Differential Change in Expression of Pulmonary ET-1 and eNOS in Rats After Chronic Left Ventricular Pressure Overload

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Pressure overload in the left ventricle of the heart follows a chronic and progressive course, resulting in eventual left heart failure and pulmonary hypertension (PH). The purpose of this research was to determine whether a differential pulmonary gene change of endothelin (ET)-1 and endothelial nitric oxide synthase (eNOS) occurred in adult rats with left ventricular overload. Eight groups of eight rats each were used (four rats with banding and four rats with sham operations). The rats underwent ascending aortic banding for 1 day, 2 weeks, 4 weeks, and 12 weeks before sacrifice. Significant medial hypertrophy of the pulmonary arterioles developed in two groups (4 and 12 weeks). Increased pulmonary arterial pressures were noted in three groups (1 day, 4 weeks, and 12 weeks). The aortic banding led to significant increases in pulmonary preproET-1 messenger RNA (mRNA) at 1 day and 12 weeks, and in pulmonary eNOS mRNA at 1 day and 12 weeks. In addition, there was increased pulmonary eNOS content at 1 day and 12 weeks in the banded rats, and increased lung cGMP levels were observed at 1 day. Increased lung ET-1 levels were also noted at 1 day (banded, 310 ± 12 ng/g protein; sham, 201 ± 12 ng/g protein; $P < 0.01$), 4 weeks (banded, 232 ± 12 ng/g protein; sham, 201 ± 12 ng/g protein; $P < 0.01$) and 12 weeks (banded, 242 ± 12 ng/g protein; sham, 202 ± 12 ng/g protein; $P < 0.01$). This indicates that the upregulated expression of ET-1 developed at least 4 weeks before eNOS expression in the course of PH, and, thus, medication against ET-1 could play a crucial role in treating PH with cardiac dysfunction secondary to aortic banding. Exp Biol Med 231:948–953, 2006

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

Introduction

Pulmonary arterial pressure is determined by the regulation of pulmonary vascular tone and pulmonary vascular remodeling. In many congenital heart diseases, increasing pulmonary flow or pulmonary arterial pressure leads to pulmonary vascular remodeling, altered vascular reactivity, and, eventually, pulmonary hypertension (PH; Refs. 1, 2). Pulmonary vascular remodeling is characterized by endothelial cell injury, infiltration of smooth-muscle cells into the subintima and thickening of the medial layer in proximal vessels (3). Pulmonary vascular remodeling may progress to concentric pulmonary vascular lesions, neointimal formation, and pulmonary plexogenic arteriopathy.

Pulmonary vascular tone depends on the balance achieved from at least two interactive forces, shear and stretch stress. Shear stress induces nitric oxide (NO)–dependent vasodilation (4) and stretch stress induces a myogenic response through vasoconstrictors. However, the molecular mechanisms by which endothelial vasomediators contribute to PH remain undetermined.

Two major endothelium-dependent vasomediators have been identified in the lungs, NO and endothelin (ET)-1. NO is a potent endogenous vasodilator synthesized from L-arginine, catalyzed by the enzyme endothelial NO synthase (eNOS), and another nitric types of oxide synthase (5, 6). ET-1 has vasoconstrictive and mitogenic effects (7). Altered gene expression of *eNOS* and *ET*-1 has been noted in

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animals with hypoxia- or monocrotaline-induced PH (8–10), and in patients with PH (11). In a few studies, however, gene expression of pulmonary *eNOS* and *ET*-1 in retrograde PH were investigated. We have reported enhanced expressions of *eNOS* and *ET*-1 in rats subjected to aortic banding (12). However, most human patients undergoing histologic examination already show clinical signs of advanced PH, making it difficult to evaluate transitional alternations in expression of eNOS and ET-1. In this study, we further examined the time course of these change in adult rats undergoing an aortic banding to result in left ventricular dysfunction through pressure overload for 1 day, 2 weeks, 4 weeks, and 12 weeks.

