Effects of Sildenafil on Pulmonary Hypertension and Levels of ET-1, eNOS, and cGMP in Aorta-Banded Rats

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Sildenafil, an oral phosphodiesterase Type 5 inhibitor, has vasodilatory effects through a cGMP-dependent mechanism. We previously showed that aortic banding could result in left ventricular overloading and pulmonary hypertension (PH). In this study, we investigated whether early administration of sildenafil, either immediately after or 2 weeks after aortic banding, could ameliorate the development of PH and alter gene expression of endothelin (ET)-1 and endothelial nitric oxide synthase (eNOS), and alter the levels of cGMP in rats undergoing an ascending aortic banding. Rats ($n = 32$) were divided into sham-operated and banding groups with or without treatment. The banded rats were further divided into three groups: (i) receiving saline on Days 1–28 (AOB_{28}; $n = 8$), (ii) receiving saline on Days 1–14 followed by treatment with 50 mg/kg/day sildenafil on Days 15–28 (AOB_{28}/Sil_{15-28}; $n = 8$), and (iii) receiving 50 mg/kg/day sildenafil on days 1–28 (AOB_{28}/Sil_{1-28}; $n = 8$). The sham-operated rats were administrated saline on Days 1–28 ($n = 8$).

Four weeks after banding, there was a significant development of PH with pulmonary vascular remodeling. Although both sildenafil-treatment groups had significant increases in cGMP and had reductions in the thickening in the medial layer of pulmonary arteriole, notable attenuation of PH occurred only in the AOB_{28}/Sil_{1-28} group. *PreproET*-1 and *eNOS* messenger RNA (mRNA) expressions were measured by competitive reverse transcription polymerase chain reaction, and eNOS protein was determined by Western blotting. Sildenafil did not alter the elevated *ET*-1 or *preproET*-1 mRNA in banded rats. Interestingly, pulmonary eNOS increased in the AOB_{28}/Sil_{1-28} group. In conclusion, early treatment with sildenafil inhibited the rise in pulmonary arterial pressure and pulmonary vascular remodeling in PH secondary to heart failure, and cGMP, but not ET-1, might be involved. Clinically, early repeated administration of sildenafil may offer an alternative in protecting against PH in heart failure. Exp Biol Med 231:942–947, 2006

Key words: endothelial nitric oxide synthase; endothelin-1; pulmonary vascular remodeling; pulmonary hypertension; aortic banding; sildenafil

Introduction

In congenital heart disease, endothelial dysfunction plays an important role in progressive pulmonary vascular remodeling. Pulmonary hypertension (PH) develops after a chronic increase in pulmonary flow or pulmonary arterial pressure (1, 2). In congenital heart disease, it is crucial to evaluate the severity or reversibility of PH, to prevent further development of disease.

To date, the molecular mechanisms by which pulmonary endothelium vasomediators contribute to PH remain undetermined. In the lung, both nitric oxide (NO) and endothelin (ET)-1 have been identified as major endothelium-dependent vasomediators. NO is a potent, endogenous vasodilator and growth inhibitor. The production of NO is catalyzed by the enzyme, endothelial NO synthase (eNOS), and its action is mediated through cGMP (3). By contrast, ET-1 is a vasoconstrictor and growth promotor.

The interactive mechanism between these two vasomediators is still not clearly resolved. However, it has been shown that ET-1 could induce vasolaxation through

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increasing eNOS activity after binding to the ET_B receptor. Altered gene expressions of *eNOS* and *ET*-1 have been noted in animals with PH induced by hypoxia or monocrotaline (4–8) and in patients with PH (9). However, few studies have examined gene expression of pulmonary *eNOS* and *ET*-1 in retrograde PH using animal models of heart failure secondary to left ventricular pressure overload (10). In previous studies, we reported enhanced expressions of eNOS and ET-1 in rats subjected to aortic banding (11, 12).

Sildenafil is an oral phosphodiesterase Type 5 inhibitor, which has been used for the treatment of erectile dysfunction in men. Interestingly, Type 5 phosphodiesterase is abundantly expressed not only in the corpus cavernosum, but also in the lung (12), especially in vascular smooth-muscle cells. Therefore, we examined the effect of long-term administration of sildenafil on the development of PH and gene expression of ET-1, eNOS, and cGMP in the lung of rats with left ventricular overloading.

