The Effects of Debanding on the Lung Expression of ET-1, eNOS, and cGMP in Rats with Left Ventricular Pressure Overload

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Pulmonary hypertension (PH) usually develops secondary to left ventricular (LV) dysfunction; therefore, it is also called retrograde PH. To investigate our hypothesis that PH is at least partially reversible, as in some congenital heart diseases, in a rat model we investigated whether release of constriction could attenuate pulmonary vascular remodeling and change the expression of endothelin (ET)-1 and endothelial nitric oxide synthase (eNOS). We used rats with LV dysfunction produced by an ascending aortic banding. In this study, there were four groups enrolled: 4-weeks banded (AOB_{1-28}; $n=7$), 7-weeks banded (AOB_{1-49}; $n=7$), debanded groups (AOB_{1-28}/DeB_{29-49}; $n=7$), and rats receiving a sham operation ($n=7$). Subsequently, there was significant attenuation of medial hypertrophy in pulmonary arterioles and reversal of PH in the AOB_{1-28}/DeB_{29-49} group (sham, 19 ± 1.3 mm Hg; AOB_{1-28}, 31 ± 2.7 mm Hg; AOB_{1-49}, 32 ± 2.7 mm Hg; and AOB_{1-28}/DeB_{29-49}, 20 ± 1.3 mm Hg). *PreproET*-1 mRNA and *eNOS* mRNA were measured by competitive reverse transcriptase (RT) polymerase chain reaction (PCR), and eNOS was measured by Western blotting. Compared with the banded groups, debanding significantly decreased pulmonary *preproET*-1 mRNA, pulmonary ET-1 (sham, 210 ± 12 pg/g protein; AOB_{1-28}, 242 ± 12 pg/g protein; AOB_{1-49}, 370 ± 49 pg/g protein; and AOB_{1-28}/DeB_{29-49}, 206 ± 1.9 pg/g protein), and plasma ET-1 levels (sham, 10.1 ± 1.5 pg/ml; AOB_{1-28}, 13.4 ± 2.0 pg/ml; AOB_{1-49}, 15.4 ± 2.0 pg/ml; and AOB_{1-28}/DeB_{29-49}, 10.3 ± 0.9 pg/ml protein). Debanding could not, however, alter pulmonary eNOS, *eNOS* mRNA, or cGMP. These findings suggest that pulmonary vascular remodeling, increased pulmonary arterial pressure, and upregulation of *ET*-1 gene expression are all reversible. We infer that it is the upregulated gene expression of *ET*-1, not eNOS, that is closely related to the development of the PH secondary to 4 weeks of aortic banding. Exp Biol Med 231:954–959, 2006

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

Introduction

Pulmonary hypertension (PH) is defined as any disorder characterized by abnormally increased pulmonary arterial pressure. It may result from pulmonary vascular or pulmonary parenchymal factors. To date, the molecular mechanisms by which endothelial vasomediators contribute to PH are unknown. Nitric oxide (NO) and endothelin (ET)-1 have been identified as major endothelium-dependent vasomediators in the lungs. NO is a potent endogenous vasodilator produced by the enzyme, endothelial NO synthase (eNOS; Ref. 1). The cytoskeleton reorganizes and endothelial cells change shape if stimulation is continuously exerted on the cells (2). In contrast, ET-1 has vasoconstrictive and mitogenic effects. It also can

This work was supported by Grant NSC93-2314-B-037-049 from the National Science Council of the Republic of China.

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Received September 28, 2005.
Accepted October 13, 2005.