Materials and Methods

Animal Model. Protocols were approved by the animal research committee of Kaohsiung Medical University, Kaohsiung, Taiwan. Male Wistar rats (6-weeks old, weighing ~220 g) were randomly assigned to aortic banding or sham operation. Aortic banding was performed as previously described (12, 13). Sham-operated animals underwent the same procedure, except that the aorta was not banded. 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) 1 day after the operation. One day, 2 weeks, 4 weeks, and 12 weeks after the operation, rats were again ventilated under similar anesthesia, and sacrificed by removing the ventilator after hemodynamic data were recorded.

Measurement of Systemic and Pulmonary Arterial Pressure. Using a cut-down procedure, a catheter (PE-50) was inserted into the femoral artery to allow pressure recording. A parasternal thoracotomy was performed and a catheter (PE-10) was inserted into the main pulmonary artery *via* the right ventricular outflow tract. The catheter was connected to pressure transducers. 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 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.

Tissue Preparation. Immediately after sacrifice, the rat lung and heart were rapidly perfused with normal saline and removed. The right ventricle was isolated by dissection along its septal insertion. Three pieces of lung tissue from different lobes were excised and immersed in 10% formalin for 24 hrs and stained with hematoxylin and eosin. One half of the remaining lung tissue was homogenized 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* ethylenediamine tetraacetic acid, 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. The homogenates were centrifuged at 14,000 *g* for 30 mins at 4°C. 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).

Measurement of 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, and centrifuged (2000 *g*, 15 mins, 4°C). The supernatant was dried under a steam of nitrogen at 60°C. The ET-1 protein in lung was measured using an enzyme immunoassay kit (Biomedica Group, Wien, Austria), and the cGMP content in lung and plasma was measured using a radioimmunoassay kit (Amersham Bioscience, England, UK).

Competitive Reverse Transcription (RT) Polymerase Chain Reaction (PCR). Total lung RNA was extracted using an acid guanidinium thiocyanate plus phenol plus chloroform mixture (14). Two competitive templates, mimic-preproET-1 and mimic-eNOS, were constructed as described previously (12) for measuring the levels of messenger RNA (mRNA) for preproET-1 and eNOS, respectively.

Immunohistochemistry for ET-1 and eNOS. Immunohistochemistry was performed on 5-μm-thick tissue sections fixed in formalin and embedded in paraffin. Slides were deparaffinized, hydrated, and treated with 0.3% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. Slides were then washed with phosphate-buffered saline (PBS) and incubated with antibodies to eNOS (1:100) or ET-1 (1:100; Oncogene, Boston, MA) for 1 hr at room temperature. Afterwards, slides were washed for 30 mins with PBS and incubated with biotinylated link antibody (DAKO, Glostrup, Denmark). Specimens were again washed with PBS, incubated for 30 mins with peroxidase-labeled streptavidin (DAKO), 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 with 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. $P < 0.05$ was considered statistically significant.

Results

Mean systemic arterial pressure was significantly decreased (mean ± SEM) in the banded rats at 1 day