Materials and Methods

Animal Model. All protocols were approved by the animal research committee of Kaohsiung Medical University. Male Wistar rats (6-weeks old, weighing approximately 220 g) were randomly assigned to aortic banding or sham operation, as previously described (11).

In summary, a left parasternal thoracotomy in the fourth intercostal space was performed after animals were anesthetized and orotracheally ventilated using rodent respirators (Harvard, South Natick, MA). A blunt, sheathed 19-gauge hypodermic needle was placed along the axis of the ascending aorta, and a length of 3–0 nylon suture was tied around the aorta. The sheathed hypodermic needle was removed, leaving a stenosis in the ascending aorta approximately 1 cm distal to the aortic valve. The procedure took less than 30 mins for each rat. Sham-operated animals underwent the same operation, except that the aorta was not banded.

Rats were individually housed in a 12:12-hr light:dark–controlled room and fed regular rat diet. Effective aortic banding was indicated by a pressure gradient of approximately 40 mm Hg, determined by transthoracic echocardiography (4500 Model, 8-MHz transducer; Philips, Eindhoven, The Netherlands) at Day 1.

Rats ($n = 32$) were divided into sham-operated and banding groups, with or without treatment of sildenafil (50 mg/kg/day, twice daily, given either from Day 1 to Day 28, or from Day 15 to Day 28). This protocol resulted in the creation of four groups: sham-operated administered saline (sham-operated; $n = 8$), banded rats administered saline (AOB_{28}; $n = 8$), banded rats administered sildenafil for 4 weeks ($AOB_{28}/Sil_{1–28}$; $n = 8$) and banded rats administered sildenafil for 2 weeks ($AOB_{28}/Sil_{15–28}$; $n = 8$). At 4 weeks, all rats were again ventilated and sacrificed by removing the ventilator after checking hemodynamic data.

Measurement of Systemic and Pulmonary Arterial Pressures. Using a cut-down procedure, a PE-50 catheter was inserted into the femoral artery to record pressures. After the left parasternal thoracotomy was performed, a PE-10 catheter was inserted into the main pulmonary artery *via* the right ventricular outflow tract. The catheter was connected to a pressure transducer. The system was filled and flushed with less than 2 ml of heparin solution (1000 IU/ml). Pulmonary and femoral arterial pressures were recorded simultaneously by a polygraph system.

To determine ET-1 levels, 2 ml of blood sample was withdrawn from pulmonary arterial cannulae, collected in a chilled syringe and transferred to a polypropylene tube containing ethylenediamine tetraacetic acid (EDTA) (1 mg/ml of blood) and aprotinin (500 KIU/ml of blood) at 4°C. Samples were centrifuged at 2000 *g* for 15 mins at 4°C. Plasma was stored at –70°C until assay.

Tissue Preparation. After rats were sacrificed, the lung and heart were rapidly perfused with normal saline, removed and dissected. The banded aortal segment was dissected and examined under an operating microscope to confirm banding effectiveness. The right ventricle was dissected along its septal insertion and isolated to determine medial wall thickness as follows: percent wall thickness = ([medial thickness × 2]/external diameter) × 100. Three pieces of lung tissue from different lobes were excised, immersed in 10% formalin for 24 hrs, and stained with hematoxylin-eosin (H-E). One-half of the remaining lung tissue was homogenated for RNA extraction, and the other half was frozen in liquid nitrogen and stored at –70°C for Western blot analysis.

Western Blot Analysis for eNOS. The lung tissue was homogenized in a buffer containing 50 m*M* Tris/HCl, pH 7.4; 0.1 m*M* EDTA; 0.1 m*M* EGTA; 100 μ*M* phenylmethylsulfonyl fluoride; 10 μ*M* leupeptin; 1 μ*M* pepstatin A; and 4.5 μg/ml aprotinin. Homogenates were centrifuged (14,000 *g*, 4°C for 30 mins). One hundred micrograms of protein from the supernatant was separated on a 6% denaturing polyacrylamide gel and electrotransferred to an Immobilon-P transfer membrane (Amersham, Arlington Heights, IL). The eNOS protein band was detected by chemiluminescence using a rabbit antibody to eNOS (Santa Cruz Biotechnology, Santa Cruz, CA). To normalize protein loading, the expression of β-actin was also determined using antibodies to actin (Santa Cruz Biotechnology).