1535-3702/06/2316-0954$15.00

stimulate the production of growth factors and potentiate the effects of transforming growth factor and platelet-derived growth factor. Therefore, ET-1 seems to play an important role in the regulation of pulmonary vascular tone (3). In addition, it is reported that the pathogenesis of PH is related to the endothelial dysfunction, resulting in an imbalance between endothelium-dependent pulmonary vasodilation and vasodilation in PH (4). Altered gene expression of eNOS and ET-1 has been noted both in animals with PH induced by hypoxia or monocrotaline (MCT; Refs. 5–7) and in human patients with PH (8). However, few studies have examined gene expression of pulmonary eNOS and ET-1 in retrograde PH in animal heart failure models (9). We have reported altered expressions of eNOS and ET-1 in Wistar rats with PH secondary to left ventricular (LV) dysfunction after aortic banding (10). Additionally, some authors observed that release of the aortic constriction could completely or partially reverse LV hypertrophy, chamber dilation, and dysfunction with a distinct time course. In addition, complete restoration of LV structure and function was possible in animals subject to 4 weeks of aortic banding (11).

Using aortic-banded rats as the study organism, we postulated that LV dysfunction regression could result from decreased pulmonary arterial pressure after debanding. We also investigated whether debanding reverses the expression of eNOS and ET-1.

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 groups. As previously described (10), 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 dark:light cycle–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 1 day.

At 4 weeks, seven banded rats were again ventilated and sacrificed by removing the ventilator after checking hemodynamic data. All other banded rats were ventilated under similar anesthesia, and randomly assigned to receive either a debanding procedure, or a simple thoracotomy. At 4 weeks the sham-operated rats also underwent simple thoracotomy, but without banding or debanding. All the rats were subsequently given continuous feed under the same conditions for an additional 21 days. At 7 weeks, the rats were again ventilated and sacrificed 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 pressure. 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 artery pressures were recorded simultaneously by a polygraph system. To assay for ET-1 levels, 2-ml samples were aspirated from pulmonary arterial cannulae, collected in chilled syringes, and transferred to polypropylene tubes 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* at 4°C for 15 mins. Plasma was stored at −70°C.

Tissue Preparation. After rats were sacrificed by removing the ventilator at 4 weeks or 7 weeks, the lung and heart were rapidly perfused with normal saline and removed. The banded aortal segment was dissected and examined under an operating microscope. The right ventricle was isolated by dissection along its septal insertion. Meanwhile, three pieces of lung tissue from different lobes were excised and immersed in 10% formalin for 24 hrs. Hematoxylin-eosin staining was subsequently performed. 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. Rat lung tissues were homogenized in a buffer containing 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 in 50 m*M* Tris/HCl, pH 7.4. Homogenates were centrifuged at 14,000 *g* at 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 against 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).

Measurement for ET-1 and cGMP. Plasma samples (0.8–1.0 ml) were acidified with 0.6% trifluoroacetic acid to give a 10% w/v homogenate. They were then centrifuged at 2000 *g* at 4°C for 15 mins, and the