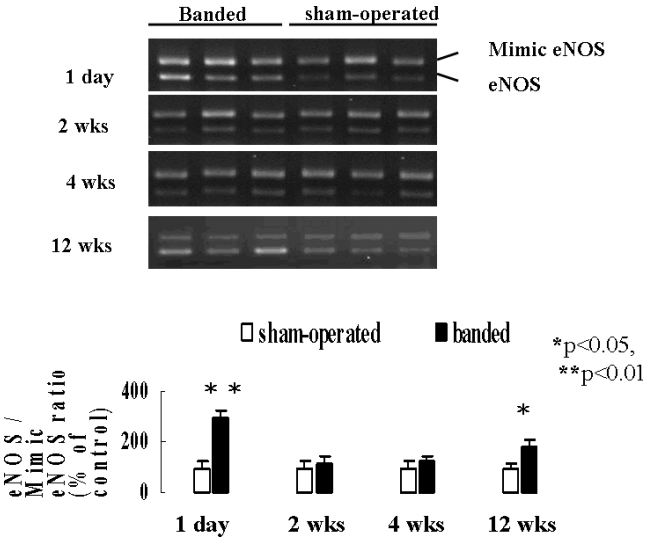

Figure 1. Expression of *eNOS* mRNA in lung tissues. Total RNA was extracted from the lung tissues, and RT-PCR was performed (upper panel). The lower panel shows normalized ratios of eNOS/mimic eNOS. There were significant increases in *eNOS* mRNA in the lungs of banded rats at both 1 day and 12 weeks, compared with the sham-operated rats. Values represent mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

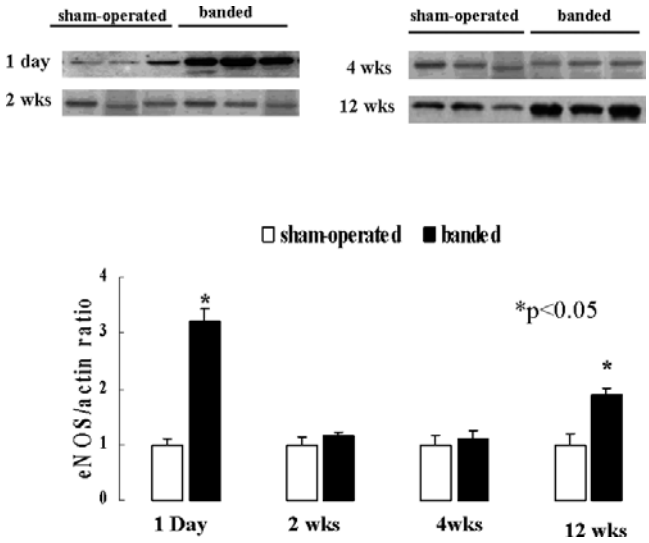

Figure 2. Western blot analysis of eNOS protein expression in the lungs of sham-operated and banded rats. The lower panel shows normalized ratios of eNOS/actin determined by densitometry. There were significant increases in eNOS in the lung of banded rats compared with sham-operated rats at both 1 day and 12 weeks. Values represent mean ± SEM. $^*P < 0.05$.

(banded, 78.0 ± 3.1 mm Hg; sham, 119.0 ± 3.4 mm Hg; $P < 0.01$; $n = 8$ for each group) and at 12 weeks (banded, 105.1 ± 3.6 mm Hg; sham, 117.2 ± 3.4 mm Hg; $P < 0.01$; $n = 8$ for each group), and mean pulmonary arterial pressures significantly increased at 1 day (banded, 45 ± 2.1 mm Hg; sham, 19.0 ± 2.1 mm Hg; $P < 0.05$; $n = 8$ for each group), 4 weeks (banded, 32 ± 2.1 mm Hg; sham, 20 ± 2.3 mm Hg; $P < 0.05$; $n = 8$ for each group) and 12 weeks (banded, 39 ± 2.1 mm Hg; sham, 20 ± 2.3 mm Hg; $P < 0.05$; $n = 8$ for each group). Histologically, the basic architecture of the lung respiratory apparatus remained normal in both groups. This included such structures as the bronchioles and alveoli at all times (1 day and 2, 4, and 12 weeks). However, pulmonary arterioles and artery showed significant medial hypertrophy at 4 and 12 weeks.

Expression of pulmonary eNOS mRNA in banded rats was significantly higher than sham at two times, at 1 day (banded, 3.10 ± 0.24; sham, 1.00 ± 0.23; $P < 0.01$) and 12 weeks (banded 1.84 ± 0.21, sham, 1.0 ± 0.12; $P < 0.05$), but not at 2 weeks or 4 weeks (Fig. 1). Western blot analysis revealed that pulmonary eNOS content significantly increased in banded rats at both 1 day (banded 3.20 ± 0.24; sham, 1.0 ± 0.23; $P < 0.05$) and 12 weeks (banded, 1.90 ± .21; sham, 1.0 ± 0.12; $P < 0.05$), but not at 2 weeks or 4 weeks (Fig. 2).

