vascular endothelial growth factor ; Endothelial nitric oxide synthase ; Pulmonary hypertension.

## Introduction

Pulmonary arterial hypertension (PAH) is a serious disease that's characterized by progressive increases in the pulmonary arterial pressure and it ultimately leads right heart failure.<sup>1)</sup> Increased vascular tone and chronic vascular remodeling are assumed to be the underlining pathogenic mechanisms. The majority of patients with PAH show a grave prognosis with a mean survival of approximately 3 years from the time of diagnosis.<sup>2-4)</sup> Although there is no curative treatment for PAH, the newer medical therapies have been shown to improve

survival, exercise tolerance, the hemodynamics, the echocardiographic parameters and the quality of life measures.<sup>5)</sup> During the past 2 decades, both survival and quality of life have improved for patients with PAH because of the introduction of therapies such as intravenous prostacyclin, endothelin receptor blockers and phosphodiesterase inhibitors.<sup>5)</sup> New studies have recently been performed to develop treatments based on the pathogenesis of PAH.<sup>6)</sup>

Resveratrol (RVT) is a natural polyphenolic phytoalexin in red wine, and it exerts a wide variety of beneficial biological effects, and especially cardiovascular protection.<sup>7)</sup> RVT also has been demonstrated to be capable of protecting against cardiovascular disease because of its antioxidant and anti-inflammatory effects.<sup>8)</sup> RVT has recently been found to protect the heart in a nitric oxide (NO) dependent manner and to inhibit vascular remodeling.<sup>9)10)</sup> RVT also upregulates vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS).<sup>11)</sup>

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VEGF is a potent endothelial cell mitogen with angiogenic, survival and differentiation effects.<sup>12)13)</sup> Moreover, several studies have revealed that the over-expression or gene transfer of VEGF effectively prevents the development of pulmonary hypertension.<sup>14)15)</sup>

In this study, we hypothesized that RVT treatment might attenuate pulmonary hypertension via the up-regulation of VEGF, iNOS and/or eNOS in monocrotaline (MCT)-induced pulmonary hypertension.

## Materials and Methods

### Experimental protocol

Thirty male Sprague Dawley rats (250-350 g) were randomly divided into three groups: group 1 was injected with distilled water (the control group, n=6), group 2 was treated with MCT (the MCT group, MCT was injected at a single dose of 60 mg/kg, sc, n=12), and group 3 was MCT treated rats (at the same dose and schedule as above) that were also treated with RVT (the RVT group, 5 mg/kg/day ip, daily; Sigma, St. Louis, MO, USA) for 28 days from the onset of MCT treatment (n=12). The rats in all three groups were kept in the same room and subjected to the same light-dark cycle. After 28 days, the tissue samples were obtained for morphometric analysis and Western blotting. The experimental procedures used in this study were reviewed and approved by the Animal Care and Use Committee of Dongguk University. The animal care and use were in accordance with the guidelines of the National Institutes of Health (Bethesda, MD).

### Assessment of right ventricular hypertrophy

After 4 weeks, the rats were euthanized by anesthetic overdose and then the right ventricle (RV) free wall was dissected from the left ventricle (LV) and septum (S), and this was weighed separately on an analytic scale. The RV remodeling was assessed by the RV-to-LV plus S weight ratio.

### Assessment of pulmonary artery remodeling

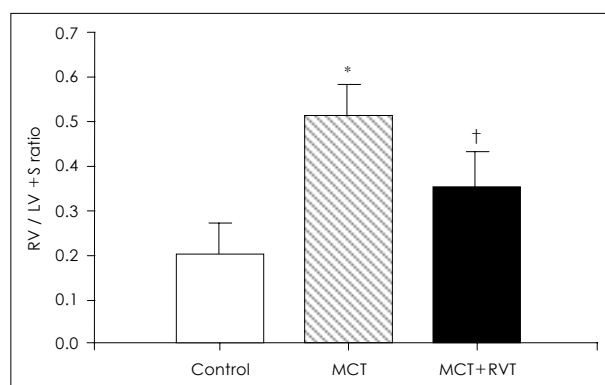
For analyzing the remodeling of the small pulmonary arterioles, the left lung was fixed with a transcardiac infusion of 4% paraformaldehyde. The perfused left lung was removed and then paraffin-embedded. Serial coronal sections 5  $\mu$ m thick were obtained at the left lower zone. Following deparaffinization, the sections were stained with the hematoxylin-eosin. The wall thickness (WT) of the pulmonary arteriole was measured with the size being 50-100  $\mu$ m. The WT ratio, which is an index of medial wall hypertrophy, was determined from the average data of 10 to 15 fields per slice and it was calculated as below:  $[WT\% = (\text{external diameter} - \text{internal diameter}) / \text{external diameter} \times 100]$ .