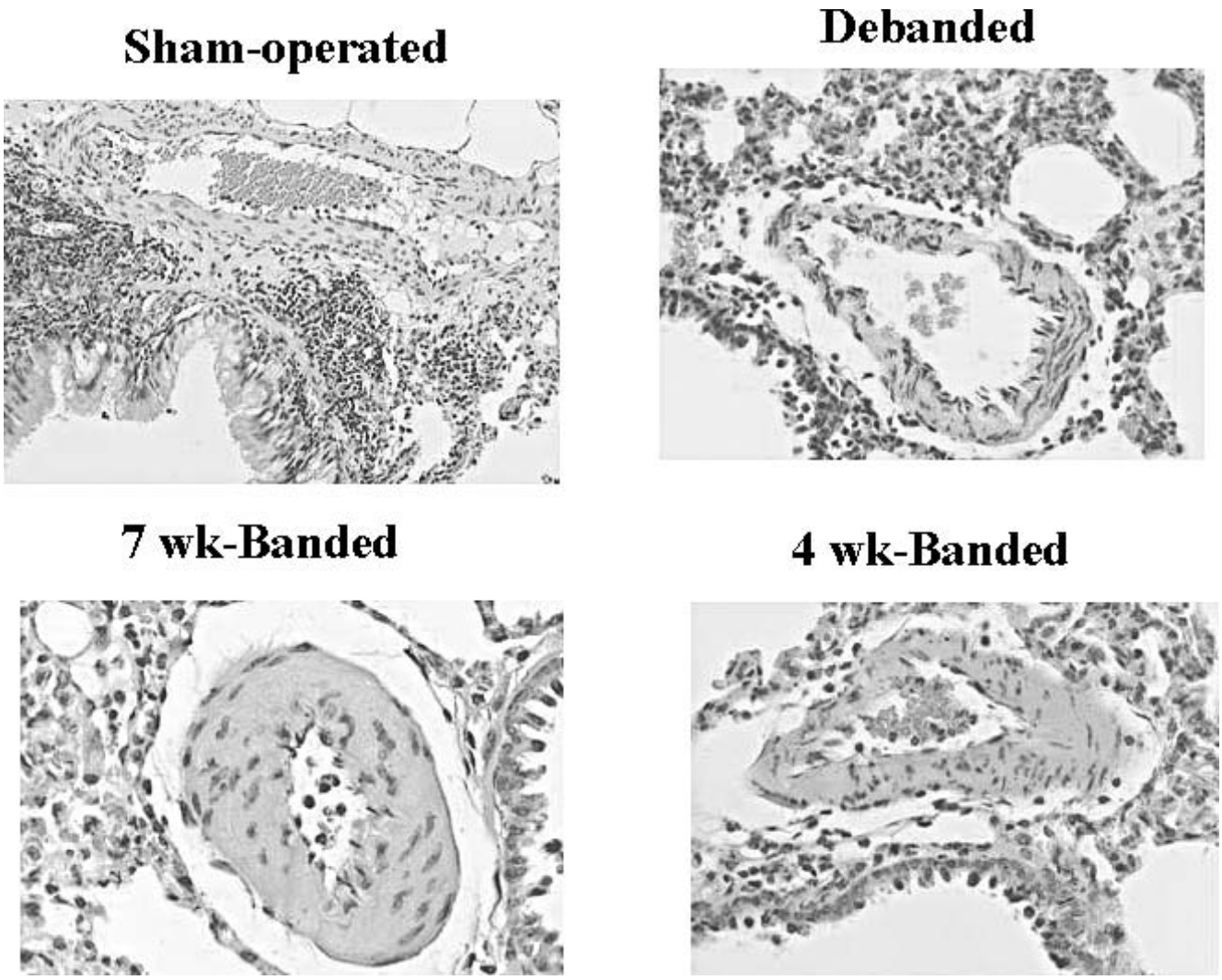

Figure 1. Hematoxylin-eosin staining of lung tissue. Pulmonary arteriole muscular layer was thickened in banded rats at 4 and 7 weeks, as compared with sham-operated and debanded rats. Magnification: ×200.

supernatant was dried under a steam of nitrogen at 60°C. An enzyme immunoassay kit (Biomedica Group, Wien, Austria) was used to measure ET-1 content in lung tissue. The cGMP content in plasma and lung tissue was measured with a radioimmunoassay kit (Amersham Bioscience, Chalfont, England, UK).

Competitive Reverse Transcription (RT) Polymerase Chain Reaction (PCR). Total lung RNA was extracted using an acid guanidinium thiocyanate-phenol-chloroform mixture mixture (12). Two competitive templates, mimic–preproET-1 and mimic-eNOS, were constructed, as described previously (10), to measure the levels of mRNA for *preproET*-1 and *eNOS*, respectively.

Immunohistochemistry for ET-1 and eNOS. Immunohistochemistry was performed using 5-μm formalin-fixed and paraffin-embedded tissue sections. 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 with antibodies to eNOS (1:100 dilution) or ET-1 (1:100 dilution; Oncogene, Boston, MA) for 1 hr at room temperature. Afterward, slides were washed with PBS and incubated with a biotinylated linking antibody (DAKO, Glostrup, Denmark) for 30 mins. Specimens were once again washed with PBS, incubated with peroxidase-labeled streptavidin (DAKO) for 30 mins, and examined under a light microscope after incubation with diaminobenzidine (Sigma, St. Louis, MO) and Mayer's hematoxylin counterstain.