There was a marked increase in eNOS immunoreactivity in the endothelium of pulmonary arterioles of the banded rats at both 1 days and 12 weeks, but not at 2 weeks or 4 weeks (Fig. 3). There were no significant differences of plasma cGMP levels between sham and banded rats. However, there were significantly increased lung cGMP levels in banded rats at 1 day (aortic banding, 5.2 ± 0.33 pmol/g protein; sham, 3.1 ± 0.16 pmol/g protein), however, again, not at 2 weeks (3.1 ± 0.16 pmol/g protein), 4 weeks (3.7 ± 0.46 pmol/g protein), or 12 weeks (3.4 ± 0.36 pmol/g protein).

In contrast, expression of pulmonary preproET-1 mRNA significantly increased in banded rats in three of the four time periods. Increases were found at 1 day (banded, 2.11 ± 0.13; sham, 1.00 ± 0.14; $P < 0.01$), 4 weeks (banded 1.87 ± 0.13; sham, 1.0± 0.15; $P < 0.05$) and 12 weeks (banded, 2.32 ± 0.13; sham, 1.0 ± 0.12; $P < 0.01$), but not at 2 weeks (Fig. 4). Lung ET-1 content was significantly increased at 1 day ($P < 0.01$), 4 weeks ($P < 0.01$), and 12 weeks ($P < 0.01$), but not at 2 weeks.

There was also an increase in ET-1 immunoreactivity in the endothelium of the pulmonary arterioles of aorta-banded rats at 1 day, 4 weeks, and 12 weeks (Fig. 5), but not at 2 weeks.

Discussion

Heart failure and PH coexist frequently and contribute to each other. In this study, hearts of aorta-banded rats underwent a transition from stable compensated hypertrophy to heart failure. Retrograde PH develops along with this process (15). Our results are consistent with studies showing a significant rise in eNOS mRNA and eNOS protein in rat lung as early as 24 hrs after abdominal aorta banding (16). It was, therefore, proposed that the ascending aortic banding could result in acute left ventricle failure and PH in 1 day. If the left heart survives the acute pressure

