Cervical Ganglion Block Attenuates the Progression of Pulmonary Hypertension via Nitric Oxide and Arginase Pathways

Sungwon Na, Ok Soo Kim, Sungwoo Ryoo, Taedong Kweon, Yong Seon Choi, Hyo Sup Shim, Young Jun Oh

Abstract—It has been recognized that the sympathetic nervous system is activated in pulmonary arterial hypertension (PAH), and abnormal sympathetic hyperactivity leads to worsening of PAH via endothelial dysfunction. The purpose of this study was to examine whether sympathetic ganglion block (SGB) can treat PAH by increasing the availability of nitric oxide (NO). PAH was induced in rats by 50 mg/kg of subcutaneous monocrotaline. After 2 weeks, daily injections of ropivacaine into the left superior cervical ganglion were repeated for 14 days (monocrotaline-SGB group). Monocrotaline group received sham SGB with saline, whereas control group received saline instead of monocrotaline. PAH was evident in monocrotaline group, with right ventricular systolic pressures (47±4 mmHg) that were higher than those of controls (17±2 mmHg), whereas SGB significantly attenuated monocrotaline-induced PAH (35±4 mmHg). The right/left ventricular mass ratios exhibited similar changes to those seen with right ventricular pressures. Heart rate variability showed significantly higher sympathetic activity in the monocrotaline group. Microscopy revealed a higher proportion of muscular arteries with thicker medial walls in the monocrotaline group, which was attenuated by SGB. Monocrotaline induced arginase hyperactivity, which was in turn decreased by SGB-induced endothelial NO synthase activation. SGB restored monocrotaline-induced hypoactivity of superoxide dismutase. In conclusion, SGB could suppress PAH and the remodeling of pulmonary arteries via inactivation of arginase and reciprocal elevation of NO bioavailability, thus attenuating disproportionate hyperactivation of the sympathetic nervous system.

Key Words: autonomic pathways • hypertension, pulmonary • nitric oxide • sympathetic nervous system

Although subcategories of pulmonary arterial hypertension (PAH) differ in their underlying pathogenesis, all share the common feature of progressively increased right ventricular afterload from excessive pulmonary vascular remodeling affecting all vessel layers, eventually leading to right heart failure and premature death. Pulmonary vascular changes in PAH have been associated with underlying pulmonary, left ventricular, or thromboembolic diseases; however, the pathogenesis of PAH in a large proportion of patients remains largely unclear.

An imbalance of vasoactive mediators has been known to play an important role in the progression of PAH. In particular, reduced nitric oxide (NO) contributes to worsening of PAH. NO is a vasoprotective molecule synthesized by endothelial NO synthase (eNOS), which is the predominant isoform of NOS. NO dilates blood vessels by stimulating guanylyl cyclase and also exerts anti-inflammatory and antithrombotic effects by inhibiting leukocyte adhesion and platelet aggregation. In addition to NO, arginase activity is known to be inadequately increased in PAH. Arginase shares the substrate l-arginine with eNOS, resulting in competitive inhibition of eNOS and subsequent vascular endothelial dysfunction. Considering the rich sympathetic nerve endings in pulmonary vasculature, there is increasing awareness of right heart failure because of PAH affecting the neurohormonal system. Although sympathetic overactivation could be interpreted as a result of circulatory failure from PAH, those patients showed increased sympathetic nerve traffic compared with the control group. In addition, sympathetic hyperactivity has been shown to act as an independent prognostic indicator in patients with PAH, in that those with increased sympathetic activity had decreased functional capacity compared with those with normal sympathetic tone. Although sympathetic activity was not always demonstrated using catecholamine levels, autonomic impairment as assessed by heart rate variability was observed in patients with PAH. In addition, impaired autonomic control, as well as endothelial dysfunction, is a marker...
of cardiovascular risk. The endothelium acts as a paracrine organ, secreting vasoactive mediators under neurohumoral control. It has been demonstrated that autonomic nerves and the endothelium work together to maintain vascular tone, creating a tonic balance between the release of vasodilating factors from the vascular endothelium and vasoconstricting factors from the sympathetic nerve terminals. Therefore, the presence of endothelial dysfunction and increased sympathetic tone create a hyperactive cycle that results in worsening of cardiovascular disease. Although to date there is not enough clinical evidence to support their use as routine therapy, sympathetic blocks in conjunction with sympatholytic drugs have been reported to attenuate the severity of symptoms and slow the progression of cardiovascular diseases including coronary artery disease, heart failure, and arrhythmias. According to the previous reports, there is a possibility that direct sympathetic nerve blocks are effective in patients with myocardial ischemia or systemic hypertension.