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

Results

Rats were divided into four groups, the sham-operated group ($n = 7$), $AOB_{1–28}$ group ($n = 7$), $AOB_{1–49}$ group ($n =$

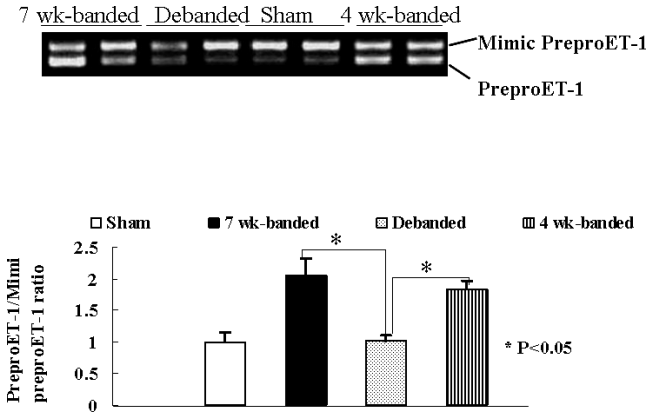

Figure 2. Expression of *preproET*-1 mRNA in lung tissues. Total RNA was extracted from lung tissues, and RT-PCR was performed (upper panel). The lower panel shows normalized ratios of preproET-1/mimic–preproET-1. There was a significant decrease in *preproET*-1 mRNA in the lung of debanded rats, compared with the banded rats at 4 or 7 weeks. Values represent mean ± SEM. $^*P < 0.05$.

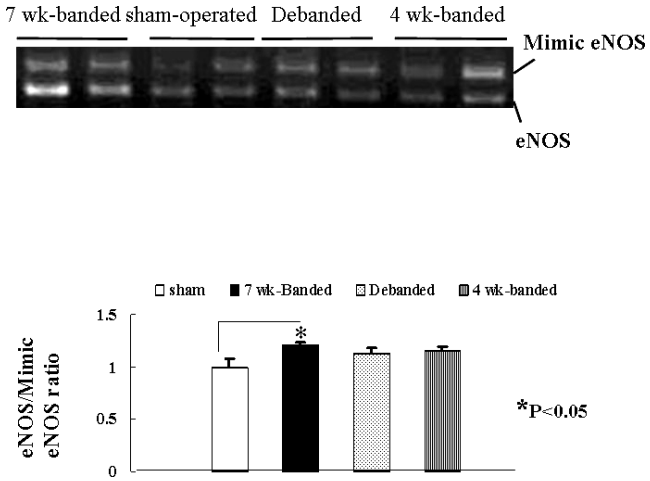

Figure 3. Expression of *eNOS* mRNA in lung tissues. Total RNA was extracted from lung tissues, and RT-PCR was performed (upper panel). The lower panel shows normalized ratios of eNOS/mimic eNOS. Significant increases were found in *eNOS* mRNA in the lungs of banded rats at 4 and 7 weeks and in debanded rats compared with the sham-operated rats. Values represent mean ± SEM. $^*P < 0.05$.