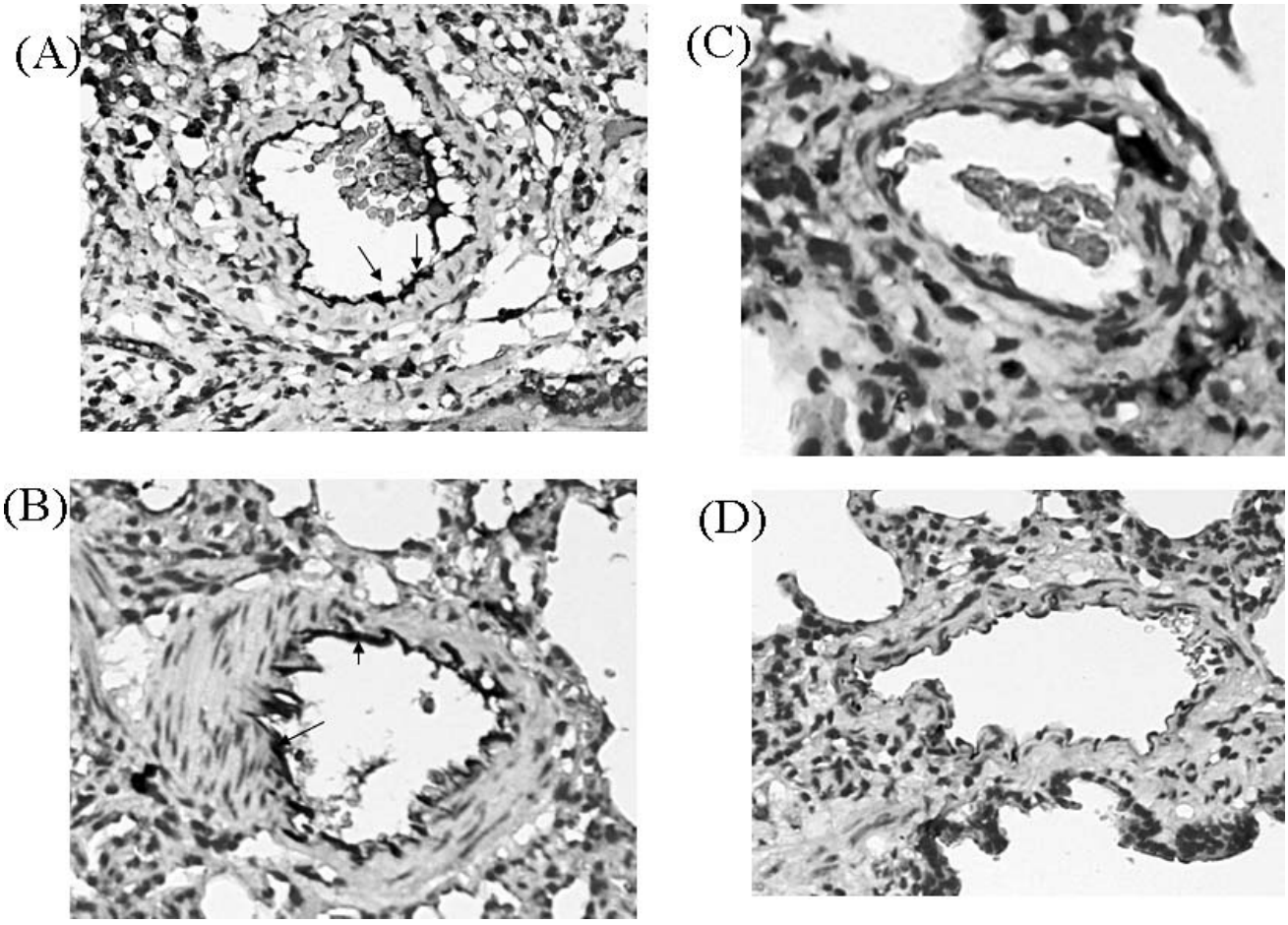

Figure 3. Immunoreactivity for eNOS. Increased eNOS immunoreactivity (arrows) was prominent in the endothelium of the pulmonary artery in banded rats at 1 day (A) and 12 weeks (B), compared with the sham-operated rats at 1 day (C) and 12 weeks (D). Magnification: ×200.

overload, it undergoes compensated hypertrophy. Therefore, the pulmonary arterial pressure could return to normal at 2 weeks, and could progress to PH whereas cardiac dysfunction developed after 4 weeks.

In vivo, increased lung eNOS levels have been reported in both study animals and in humans. In animal models, increased lung eNOS levels are observed in hypoxia-induced PH (8) and in high pulmonary flow (17). It is also observed in humans with PH (18). Upregulated *eNOS* mRNA levels and eNOS expression have been shown to correlate with vascular remodeling in rat lungs exposed to acute hypoxia (9). Moreover, in rats subjected to chronic hypoxia, increased *eNOS* gene expression has been noted in lungs, and *de novo* expression of eNOS protein has been found in microvascular endothelium (8). Our results also reveal that banding can upregulate eNOS expression as early as 12 weeks. However, other reports have found decreased *eNOS* mRNA and eNOS protein in lungs of pulmonary hypertensive rats with heart failure induced by coronary artery ligation (19). The discrepancy is probably related to the different transitional changes, that is, hypertrophic left ventricle or dilated left ventricle in both heart failure models.