Given the relationship between sympathetic nervous system hyperactivity and endothelial dysfunction in patients with PAH, blockade of sympathetic hyperactivity may improve vascular reactivity by preserving endothelial function. To date, however, there is limited amount of data on the therapeutic effects of sympathetic ganglion block (SGB) on PAH. The purpose of the present study is to assess the therapeutic effects of SGB in a monocrotaline-induced PAH rat model. In parallel, we investigated whether SGB could attenuate the changes in arginase and NO bioavailability, as well as oxidative stress caused by monocrotaline.

Methods

Detailed methods about animal preparation, experimental design, SGB, heart rate variability, hemodynamic analysis, right ventricular mass, pulmonary vessel morphometry, and NO (synthase)/arginase/cGMP/oxidative stress assay are described in the online-only Data Supplement.

Results

Right Ventricular Systolic Pressure and Right Ventricular/Left Ventricular Mass Ratio

Right ventricular systolic pressure was significantly increased after monocrotaline administration compared with the control group, whereas this change was attenuated by SGB (47±4, 17±2, and 35±4 mmHg, respectively; Figure 1A and 1B). Similarly, the right ventricular/left ventricular ratio, which was increased 1.6× in monocrotaline-treated rats compared with control rats, was markedly reduced by treatment with SGB (49±2, 26±1, and 38±2%, respectively; Figure 1C).

Heart Rate Variability

Although total power and high frequency (HF) showed significant reductions 4 weeks after monocrotaline treatment compared with baseline and control group values, low frequency (LF) was comparable with that of the control group (Table). The LF/HF ratio, which represents sympathovagal balance, was significantly increased in monocrotaline-treated rats compared with baseline and control group values. The monocrotaline-SGB group had a significantly lower LF/HF ratio compared with those of the monocrotaline and control groups.

NO Bioavailability and cGMP

Plasma nitrite (NO$_2^-$) levels were significantly reduced in monocrotaline-treated rats compared with the control and monocrotaline-SGB groups (Figure 2A). Plasma nitrite was highest overall in the monocrotaline-SGB group. The level of cGMP of lung tissue, which was significantly decreased in the monocrotaline group compared with the control group, was restored after SGB treatment (Figure 2B).

eNOS and Arginase Expression

The expression of eNOS of lung tissue was significantly decreased in the monocrotaline group compared with the control group but was significantly increased in the monocrotaline-SGB group compared with the other 2 groups (Figure 3A). The expression of arginase 1 of lung tissue was comparable among all 3 groups (Figure 3B). Arginase 2 expression of lung
tissue, however, was significantly higher in the monocrotaline group. The monocrotaline-SGB and control groups showed similar levels of expression of arginase 2.

**eNOS and Arginase Activity**

eNOS activity of lung tissue was significantly reduced in the monocrotaline group compared with the control group, whereas it was increased in the monocrotaline-SGB group (Figure 4A). Arginase activity of lung tissue was significantly increased in the monocrotaline group, whereas it was significantly decreased in the monocrotaline-SGB group (Figure 4B).

### Table. Heart Rate Variability

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>MCT Group</th>
<th>MCT-SGB Group</th>
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<tr>
<td></td>
<td>Baseline</td>
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<td>Baseline</td>
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<td>HR, bpm</td>
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<tr>
<td>319±9</td>
<td>324±7</td>
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<tr>
<td>HF, ms²</td>
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<td>LF/HF ratio</td>
<td>1.9±0.4</td>
<td>1.5±0.3</td>
<td>1.8±0.4</td>
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</table>

Data are presented as mean±SEM. bpm indicates beats per minute; HF, high frequency; HR, heart rate; HRV, heart rate variability; LF, low frequency; MCT, monocrotaline; SGB, sympathetic ganglion block; and TP, total power.

*P<0.05 compared with baseline; †P<0.05 compared with the control group after 4 wk; and ‡P<0.05 compared with the MCT group after 4 wk.