7), and $AOB_{1–28}/DeB_{29–49}$ group ($n = 7$). The mean pulmonary arterial pressure was significantly lower in the sham-operated group (sham, 19 ± 1.3 mm Hg; $AOB_{1–28}$, 31 ± 2.7 mm Hg; $AOB_{1–49}$, 32 ± 2.7 mm Hg; $P < 0.05$), and significantly lowered (to 20 ± 1.3 mm Hg) in the $AOB_{1–28}/DeB_{29–49}$ group, as compared with the $AOB_{1–28}$ group ($P < 0.05$). In the sham-operated, $AOB_{1–49}$, and $AOB_{1–28}/DeB_{29–49}$ groups, basic architecture of the lung respiratory apparatus, such as the bronchioles and alveoli, seemed to be histologically normal. The predominant change was the development of medial thickening in the pulmonary arterioles in the $AOB_{1–28}$ and $AOB_{1–49}$ groups. In addition, there was significant attenuation of medial hypertrophy in pulmonary arterioles in the $AOB_{1–28}/DeB_{29–49}$ group, as compared with either the $AOB_{1–28}$ or the $AOB_{1–49}$ group (Fig. 1).

The pulmonary *preproET*-1 mRNA levels from the two $AOB_{1–28}$ (1.83 ± 0.13; $P < 0.05$) and $AOB_{1–49}$ (2.05 ± 0.27; $P < 0.01$) groups was significantly higher than the sham-operated group (1.00 ± 0.16). Pulmonary *preproET*-1 mRNA was also significantly reduced, to 1.03 ± 0.09, in the $AOB_{1–28}/DeB_{29–49}$ group ($P < 0.05$), as compared with the $AOB_{1–28}$ group (Fig. 2).

Pulmonary ET-1 content in $AOB_{1–28}$ and $AOB_{1–49}$ groups was significantly increased compared with the sham-operated group (sham, 210 ± 12 pg/g protein; $AOB_{1–28}$, 242 ± 12 pg/g protein; $AOB_{1–49}$, 370 ± 49 pg/g protein; $P < 0.01$). In addition, pulmonary ET-1 content was significantly decreased, to 206 ± 1.9 pg/g protein, in the $AOB_{1–28}/DeB_{29–49}$ group compared with the $AOB_{1–28}$ group ($P < 0.01$). Plasma ET-1 in the $AOB_{1–49}$ group was significantly higher than in the sham-operated group (sham, 10.1 ± 1.5 pg/ml; $AOB_{1–49}$, 15.4 ± 2.0 pg/ml; $P < 0.05$), and, in the $AOB_{1–28}/DeB_{29–49}$ group, it was significantly lower (10.3 ± 0.9 pg/ml protein) than in the $AOB_{1–28}$ group ($P < 0.05$).

In contrast, the debanding did not alter pulmonary *eNOS* mRNA levels significantly in the $AOB_{1–28}/DeB_{29–49}$ group (1.13 ± 0.05), compared with $AOB_{1–28}$ (1.15 ± 0.05). However, there was increased pulmonary *eNOS* mRNA in the $AOB_{1–49}$ group compared with the sham-operated group (sham, 1.0 ± 0.07; $AOB_{1–49}$, 1.22 ± 0.03; $P < 0.05$; Fig. 3). In addition, in none of the four groups was there a significant difference either in pulmonary eNOS (result not shown), or in pulmonary cGMP (sham, 4.0 ± 0.21 pmol/g protein; $AOB_{1–28}/DeB_{29–49}$, 3.6 ± .16 pmol/g protein; $AOB_{1–49}$, 3.7 ± 0.17 pmol/g protein; and $AOB_{1–28}/DeB_{29–49}$, 3.8 ± .16 pmol/g protein).

Although for eNOS immunoreactivity, no group was significantly different (data not shown), differences were found with ET-1 immunoreactivity. Endothelia of pulmonary arterioles in both banded groups had significant enhancements, and there was a marked attenuation in the endothelia of pulmonary arterioles in the $AOB_{1–28}/DeB_{29–49}$ group as compared with the $AOB_{1–28}$ and $AOB_{1–49}$ groups (Fig. 4).