Measurements for ET-1 and cGMP. Plasma samples (0.8–1.0 ml) were acidified with 0.6% trifluoroacetic acid and centrifuged (2000 *g*, 4°C for 15 mins). The supernatant was dried under a steam of nitrogen at 60°C.

The plasma and lung tissue levels of ET-1 and cGMP were measured. ET-1 and cGMP were determined using an enzyme immunoassay kit (Biomedica Group, Wien, Austria) and a radioimmunoassay kit (Amersham Bioscience, Chalfont, England, UK), respectively.

Competitive Reverse Transcription (RT) Poly-

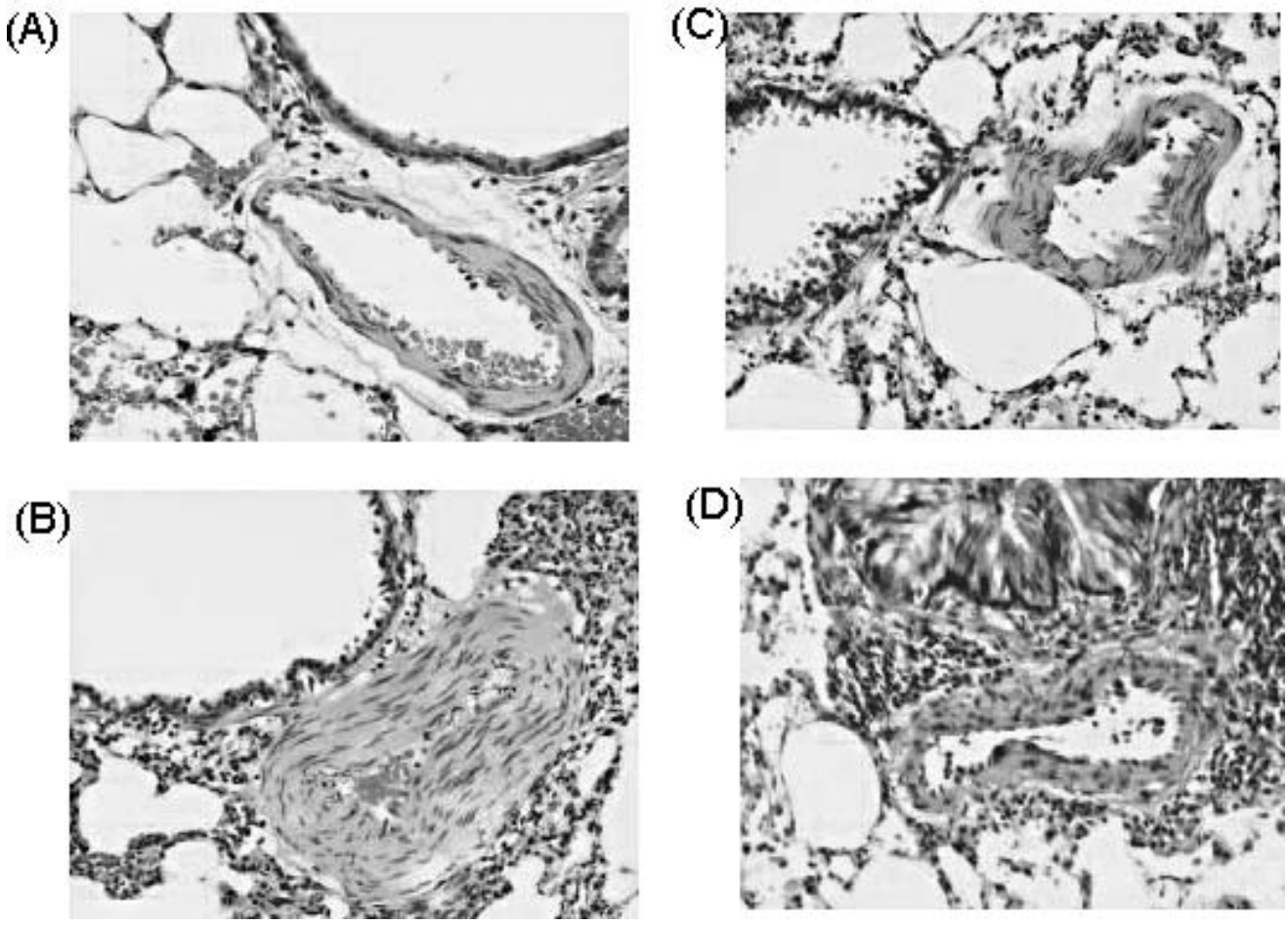

Figure 1. Hematoxylin-eosin staining of lung tissue. The pulmonary arteriole muscular layer was thickened in banded rats at 4 weeks (B), as compared with sham-operated (A) and banded rats fed with sildenafil from Day 15 to Day 28 (C), and from Day 1 to Day 28 (D). Magnification: ×200.

