Reduction Hypoxic Pulmonary Vascular Remodeling by Nitric Oxide From the Endothelium

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Abstract—We examined whether overproduction of endogenous nitric oxide (NO) can prevent hypoxia-induced pulmonary hypertension and vascular remodeling by using endothelial NO-overexpressing (eNOS-Tg) mice. Male eNOS-Tg mice and their littermates (wild-type, WT) were maintained in normoxic or 10% hypoxic condition for 3 weeks. In normoxia, eNOS protein levels, Ca$^{2+}$-dependent NOS activity, and cGMP levels in the lung of eNOS-Tg mice were higher than those of WT mice. Activity of eNOS and cGMP production in the lung did not change significantly by hypoxic exposure in either genotype. Chronic hypoxia did not induce iNOS expression nor increase its activity in either genotype. Plasma and lung endothelin-1 levels were increased by chronic hypoxia, but these levels were not significantly different between the 2 genotypes. In hemodynamic analysis, right ventricular systolic pressure (RVSP) in eNOS-Tg mice was similar to that in WT mice in normoxia. Chronic hypoxia increased RVSP and induced right ventricular hypertrophy in both genotypes; however, the degrees of these increases were significantly smaller in eNOS-Tg mice. Histological examination revealed that hypoxic mice showed medial wall thickening in pulmonary arteries. However, the increase of the wall thickening in small arteries (diameter <80 μm) by chronic hypoxia was inhibited in eNOS-Tg mice. Furthermore, muscularization of small arterioles was significantly attenuated in eNOS-Tg mice. Thus, we demonstrated directly that overproduction of eNOS-derived NO can inhibit not only the increase in RVSP associated with pulmonary hypertension but also remodeling of the pulmonary vasculature and right ventricular hypertrophy induced by chronic hypoxia. (Hypertension. 2001;37:322-327.)

Key Words: nitric oxide ■ nitric oxide synthase ■ hypoxia ■ hypertension, experimental ■ remodeling

Pulmonary hypertension, characterized by elevated pulmonary blood pressure, pulmonary vascular remodeling, and right ventricular hypertrophy, is a common complication of chronic lung disease and heart failure. Although the pathogenesis remains poorly understood, hypoxia is regarded as one of the critical factors that causes pulmonary hypertension and aggravates pathophysiological conditions. Hypoxic exposure induces pulmonary vasoconstriction and elevates pulmonary arterial pressure, which is a mechanical stimulus of smooth muscle cells (SMC). The persistent hypoxic exposure also induces the alterations of pulmonary vascular structure in experimental animal models and patients with pulmonary hypertension. Altered production and release of potent vasoactive substances by the endothelium are involved in the development of pulmonary vascular remodeling. Several vasoactive substances have growth-regulating properties, and pulmonary vascular remodeling could result from an imbalance between growth-inhibitory vasodilators and growth-promoting vasoconstrictors.

Nitric oxide (NO) synthesized by endothelial NO synthase (eNOS) is a potent vasodilator and is considered to play an important role in regulating pulmonary vascular tone. In recent studies of gene-engineered mice, eNOS-deficient mice showed mild pulmonary hypertension, and chronic hypoxia augmented the increase in pulmonary arterial pressure and pulmonary vascular remodeling. Therefore, the reduced production of eNOS-derived NO causes pulmonary hypertension and aggravates pathophysiological conditions. NO produced by eNOS is thought to serve as a protective factor against hypoxia in the pulmonary vasculature. Although endothelium-dependent vasodilatory response is shown to be impaired in pulmonary hypertension, there are inconsistent reports on the expression of eNOS in the vessels in pulmonary hypertension. Regarding experimental hypoxia-induced pulmonary hypertension, most studies demonstrated the upregulation of eNOS in pulmonary arteries. Therefore, it is considered that upregulation of eNOS is not sufficient for overcoming the detrimental effects of chronic hypoxia on pulmonary vasculature. Although inhaled NO and gene transfer of eNOS into the lung were demonstrated to inhibit hypoxic vasoconstriction, it is still unclear whether overproduction of endogenous eNOS-derived NO can pre-
vent pulmonary vascular remodeling and right ventricular hypertrophy associated with pulmonary hypertension.