Discussion

Rats with aortal-banding underwent a transition from stable compensated hypertrophy, to heart failure, to PH (13, 14). In the present study, debanding not only reversed the PH, but also attenuated pulmonary vascular remodeling through the restoration of LV dysfunction. It is unclear whether altered *eNOS* gene expression plays a protective or pathologic role in PH. Our results revealed that upregulated *eNOS* expression occurred in banded rats at 7 weeks, while PH developed. However, it has been reported that down-regulated pulmonary *eNOS* expression develops in coronary artery ligation–induced PH (15). Although the explanation is unclear, the discrepancy may be related to the different

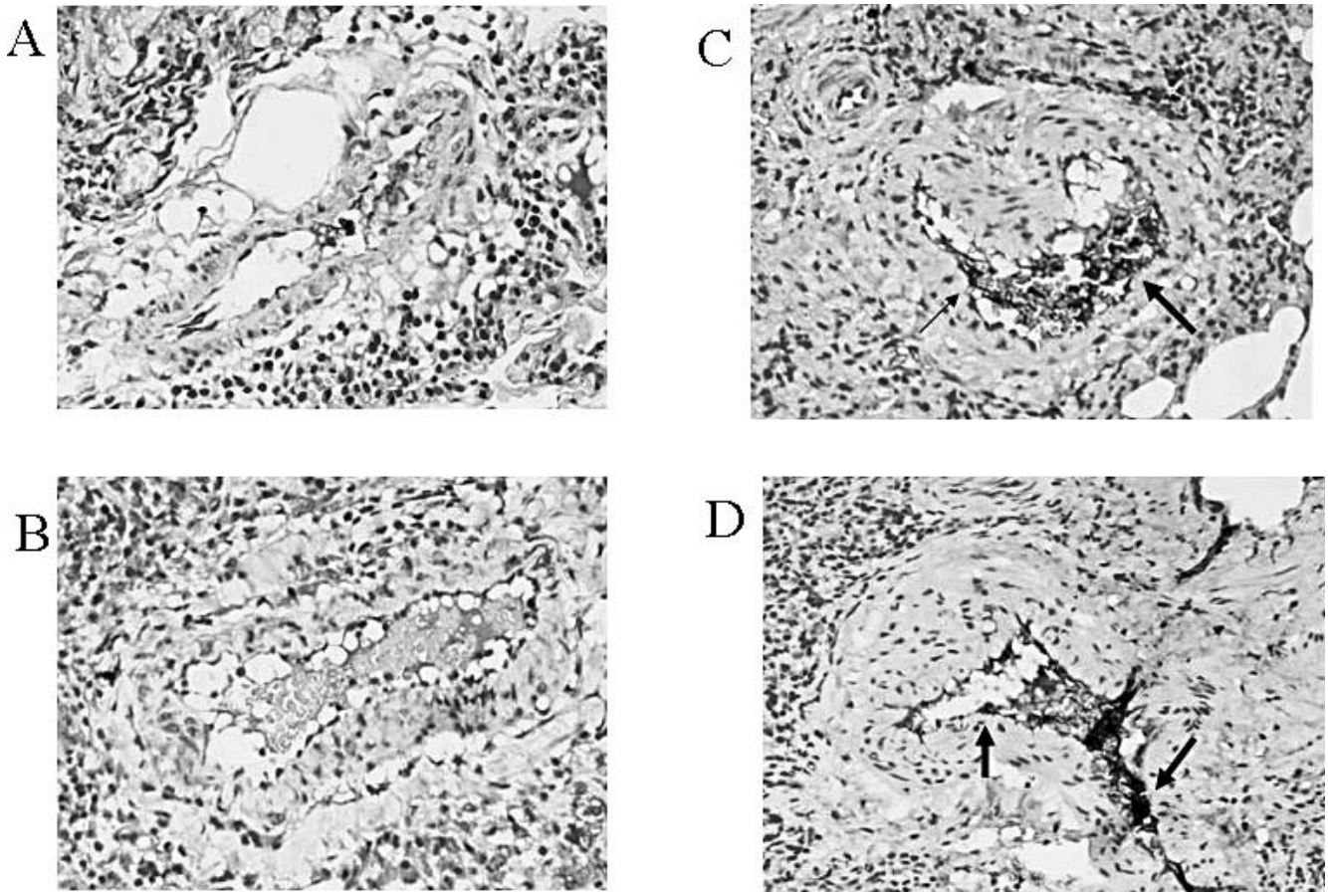

Figure 4. Immunoreactivity for ET-1. Increased ET-1 immunoreactivity (arrows) in the endothelium of the pulmonary artery in the banded rats at 4 weeks (C) and 7 (D) weeks, compared with sham-operated rats (A) or debanded rats (B). No difference in ET-1 immunoreactivity was found between debanded and sham-operated rats.

transitional changes, that is, hypertrophic left ventricle, or dilated left ventricle in both heart failure models. Steudel *et al*. (16) found hypoxia in PH with *eNOS* knockout models. It has also been reported that upregulated *eNOS* expression in lungs of hypoxia-induced PH did not reverse after hypoxia recovery (17). It has been proposed that upregulated *eNOS* expression can result from altered vascular mechanical force, or pulmonary vascular remodeling, rather than from hypoxic or nonhypoxic factors in the various PH models (18). Other reports suggest that both decreased cGMP levels in pulmonary arteries and impaired endothelium NO–mediated arterial relaxation could result from the dissociation between eNOS expression and NO production in rats with MCT-induced PH. Similarly, we found that, although debanding in rats attenuated PH progression, it did not alter *eNOS* gene expression in lungs. Unchanged lung cGMP levels were accompanied by upregulated lung eNOS; the expression of eNOS was not altered even when PH was reversed by debanding. Because cGMP levels were the same in both banded and debanded rats, and because, in banded rats at 7 weeks, PH was not offset by increased eNOS, we suggest that the gene expression of *eNOS* does not play a crucial role in the development or maintenance of PH secondary to LV failure.