merase Chain Reaction (PCR). Total lung RNA was extracted using a mixture of acid guanidinium thiocyanate-phenol-chloroform (13). Two competitive templates, mimic–preproET-1 and mimic-eNOS, were constructed, as described previously (11), for measuring the levels of messenger RNA (mRNA) for *preproET*-1 and *eNOS*, respectively.

Immunohistochemistry for ET-1 and eNOS. Immunohistochemistry was performed using 5-μm tissue sections fixed in formalin and embedded in paraffin. The slides were deparaffinized, hydrated, and treated with 0.3% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. They were then washed with phosphate-buffered saline (PBS) and incubated for 1 hr at room temperature with antibodies to eNOS (1:100 dilution) or ET-1 (1:100 dilution; both from Oncogene, Boston, MA). Afterward, the slides were washed with PBS and incubated for 30 mins with biotinylated second antibody (DAKO, Glostrup, Denmark). The specimens were washed with PBS again, incubated for 30 mins with peroxidase-labeled streptavidin (DAKO), and examined under a light microscope after incubation with diaminobenzidine (Sigma, St. Louis, MO) and counterstained with Mayer's hematoxylin.

Statistical Analysis. The results obtained from competitive RT-PCR, and Western blots were analyzed by densitometry and expressed as mean ± SEM. After an F test for the analysis of variance, statistical analysis was performed using an unpaired two-tailed *t* test, and $P < 0.05$ was considered statistically significant.

Results

In this experiment, there were four groups of eight rats each. The right ventricle weight to body weight ratio (RV/BW; g/kg body weight) in the $AOB_{28}/Sil_{1–28}$ group (0.53 ± 0.02 [mean ± SE]) was significantly lower than either the $AOB_{28}/Sil_{15–28}$ (0.62 ± 0.02; $P < 0.05$) or the AOB_{28} group (0.62 ± 0.02; $P < 0.05$). The mean systemic arterial pressure in the sham-operated group was slightly lower than the pressures obtained in the three banded groups, which had no differences among them (data not shown). Importantly, the mean pulmonary arterial pressure in $AOB_{28}/Sil_{1–28}$ group (26 ± 1.1 mm Hg) was significantly