Upregulated expression of both *preproET*-1 mRNA and ET-1 protein have been observed in lungs with hypoxia-induced PH (10), in pulmonary vascular endothelial cells of human patients with primary PH (18), and in lungs of rats with heart failure induced by coronary artery ligation (20). Similarly, our study demonstrated that both *preproET*-1 mRNA and ET-1 increased in the lungs of aorta-banded rats by 4 weeks, which was earlier than the eNOS expression.

NO can interact with ET-1. NO participates in mitigating the vasopressor activity of ET-1, inhibits translation of *preproET*-1 mRNA, augments the degradation of ET-1 protein, and reduces ET-1 formation, *via* a cGMP-dependent mechanism. At present, the interaction between eNOS and ET-1 expression and their regulatory mechanisms in PH are unresolved. Hyporesponsiveness to NO has been demonstrated in monocrotaline-induced PH (21). Bouloumie *et al.* also found that impaired endothelium-dependent vasodilation coincided with enhanced eNOS expression in rat aortas (22). Furthermore, despite an increase in basal NO production, a reduction in NO-mediated vasorelaxation of the thoracic aorta has been demonstrated in transgenic mice overexpressing eNOS, an

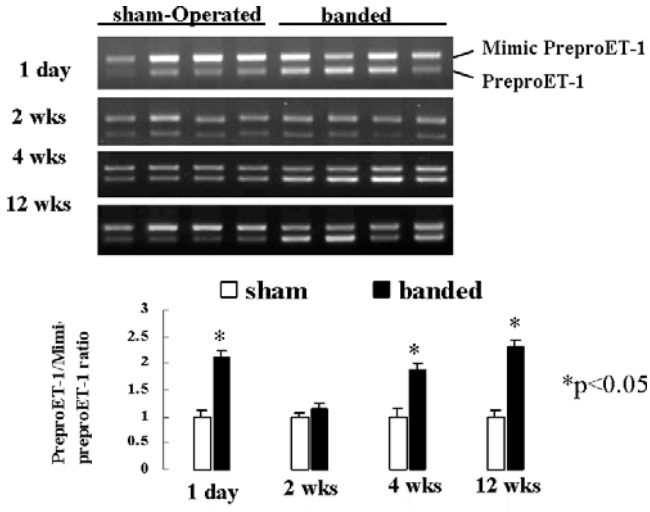

Figure 4. Expression of *preproET*-1 mRNA in lung tissues. Total RNA was extracted from lung tissue, and RT-PCR was performed (upper panel). The lower panel shows normalized ratios of preproET-1/mimic preproET-1. There were significant increases in *preproET*-1 mRNA in the lung of shunt rats at 1 day, 4 weeks, and 12 weeks, compared with the sham-operated rats. Values represent mean ± SEM. $^*P < 0.05$.

activity related to reduced soluble guanylate cyclase and cGMP-dependent protein kinase (23). It was reported that the impaired endothelium NO–mediated arterial relaxation apparently caused dissociation between eNOS expression and NO production in rats with monocrotaline-induced PH, and has resulted in decreased cGMP levels in pulmonary arteries. Similarly, our study showed that cGMP levels were unchanged despite increased eNOS expression in banded rats with PH, indicating that the upregulated eNOS expression did not completely compensate for pulmonary vasoconstriction *via* the NO-cGMP pathway. In conclusion, our results indicate that upregulated ET-1 gene expression occurs at least 4 weeks earlier than eNOS and is crucial in the mediation of PH in rats with aortic banding, and that the upregulated eNOS is not completely compatible with cGMP. Clinically, our findings offer insight regarding the use of ET-1 antagonists in treating PH in heart failure secondary to left ventricular pressure overloading.