### Western blot analysis

The left lung was removed and then snap-frozen at -70°C for Western blotting analysis. The tissue samples were homogenized in ten volumes of homogenizing buffer (0.32 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2 containing 8.5 mM leupeptin and 1 mM phenylmethylsulfonyl fluoride), for 10 s with polytron. The aliquots were stored at -70°C. Samples of homogenate were run on 7.5% polyacrylamide mini gels (Bio-rad Mini Protean). For each gel, an identical gel was run in parallel and then subjected to Coomassie staining to assure identical loading. After electrophoresis, the protein was transferred to nitrocellulose paper for 2 hours at 400 mA and 120 V in a Bio-Rad trans-blot system. After transfer, the protein bands were identified by Ponceaus S and they were destained with distilled water. The nitrocellulose sheets were washed in PBST and incubated with mouse anti-eNOS (Transduction Laboratories, Lexington, CA, USA), rabbit anti-iNOS (Transduction Laboratories, Lexington, CA, USA), and rabbit anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for overnight at 4°C. The labeling was visualized with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Little Chalfont, UK). The immunoblots' signals were developed by the ECL system and this was quantified with using Scion Image software (version 1.59).

### Statistical analysis

All data was presented as means  $\pm$  SDs and it was analyzed by ANOVA with using the procedures in the SPSS program (SPSS Inc). Statistical hypotheses were considered significant if  $p < 0.05$ .



**Fig. 1.** Ratio of the right ventricle (RV) to left ventricle (LV) plus septum (S) weight in the control, the monocrotaline (MCT)-induced pulmonary hypertension and the MCT treated with resveratrol (RVT). The RV/LV+S ratio was decreased after RVT treatment. The values are expressed as a mean  $\pm$  SD. \*: significantly different from the control group ( $p < 0.01$ ), †: significantly different from the MCT group ( $p < 0.01$ ).

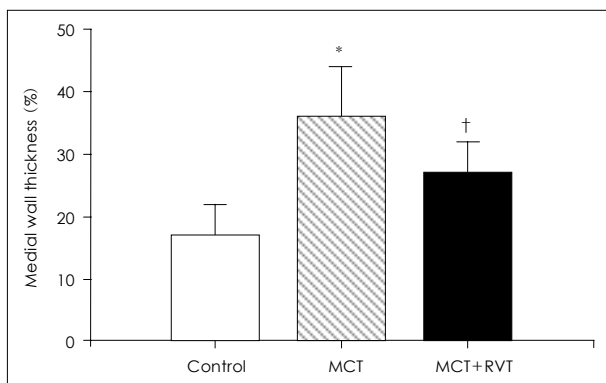
## Results

### Effects of RVT on right ventricular hypertrophy

In the MCT group, right ventricular hypertrophy developed and significant increases in the RV/LV+S ratio were noted compared with that of the controls ( $0.51 \pm 0.07$  vs.  $0.20 \pm 0.03$ , respectively,  $p < 0.01$ ), and this was markedly suppressed in the RVT treatment group ( $0.35 \pm 0.08$ ,  $p < 0.01$ ) (Fig. 1). However, RVT treatment did not suppress RV hypertrophy to the normal values.

### Effects of RVT on pulmonary artery remodeling

Histological analysis also showed that MCT treatment increased the medial wall thickness of the pulmo-



**Fig. 2.** Medial wall thickness of the pulmonary arterioles. Treatment with resveratrol (RVT) attenuated the medial wall thickness in the mon-octaline (MCT) treated rats. The values are expressed as a mean  $\pm$  SD. \*: significantly different from the control group ( $p < 0.01$ ), †: significantly different from the MCT group ( $p < 0.01$ ).

nary arterioles compared with that of the control ( $36 \pm 8\%$  vs.  $17 \pm 5\%$  respectively,  $p < 0.01$ ), and this was also significantly suppressed in the RVT treated group ( $27 \pm 5\%$ ,  $p < 0.01$ ) (Fig. 2). However, this treatment did not completely reverse small pulmonary arteriole remodeling.