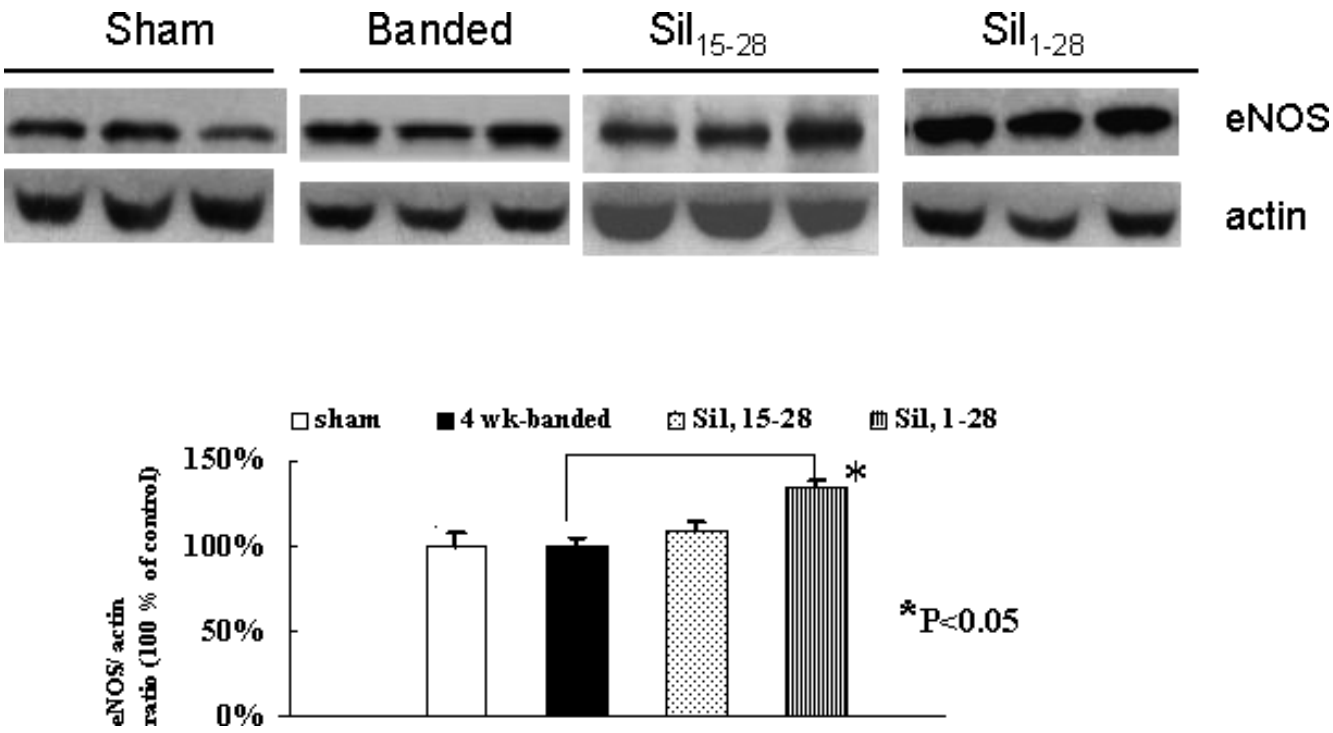

Figure 2. Western blot analysis of eNOS protein in the lung. The lower panel shows normalized ratios of eNOS:actin determined by densitometry. There was a significant increase in eNOS in the lung of 4-week-banded rats administrated sildenafil from Day 1 to Day 28 (Sil, 1–28), compared with the lung of 4-week-banded rats. Values represent mean ± SEM. *$P < 0.05$.

lower than that of the AOB_{28} group (33 ± 2.0 mm Hg; $P < 0.05$). There was, however, no difference in the mean pulmonary arterial pressure between the AOB_{28} and the $AOB_{28}/Sil_{15–28}$ groups (29 ± 1.2 mm Hg).

Histologically, the basic architecture of the lung respiratory apparatus, such as the bronchioles and alveoli, seemed to be normal in both banded and sham-operated groups. Quantitative analysis of peripheral pulmonary arterioles demonstrated that the banding generated an increase in percent wall thickness of pulmonary arteriole (sham-operated, 22 ± 1.1%; AOB_{28}, 40 ± 2.2 %; $P < 0.01$), and there was a significantly attenuated percent wall thickness of the pulmonary arteriole in the $AOB_{28}/Sil_{15–28}$ group (33 ± 1.8%; $P < 0.05$) and the $AOB_{28}/Sil_{1–28}$ group (26 ± 1.4%; $P < 0.01$), when compared with the AOB_{28} group (Fig. 1).

The pulmonary eNOS content in the $AOB_{28}/Sil_{1–28}$ group was significantly increased, compared with the AOB_{28} group ($P < 0.05$). There was also a trend toward an increase in pulmonary eNOS in the $AOB_{28}/Sil_{15–28}$ group when compared with the AOB_{28} group (Fig. 2). Similarly, there was a marked increase in eNOS immunoreactivity in the endothelium of pulmonary arterioles in the $AOB_{28}/Sil_{1–28}$ group, as compared with the other groups (Fig. 3).