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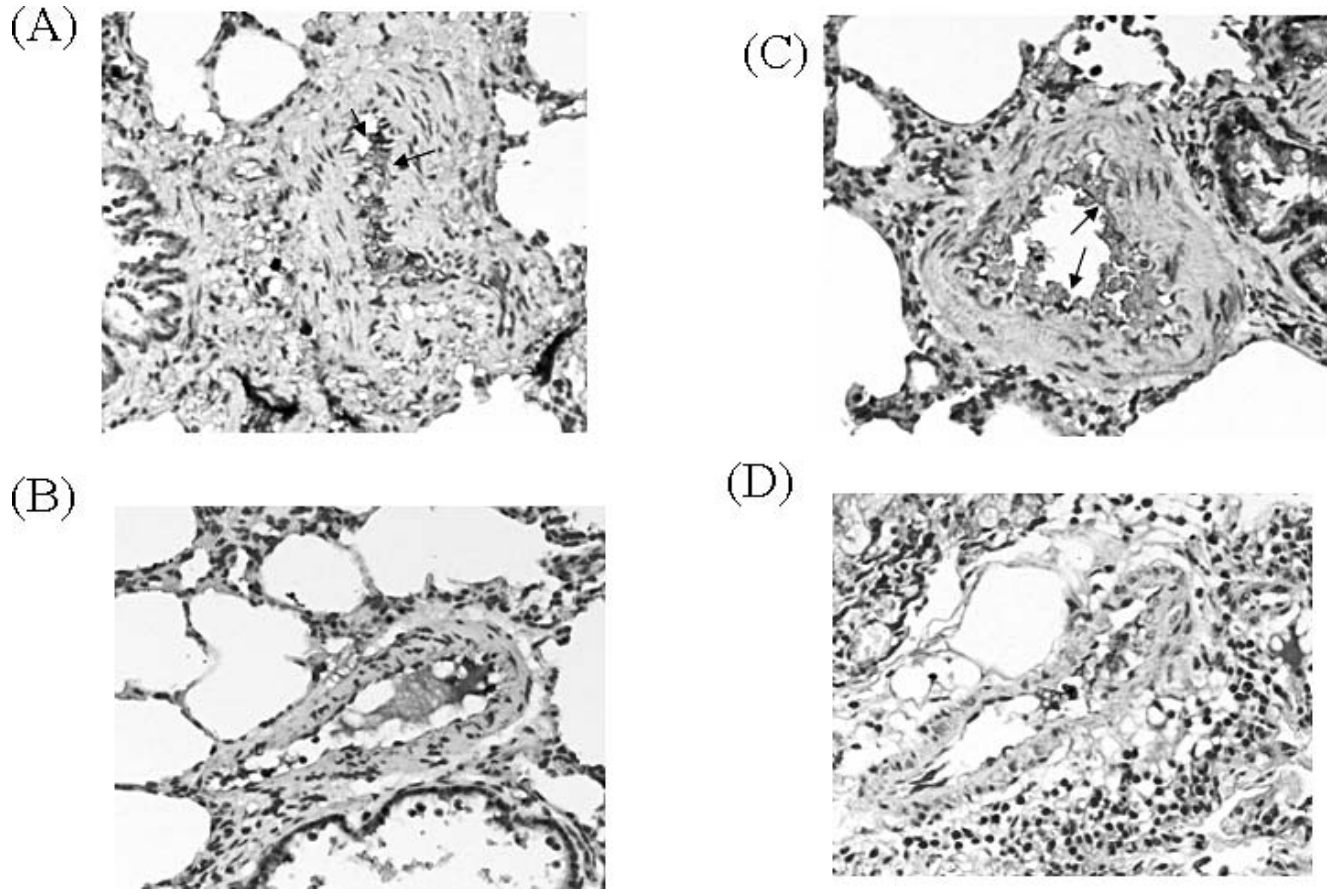

Figure 5. Immunoreactivity for ET-1. There was an increase in ET-1 immunoreactivity (arrows) in endothelia of the pulmonary arterioles of aorta-banded rats at 1 day (A) and 4 weeks (C) compared with sham-operated rats at 1 day (B) and 4 weeks (D).

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