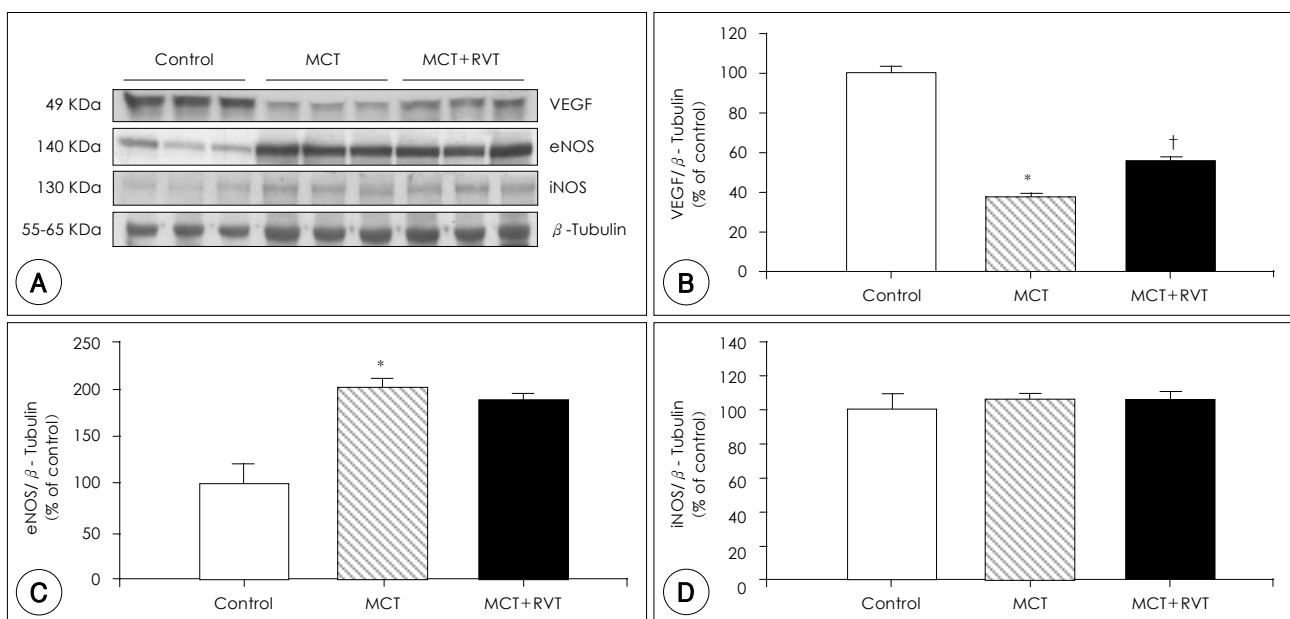
### Western blot analysis

Western blot analysis demonstrated that the expression of VEGF in the MCT group was significantly decreased by  $-62.5\%$  compared with that of the control group ( $p < 0.01$ ). However, the expression of VEGF was significantly upregulated after treatment with RVT by  $49\%$  compared with that of the MCT group ( $p < 0.01$ ) (Fig. 3A, B). The expression of eNOS was also significantly increased by  $100\%$  after MCT treatment ( $p < 0.01$ ). However, upregulation of the eNOS expression was not reversed by the RVT treatment (Fig. 3A, C). There was no significant change in the iNOS expression between the three groups (Fig. 3A, D).

## Discussion

In the present study, we demonstrated that RVT was able to attenuate the MCT-induced pulmonary hypertension and RV hypertrophy in rats, and this was partly associated with upregulation of VEGF.

RVT is a polyphenol in red wine, and it has been proved to be a major constituent that's responsible for the cardiovascular benefits associated with a moderate amount of wine consumption.<sup>7)</sup> Most of the studies



**Fig. 3.** Western blot analysis. The decreased vascular endothelial growth factor (VEGF) expression in monocrotaline (MCT) rats was upregulated after treatment with resveratrol (RVT) (A, B). The expression of endothelial nitric oxide synthase (eNOS) was increased after MCT treatment, but upregulation of eNOS could not be reversed by the RVT treatment (A, C). The expression of inducible nitric oxide synthase (iNOS) was not significantly changed between the control, the MCT treatment and the MCT with RVT treatment groups (A, D). Values are expressed as a mean  $\pm$  SD. \*: significantly different from the control ( $p < 0.01$ ), †: significantly different from the MCT group ( $p < 0.01$ ).

have focused on the beneficial effects of RVT in the prevention of atherosclerosis and coronary heart disease, but there has been no much consideration given for its possible use as a therapeutic agent for treating PAH. Our study showed that RVT effectively attenuates the MCT-induced RV hypertrophy and pulmonary hypertension that's caused by small pulmonary arteriole remodeling. The mechanisms of the beneficial effects are diverse. RVT interferes with the release of inflammatory mediators activated by polymorphonuclear leukocytes and the inflammatory cytokine released from alveolar macrophages in patients with chronic obstructive pulmonary disease.<sup>5)</sup> RVT also inhibits, in a dose-dependent manner, smooth muscle cell proliferation by inducing an increase in apoptosis.<sup>10)</sup> Recently, RVT has recently been shown to aid in cardioprotection by the enhanced expressions for iNOS, eNOS, VEGF and the kinase insert domain receptor (KDR).<sup>9)</sup>