In contrast, the increased local expression and release of ET-1 may contribute to abnormal pulmonary circulation vascular tone and structure. Previously, upregulated expression of both *preproET*-1 mRNA and ET-1 protein have been observed in lungs of hypoxia-induced PH (7), pulmonary vascular endothelial cells of patients with primary PH (19), and lungs of rats with heart failure induced by coronary artery ligation (20). Similarly, our study demonstrates that both *preproET*-1 mRNA and ET-1 increased in the lungs of aorta-banded rats at 4 and 7 weeks, and decreased after subsequent debanding. Therefore, we suggest that, in this animal model, upregulated ET-1 expression is involved closely in PH and is not compatibly offset by upregulated eNOS expression.

Further, the interactive roles of eNOS and ET-1 expression on the regulatory mechanisms in PH remain unresolved. However, NO clearly participates in mitigating vasopressor activity of ET-1 (21), inhibiting the translation of *preproET*-1 mRNA (22), augmenting the degradation of ET-1 protein (23), and reducing ET-1 formation *via* a

cGMP-dependent mechanism (24). Impaired endothelium-dependent vasodilation has also been found to coincide with enhanced eNOS expression in rat aortas (25). Despite an increase in basal NO production, a reduction in NO-mediated vasorelaxation of the thoracic aorta has been demonstrated in transgenic mice that overexpress eNOS. This activity has been related to reduced soluble guanylate cyclase and cGMP-dependent protein kinase (26).

Taking into consideration all of these findings, we infer that, in this model, upregulated eNOS expression does not completely compensate in offsetting pulmonary vasoconstriction and gene expression *via* the NO-cGMP pathway. We suggest, therefore, that *ET*-1 gene expression is upregulated earlier than *eNOS*, and that it is crucial in the mediation of PH in rats with aortic banding. Clinically, our findings offer insight regarding ET-1 antagonists in preventing the progression of PH in heart failure by LV pressure overloading.

We thank Mr. Sheng-I. Lue, Miss Yu-Ju Chen, Mr. Kou-Tong Hwang, and Miss Wan-Tzu Chen for technical assistance.

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