**Oxidative Stress**

Monocrotaline significantly impaired superoxide dismutase (SOD) activity, which was reversed in SGB-treated rats (Figure 5A). In addition, malondialdehyde and nitrotyrosine levels of lung tissue were significantly increased in monocrotaline-treated rats but were normalized after SGB treatment (Figure 5B and 5C).

**Histology**

Compared with the control group, monocrotaline induced significant increases in the proportions of partially muscular

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**Figure 2.** Nitrite (NO₂⁻; A) and cGMP (B). Plasma nitrite measured by the Griess reagent was significantly decreased in monocrotaline (MCT)-treated rats, whereas sympathetic ganglion block (SGB) in rats with pulmonary arterial hypertension (MCT-SGB group) significantly increased plasma nitrite levels compared with the control and MCT groups. cGMP of lung tissue as a marker of nitric oxide production was significantly decreased after MCT treatment (MCT group) compared with that of the control group. This change was reversed after SGB (MCT-SGB group). *P<0.05; **P<0.01; and ***P<0.001.

**Figure 3.** Endothelial nitric oxide synthase (eNOS) and arginase expression. The expression of eNOS of lung tissue was reduced in the monocrotaline (MCT) group and was increased in the MCT-sympathetic ganglion block (SGB) group compared with the other 2 groups (A). Arginase 1 (Arg1) levels of lung tissue were not different among the groups, whereas arginase 2 was significantly increased in the MCT group (B). This increase was normalized after SGB. *P<0.05; **P<0.01; and ***P<0.001.
and muscular arteries (Figure 6A). The proportion of muscular arteries was higher in monocrotaline group compared with the control group (48% versus 7%), but it was decreased to 14% in SGB-treated rats. Meanwhile, decreased proportion of nonmuscular artery was restored in monocrotaline-SGB group (75% versus 25% versus 63%). SGB could attenuate monocrotaline-induced medial wall thickening of muscular pulmonary arteries corresponding to terminal bronchioles (10±1 versus 22±2 versus 11±1%; Figure 6B). Monocrotaline also provoked significant medial thickening of the peribronchial artery (Figure 6D) compared with that of the control group (Figure 6C). SGB attenuated monocrotaline-induced medial wall thickening (Figure 6E).

**Immunohistochemistry**

The expression and localization of eNOS and arginase 2 in the pulmonary arteries were shown in Figure 7. The expression of eNOS was reduced in the monocrotaline group and prominent in the control and monocrotaline-SGB groups, which suggests SGB-induced eNOS expression in the pulmonary vascular endothelium. In contrast, the expression of arginase 2 was prominent in the monocrotaline group but weak in the control and monocrotaline-SGB groups.

**Discussion**

The major finding of the present study was that direct blockade of the sympathetic ganglion suppresses arginase activity and reciprocally increases NO bioavailability, thereby attenuating right ventricular hypertrophy and pulmonary vascular remodeling induced by monocrotaline in PAH rat model. Oxidative stress associated with monocrotaline was also found to be lessened after the sympathetic block. These findings may help to explain the link between sympathetic hyperactivity and reduced NO bioavailability in cases of PAH, helping us to further understand its pathogenesis.

PAH is a chronic, progressive disease with several known pathogenesis. According to the current classification of this disease, the potential causes fall into 5 categories. Regardless of pathogenesis, however, all of these categories share the common features of pulmonary vascular and right ventricular remodeling that result in right ventricular overload leading to right heart failure. Several mechanisms of PAH development have been suggested including an imbalance of vasoactive mediators, cell proliferation, vascular remodeling, endothelial repair, angiogenesis, inflammation, thrombosis, oxidative stress, and right ventricular hypertrophy. To date, however, the pathogenesis of PAH cannot be fully explained by a single insult.