VEGF is a pluripotent growth and permeability factor that has a broad impact on endothelial cell function and it is capable of stimulating NO release from the vascular endothelium and increasing the local eNOS expression.<sup>14)15)</sup> It also causes potent NO-dependent vasodilation and preserves endothelium-dependent vasodilation, although the role of VEGF-mediated vasodilation in pulmonary hypertension has not been fully evaluated.<sup>15)</sup> VEGF is strongly expressed in the plexiform lesions of pulmonary tissue in the patients suffering with severe primary and secondary pulmonary hypertension and various forms of congenital heart diseases, and also in persistent pulmonary hypertension of the newborn.<sup>16)</sup> The VEGF gene and protein expression are upregulated in the RV tissue after animals were subjected to chronic hypoxic exposure.<sup>13)17)</sup> An increased VEGF expression in lung tissue may be desirable not only to promote the development of new pulmonary vessels, but also to improve endothelial function.<sup>18)19)</sup> In contrast, the VEGF gene expression appeared to be decreased in the MCT-induced rat model of pulmonary hypertension.<sup>17)</sup> Our study also revealed that the decreased expression of VEGF after MCT treatment was partly reversed after RVT treatment. These results suggest the possibility that the protective effect of RVT is partly due to the increased expression of VEGF. We did not attempt to specifically address the mechanism of the beneficial role of VEGF in this model. However, the direct actions of VEGF, including vasodilation through NO release and VEGF acting as an endothelial survival factor by inhibiting MCT-induced endothelial cell apoptosis, may contribute to the development of MCT-induced pulmonary hypertension. It may also inhibit smooth muscle cell proliferation.<sup>14)15)</sup> The mitogenic effects of VEGF enhanced endothelial regrowth and recovery. The angiogenic effects may contribute to pulmonary microvascular regeneration in the affected lungs to reduce vascular

resistance.<sup>14)</sup>

The role of NOS in pulmonary vascular disease has been contradictory. Giaid and Saleh reported the decreased expression of eNOS in patients with pulmonary hypertension and they also reported its absence in plexiform lesions,<sup>20)</sup> whereas Mason and coworkers demonstrated a high expression of eNOS in plexiform lesions.<sup>21)</sup> The enhanced expressions of eNOS were found in several experimental models of pulmonary hypertension and in patients with pulmonary hypertension.<sup>22)23)</sup> In this study, the expression of eNOS was not reversed after RVT treatment although the eNOS expression was increased in a MCT models, like that seen in a previous report.<sup>22)</sup> Therefore, the role of the increased expression of eNOS in MCT-induced pulmonary hypertension in the present study is still undetermined. However, several reports suggest that the increased level of eNOS represents a potential source of NO production and enhanced endothelium-dependent arterial dilatation, but the bioavailability of NO in the lung was compromised significantly.<sup>22)</sup> The unaltered eNOS expression after RVT treatment may be partly due to the improper dosage of RVT. The possible role of iNOS in PAH is unclear. In a MCT-induced pulmonary hypertension model, the iNOS mRNA expression in rat heart was increased.<sup>22)</sup> Yet Hampl, et al.<sup>24)</sup> revealed that the transient iNOS induction in the pulmonary vascular walls at the beginning of chronic hypoxia participated in the pathogenesis of pulmonary hypertension. However, Hoehn, et al.<sup>23)</sup> reported no changes of iNOS in rapid progressive pulmonary hypertension of the newborn. Our present study also demonstrates that the changes of iNOS expression were negligible, the same as previous reports.<sup>24)</sup> Therefore, the role of iNOS in chronic PAH is probably limited to the pathogenesis of pulmonary hypertension. Taken together, RVT treatment can not modulate the compensatory expression of NOS. This means that the protective role of RVT was not significantly dependent on the expression of NOS isoforms.

In conclusion, RVT attenuates RV hypertrophy and vascular remodeling in MCT-induced pulmonary hypertension, although RVT did not induce complete reversal of the RV hypertrophy and vascular remodeling. In addition, upregulation of the VEGF expression after RVT treatment partly contributes to the resolution of pulmonary hypertension. Therefore, we suggest that RVT may be a new strategy for the treatment of pulmonary hypertension. However, further studies are needed to determine the optimal dose of RVT, the exact mechanisms of VEGF induction and the roles of NOS in MCT-induced pulmonary hypertension.

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