Although the pulmonary cGMP in AOB_{28} group (3.1 ± 0.1 pmol/g protein) was not different from the sham-operated group (3.0 ± 0.22 pmol/g protein), repeated administration of sildenafil increased the pulmonary cGMP in both the $AOB_{28}/Sil_{1–28}$ group (4.6 ± 0.15 pmol/g protein; $P < 0.01$) and the $AOB_{28}/Sil_{15–28}$ group (3.9 ± 0.16 pmol/g protein). By contrast, there was a significant increase in *preproET*-1 mRNA in the AOB_{28} group as compared with the sham-operated group ($P < 0.05$). However, there was no significant difference in the expression of pulmonary *preproET*-1 mRNA among the AOB_{28}, $AOB_{28}/Sil_{1–28}$, and $AOB_{28}/Sil_{15–28}$ groups (data not shown). Likewise, there was a significant increase in the pulmonary ET-1 level in the AOB_{28} group (231 ± 11 ng/g protein) as compared with the sham-operated group (198 ±12 ng/g protein; $P < 0.05$), but there were no significant differences in the pulmonary ET-1 levels among the AOB_{28}, $AOB_{28}/Sil_{1–28}$ (229 ± 13 ng/g protein), and $AOB_{28}/Sil_{15–28}$ (219 ± 14 ng/g protein) groups. Consistent with these findings, there was no difference in ET-1 immunoreactivity among these three groups (data not shown).

Discussion

In various animal models, PH seems to result from pulmonary endothelial dysfunction, in which there is an imbalance between vasoconstriction and vasodilation (14). Thus, agents that can cause pulmonary vasorelaxation, such as sildenafil, may have beneficial effect for the treatment of this disease.

In the present study, we demonstrated that daily administration of sildenafil immediately after banding could attenuate pulmonary vascular remodeling in banded rats and prevent the development of PH in the $AOB_{28}/Sil_{1–28}$ group. This is consistent with other reports showing that sildenafil