There has been increasing interest in the relationship between NO and sympathetic activity, as well as their role in the development of PAH. NO is the primary mediator of vasodilation that is released from the vascular endothelium.
NO released toward the vascular lumen is a potent inhibitor of platelet aggregation and exerts anti-inflammatory effects by inhibiting leukocyte adhesion to the vessel wall. As demonstrated in earlier studies, leukocyte adhesion is the primary event in the development of atherosclerosis, and NO plays a protective role against atherogenesis as well as reciprocal changes in arginase levels. The protective effect of NO by restoring disrupted autonomic balance was demonstrated in a myocardial ischemia and hypertension animal model via gene transfer. Dawson et al transferred a neuronal type of NOS gene into cardiac cholinergic neurons in myocardial ischemia rat model. NOS gene transfer led to increased acetylcholine release and NOS activity in the right atrium and showed improved mortality. Based on the fact that sympathetic ganglion, especially, the stellate ganglion, plays the main role in controlling the autonomic nervous system, there was a study investigating the effect of NOS gene transfer into stellate ganglion on calcium transient. NOS gene transfer into the stellate ganglion improved NOS expression and cGMP levels and reduced peak calcium transient in a hypertension rat model.

Based on these reports, the present study focused on the therapeutic effects of direct SGB in a PAH rat model. Clinical application of sympathetic blockade has been expanded to patients with hormonal imbalance, psychiatric disorders, and cardiovascular diseases, and previous publications have demonstrated that sympathetic blockade can be applied repeatedly without serious adverse effects. We found that monocrotaline treatment led to a significant increase in LF/HF ratio indicating increased sympathovagal balance, whereas SGB attenuated this monocrotaline-induced autonomic balance change. Similar to the previous reports, our results also showed that sympathetic hyperactivity is involved in the pathogenesis or progression of PAH, and that the modulation of sympathetic hyperactivity may prevent development or halt the progression of PAH. Heart rate variability assesses autonomic nervous system activity by analyzing fluctuations in the RR interval on electrocardiography. Similar to the result of this study, Sanyal et al reported that total power and HF power were reduced in monocrotaline-treated rats, which indicates a decrease in parasympathetic activity. But LF/HF ratio, which reflects sympathetic activity tones in the heart, was comparable unlike our results. This discrepancy could result from the differences in the dose of monocrotaline (50 versus 80 mg/kg) and the time gap between monocrotaline injection and heart rate variability measurements (4 versus 7 weeks).

Recent data have shown that sympathetic nerve block increases local NO release in humans. There are no human or animal data, however, that examine changes in arginase activity or expression after sympathetic blockade. As we reported in this study, increased arginase activity and expression induced by monocrotaline were diminished by SGB, which may be because of increased NOS activity.

Oxygen-derived free radicals make a significant contribution to the progression of PAH. Kamezaki et al found that SOD gene transfer could ameliorate the progression of PAH in a rat model. Tempol, an SOD mimic, was also shown to have preventative effects in a hypoxia-induced PAH animal model. The sympathetic nervous system is also affected by changes in oxygen-derived free radicals. For example, the effects of angiotensin II on the sympathetic nervous system are reported to be mediated by increased oxidative stress. Miura et al used antioxidants in a myocardial ischemia model and concluded that SOD could attenuate not only myocardial stunning but also the neural stunning of sympathetic cardiac innervation. The results of the present study showed that monocrotaline suppressed antioxidant and SOD activity, which could be reversed with SGB. This suggests that direct sympathetic nerve or ganglion blockade may be a successful treatment modality for many other cardiovascular diseases that share this common pathway of sympathetic activation and endothelial dysfunction.

Although the effect of direct SGB on cardiovascular diseases was not fully investigated in humans, electric autonomic nerve stimulation has been tried to modulate cardiovascular or neurological diseases with autonomic imbalance. Considering left heart failure, imbalance of cardiac
autonomic activity is characterized by marked sympathetic hyperactivity and abnormally low parasympathetic level. Although sympathetic activation provides a compensatory response to improve cardiac function in early stages, however, upregulation of sympathetic activity and parasympathetic withdrawal is known to contribute to the progression and pathogenesis of heart failure. Based on autonomic imbalance in the heart failure, vagus nerve stimulation showed some beneficial effects in failing heart such as a decrease of ventricular work. Along with hemodynamic effects, vagal stimulation exerted anti-inflammatory and attenuation of the renin–angiotensin system. Along with direct sympathetic nerve or ganglion block, parasympathetic nerve stimulation can be another modality to improve autonomic imbalance associated with cardiovascular diseases. There are many different animal models of PAH, and the most commonly used ones are the chronic hypoxia model and the monocrotaline injury model. Monocrotaline causes direct endothelial damage, which is thought to be the mechanism of PAH associated with drugs and toxins. Considering that uncontrolled sympathetic activity could be interpreted as a response to right heart circulatory failure caused by PAH regardless of its pathogenesis, there still is a possibility that SGB can be applied to all types of pulmonary hypertension. Our results suggest that a clinical trial of SGB for the treatment of PAH in humans is necessary, and the most probable human PAH subtypes that will be treatable using this method are drug/toxin-induced and infection-related cases because they share features with the monocrotaline-induced PAH animal model. Also, further application of sympathetic block in human patients with PAH will be helpful in establishing its therapeutic effect on PAH.

**Perspectives**

PAH is an uncommon but fatal cardiovascular disorder. Current treatment options include vasodilators, phosphodiesterase inhibitors, and endothelin receptor blockers although these have offered limited efficacy. Recently, endothelial dysfunction associated with sympathetic hyperactivity has been suggested as a potential mechanism of pathogenicity of cardiovascular disease, and various modalities, such as NOS gene transfer or electric vagal stimulation, have been attempted to restore disrupted autonomic balance in these conditions. The present study demonstrates that direct SGB achieved by injecting local anesthetic into the superior cervical ganglion attenuated monocrotaline-induced progression of PAH in rats, likely via inhibition of arginase and oxidative stress and elevation of eNOS. Our results suggest that direct SGB can be applied as a novel therapeutic treatment modality for many cardiovascular diseases associated with endothelial dysfunction including PAH. Further studies are needed to validate the use of SGB in humans with PAH.

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**Disclosures**

None.

**References**


**Figure 7.** Immunohistochemical localization of endothelial nitric oxide synthase (eNOS; A to C) and arginase 2 (Arg2; D to F) in the pulmonary artery endothelium. The expression of eNOS (narrow arrow) of lung tissue was reduced in the monocrotaline (MCT) group but prominent in the control and MCT-sympathetic ganglion block (SGB) groups. The expression of Arg2 (thick arrow) of lung tissue was prominent in the MCT group but weak in the control and MCT-SGB groups. Scale bar, 100 μm.
What Is New?

• Pulmonary hypertension is associated with sympathetic hyperactivity mediated by nitric oxide and arginase.

• Sympathetic ganglion block (SGB) attenuated arginase levels and oxidative stress and improved nitric oxide bioavailability in a rat model of pulmonary hypertension.

• SGB has been used to treat pain to date, but this is the first study to apply SGB in cases of pulmonary hypertension.

What Is Relevant?

• SGB is a potential treatment for pulmonary arterial hypertension.

• We showed pathophysiological roles of arginase and nitric oxide in relation to sympathetic hyperactivity.

Summary

Our results suggest that SGB may be a novel treatment modality for pulmonary hypertension.
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Methods

Animals and experimental design
All experiments were performed in male Sprague-Dawley rats at 10 weeks of age (weight = 300 g) according to our institutional review committee guidelines. The rats were randomly divided into three groups including control (n=10), MCT (n=10), and MCT-SGB (n=10) groups. PAH was induced in MCT and MCT-SGB groups by subcutaneous injection of MCT, 50 mg/kg (Sigma-Aldrich, Lyon, France) dissolved in HCl neutralized to pH 7.0 with 0.5N NaCl. The control group received a subcutaneous injection of saline only.

Sympathetic Ganglion Block (SGB)
SGB was repeated daily over the course of 14 days starting at day 14 after MCT treatment by injecting saline or local anesthetics into the left superior cervical ganglion. Ropivacaine (0.1 mL, 0.25%) was injected daily to block the superior cervical ganglion in the MCT-SGB group. Sham injection was performed using 0.1 ml of saline daily instead of ropivacaine for the control and MCT groups. SGB and sham injections were repeated for a total of 14 days. Under inhaled general anesthesia (1.0-1.5% isoflurane), the largest transverse cervical process of C1 was located and used as a landmark. A short-beveled 25-gauge needle attached to a 1 mL syringe was inserted using a sagittal approach, and advanced in the anteroposterior direction toward the transverse process of C2 along the thyroid cartilage. After confirming that there was no backflow of blood into the syringe, ropivacaine or saline was slowly injected. Sympathetic blockades were confirmed by ptosis of the eye on the side in which the injection was given.