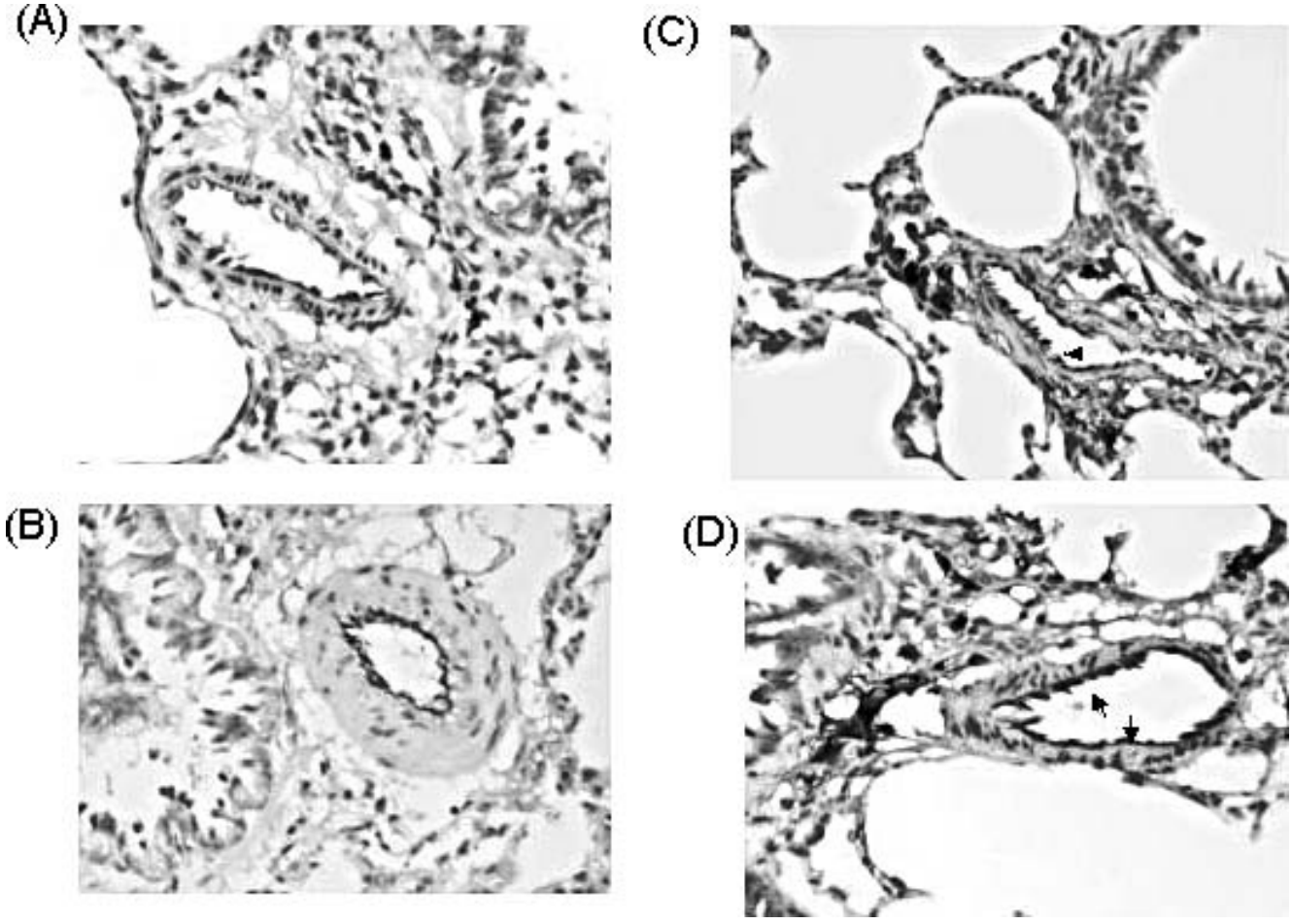

Figure 3. Immunoreactivity for eNOS. Increased eNOS immunoreactivity (arrows) in the endothelium of pulmonary artery in the 4-week-banded rats administrated sildenafil from Day 1 to Day 28 (D), compared with sham-operated rats (A) or 4-week-banded rats (B) and 4-week-banded rats administrated sildenafil from Day 15 to Day 28 (C). No difference in eNOS immunoreactivity was found between the untreated banded and sham-operated rats.

could attenuate PH in humans and mice (15) and offers a novel approach for the treatment of this condition.

Recently, the efficacy of sildenafil for the treatment of PH was demonstrated in a placebo-controlled, randomized study of 10 healthy volunteers (16). Other studies have revealed that sildenafil could attenuate the rise in pulmonary arterial pressure associated with hypoxic challenge without affecting the systemic blood pressure (15, 16). Moreover, sildenafil also seems to be as effective as inhaled NO in assessing acute vasoreactivity in patients with PH (16), and is known to improve pulmonary hemodynamics in patients with primary PH (17, 18). In a randomized, open-labeled clinical trial, the acute hemodynamic effects of combination therapy with sildenafil and/or inhaled iloprost were assessed in a mixed population of 30 patients with primary PH or chronic thromboembolic PH (19), and a decrease in pulmonary vascular resistance was found after a 1-hr treatment.

Our study showed that repeated daily administration of sildenafil could upregulate pulmonary expression of eNOS, but not ET-1, and could prevent the development of PH secondary to heart failure. Because the so-called NO axis (the eNOS-NO-cGMP pathway) is thought to contribute to the sildenafil response, induction of eNOS is an essential component of the signaling mechanism for the sildenafil-induced delayed preconditioning, which could mediate cardioprotection (20). Other experimental data further support the role of sildenafil in directly protecting against necrosis and apoptosis of the myocardium through the NO pathway (21).

In our study, daily administration of sildenafil beginning on Day 15 also attenuated pulmonary vascular remodeling, although gene expression of pulmonary *eNOS* was not upregulated through 4 weeks of banding. The reason for this discrepancy is not clear at the present and remains to be investigated.

However, NO is known to participate in mitigating vasopressor activity of ET-1 (22), to inhibit the translation of *preproET*-1 mRNA (23), to augment the degradation of ET-1 protein (24), and to reduce ET-1 formation (14). Kourembanas *et al*. (24) reported that increased expression of ET-1 was converted to normal if inhaled NO was

administered in hypoxia-induced PH animal models, and suggested that both ET-1 and NO can regulate each other through an autocrine feedback loop (25). Afterward, the altered expression of ET-1 could be reversed if the production of NO was reduced by a NOS inhibitor. In addition, it was reported that an increase of ET-1 protein could be accompanied by a decreased production of NO in monocrotaline-induced PH. Because there is a reciprocal regulation between the NO axis and the ET-1 pathway, the fact that the expression of ET-1 was not upregulated in banded rats administrated with sildenafil in our study may imply that there was probably an impaired interaction between ET-1 and eNOS or an impaired activity of eNOS.

In conclusion, our results show that long-term administration of sildenafil attenuates pulmonary vascular remodeling and the development of PH through a cGMP-dependent, but not ET-1–dependent, pathway. Our findings offer insight regarding the repeated use of sildenafil in treating or protecting against PH in heart failure secondary to left ventricular pressure overloading.

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