Heart Rate Variability (HRV)
Twenty-eight days after MCT treatment, animals were anesthetized with 1.0-1.5% isoflurane inhalation. Rats were mechanically ventilated (control mode; tidal volume of 0.5 mL/100 g body weight; respiratory rate of 55–60 breaths/minute) with a volume-driven small-animal ventilator (model 665A; Harvard Apparatus, Holliston, MA, USA). Oxygenated, humidified air maintained at 37°C was delivered to the animals. Cardiac autonomic function was assessed by frequency-domain HRV analysis of 300 seconds of normal RR intervals. HRV data was acquired by a PowerLab 8/30 Data Acquisition system (ADInstruments, Sidney, Australia) and analyzed using LabChart v7 software (ADInstruments, Sidney, Australia) at 1,024 Hz. The fast Fourier transformation was used to perform power spectral density analysis according to Welch’s method. Two major power spectrum components were obtained; the high frequency (HF, 0.73-2.0 Hz) power spectrum which represents parasympathetic nervous system activity, and the low frequency (LF, 0.04-0.73 Hz) power spectrum which represents both sympathetic and parasympathetic nervous system activity. The LF/HF ratio was calculated to evaluate the balance between sympathetic and parasympathetic activity. The spectral components of HRV were calculated as absolute units (ms²). HRV measurement was repeated 28 days after treatment, and compared with the baseline data acquired prior to treatment.

Right ventricular systolic pressure (RVSP) and right ventricular hypertrophy
After HRV evaluation, right ventricular catheterization was performed under general anesthesia using inhaled isoflurane. The thorax was surgically opened and the heart was exposed. A 26-gauge needle was inserted into the right ventricle and connected to the PowerLab system for pressure measurements. RVSP data are presented as the mean of 10 measurements.
After all hemodynamic assessments were complete, the rats were euthanized by exsanguination. The heart and lungs were harvested and dissected from connective tissues in Kreb's solution. The heart was further dissected and weighed in order to calculate the right ventricular hypertrophy index (RV/LV ratio: ratio of right ventricular free wall weight to left ventricular free wall and septal weight).

**Griess nitrite assay**

Blood was collected in tubes containing citrate and EDTA anticoagulants. The tubes were then centrifuged for 15 minutes at 1000 g. To measure nitrite content, plasma was incubated with 100 µl of Griess reagent (1% sulfanilamide in 0.1 mol/l of HCl and 0.1% N-(1-naphthyl) ethylene diamine dihydrochloride (Sigma-Aldrich, St Louis, MO, USA)) at room temperature for 10 minutes. The absorbance was then measured at 540 nm using a microplate reader. The nitrite content was calculated based on a standard curve constructed using NaNO₂.

**Western blot**

Lung tissue was homogenized in ice-cold Proprep (Intron, Seoul, Korea), and the extracts were loaded onto 8-10% SDS-polyacrylamide gel. The proteins were then transferred onto polyvinyl-difluoride membranes (0.2 µm: Immun-Blot, Bio-Rad, CA, USA). Membranes were blocked using 5% (wt/vol) nonfat milk in Tris-buffered saline with 0.1% Tween-20. The blots were incubated overnight at 4°C with primary antibodies against eNOS (Santa Cruz Biotechnology, CA, USA), iNOS (Abcam®, UK), ARG1 (R&D Systems, MN, USA), and ARG2 (Santa Cruz Biotechnology) at 1:1,000 dilution. After incubation, the membranes were incubated with secondary anti-rabbit antibody at 1:5,000 dilution. Protein expression was normalized to β-actin (1:1,000 dilution). Bands were subsequently detected using a chemiluminescence assay (ECL Plus, Amersham Biosciences, CA, USA).

**eNOS activity**

eNOS activity was assayed using a rat eNOS ELISA kit (Yuhan EIAabsence, Korea). In brief, the lung tissue supernatants were homogenized in ice-cold Proprep, and then incubated in a 96 well plate containing co-factors and the substrate, L-arginine, for 1 hour at 37°C. After the incubation period, the reaction was quenched by the addition of stop solution. The concentration of the nitrites and nitrates in the reaction mixture was determined using a colorimetric method (530 nm) to evaluate eNOS activity.

**cGMP**

The ELISA used in this study was compatible with cGMP samples that were treated with hydrochloric acid to eliminate endogenous phosphodiesterase activity. Lung tissue in this matrix can be measured directly without further treatment. Tissue samples were frozen in liquid nitrogen and then ground into a fine powder under liquid nitrogen in a stainless steel mortar. After the liquid nitrogen had evaporated, the frozen tissue was weighed and homogenized in 10 volumes of 0.1M HCl, and was then centrifuged at > 600 g at room temperature. The samples were then diluted in 0.1M HCl. Activity was measured using a rat cGMP ELISA kit (CUSANBIO biotech, DE, USA), and density was determined using a 450 nm filter.

**Arginase activity**

Intracellular arginase activity was measured in lung cell lysates using a Quantichrom arginase assay kit (BioAssay Systems, CA, USA), which detects the conversion of arginine to urea by
arginase. After total protein quantification was completed, the samples were incubated with arginine buffer at 37°C for 2 hours. Density was determined using a 430 nm filter.

**Oxidative stress**

To measure oxidative stress in the lung tissue, biochemical evaluation using lipid peroxidation was performed. An Oxiselect™ SOD activity assay kit (Cell Biolabs, Inc. CA, USA) was used to assess superoxide dismutase (SOD) activity in the lung supernatant. SOD exerts its antioxidant effects by catalyzing the conversion of superoxide into oxygen and hydrogen peroxide. The lung tissue supernatant was incubated in xanthine oxidase solution for 1 hour at 37 °C. Absorbance was then read at 490 nm to measure superoxide anions. The activity of SOD was determined by the inhibition of chromogen reduction. SOD activity is expressed as a percentage.

Malondialdehyde (MDA) is a stable product of lipid peroxidation, which is regarded as an index of oxygen free radical production. Nitrotyrosine is a product of NO and superoxide. Lung tissue MDA and nitrotyrosine were analyzed using an OxiSelect™ MDA Adduct ELISA kit and OxiSelect™ nitrotyrosine ELISA kit (Cell Biolabs, Inc. CA, USA). Absorbance was read using a spectrophotometer at a 450 nm wavelength after the addition of stop solution to halt the enzymatic reaction.

**Microscopy**

For microscopic evaluation, lung tissue from the left lower lobe was fixed by immersion in 3% paraformaldehyde. This tissue was then embedded in paraffin, sectioned into 4 µm slices, and stained with hematoxylin and eosin. All slides were analyzed under light microscopy by a pathologist who was blinded to the experimental groups. Microscopy and photography were performed using a Nikon microscope with a Nikon D1 camera attached to the phototube at magnifications of X100 - X400. In each rat, 40 to 50 arteries were examined and categorized as nonmuscular, partially muscular, or muscular. The pulmonary artery was regarded as muscular if it had a complete medial coat of muscle. A partially muscular artery was one with only a crescent of muscle in the medial layer. A nonmuscular artery, on the other hand, showed no apparent muscular layers. The medial wall thickness of muscular pulmonary arteries was measured, ranging from 50 to 150 µm in external diameter. Media thickness was defined as the percentage of the distance between the lamina elastic interna and the lamina elastic externa over the diameter.

**Immunohistochemistry**

To examine the expression and localization of eNOS and arginase 2 in the pulmonary vascular endothelium, paraffin embedded lung tissues were deparaffinized, heated in citrate buffer (0.01 M, pH 6.0) in a microwave for 3 minutes, and treated with peroxidase-blocking solution for 30 minutes (Dako, S2023). After three washes in phosphate buffered saline (PBS), the sections were incubated overnight at 4°C with primary rabbit anti-eNOS antibody (dilution 1: 100) or primary rabbit anti-arginase 2 antibody (1:100). After three washes in PBS, the sections were incubated for 45 minutes at room temperature with secondary antibody (Dako real EnVision™, K5007), washed three times in PBS, and then treated with diaminobenzidine (Dako, K5007).

**Statistical analysis**

Data are expressed as means ± SEM or as numbers of subjects. Nonparametric data such as the proportion of muscular pulmonary arteries were analyzed using chi-square tests. The change in HRV before and after treatment was compared using a paired t-test. Additionally,
ANOVA with Tukey’s multiple comparison was performed to compare parametric variables among the three groups. P-values < 0.05 were considered to be statistically significant.