We have reported previously that eNOS-overexpressing (eNOS-Tg) mice showed systemic hypotension caused by overproduction of NO derived from the endothelium. In these eNOS-Tg mice, eNOS protein levels and activity are significantly elevated in the lung compared with wild-type (WT) mice. This study was undertaken to examine the effects of endogenous NO overproduction on chronic hypoxia-induced pulmonary hypertension and vascular remodeling in eNOS-Tg mice.

**Methods**

**Animals**

Endothelial NOS-Tg mice, driven by the preproendothelin-1 (ppET-1) promoter, were derived from the same genetic background (C57BL/6) as previously described. These eNOS-Tg (n=79) and their littersmates, WT (n=77) mice, were exposed to hypoxia or normoxia for 3 weeks. For chronic hypoxia–induced pulmonary hypertension, mice were housed in an acrylic chamber with nonrebreathing adsorption-type oxygen concentrators to utilize excreted gas mixture of 10% O₂ to 90% N₂, which was produced by remodeling adsorption-type oxygen concentrators to utilize exhaust air (Teijin). Normoxic control mice were maintained in plastic cages in ambient air. In a separate experiment, N³-nitro-L-arginine methyl ester (L-NAME), a potent inhibitor of NO synthase, was administered in drinking water (1 mg/mL) 1 week before normoxic or hypoxic exposure and continued throughout the experimental period (4 weeks). All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine.

**Hemodynamic Measurements**

After 3 weeks of hypoxic or normoxic exposure, mice were weighed and anesthetized with sodium pentobarbital (80 µg/kg IP). A tracheostomy was performed, and the animal was mechanically ventilated (Harvard small animal ventilator model 687) with 10% O₂ for the hypoxic group and with room air for the normoxic group. For measuring systemic arterial pressure (SAP), a catheter was introduced into right femoral artery as described previously. After the chest was opened, a 27-gauge stainless steel needle attached to a pressure transducer was inserted directly into the right ventricle to measure right ventricular systolic pressure (RVSP) as an estimate of pulmonary arterial systolic pressure. SAP, RVSP, and heart rate (HR) were recorded on a Macintosh computer with the MacLab system (Bioresearch Center).

**Tissue Preparation, Weight Analysis, and Hematocrit Analysis**

After measurement of hemodynamic parameters, total blood was collected from the right ventricles and hematocrit values were obtained. The lung was removed and rinsed with PBS, blotted dry, and weighed. The heart was resected, both atria were removed, and the RV free wall was separated from the left ventricle and septum. Each of the ventricles was rinsed with PBS, blotted, and weighed.

**Analysis of NOS Protein and Measurement of Pulmonary cGMP Levels**

To examine the expression of eNOS protein in the lung, immunoblot analysis was performed with the use of a polyclonal anti-eNOS antibody (Transduction Laboratories), which recognizes both bovine and murine eNOS protein. NOS enzymatic activity was measured by the conversion of [³H]-L-arginine to [³H]-L-citrulline, and lung cGMP levels were measured with an enzyme immunoassay kit (Amersham) as described previously.

**Measurement of Plasma and Pulmonary ET-1 Protein Levels**

In 3 to 7 animals of each group, plasma and lung ET-1 protein was extracted with a Seppak C-18 column (Waters) and measured with a sandwich enzyme immunoassay kit (Peninsula Laboratories) as described previously.

**Morphological Examination (Immunohistochemistry and Image Analysis)**

To examine the vascular structural alterations and the expression of inducible NOS (iNOS), immunohistochemical staining was carried out by the labeled streptavidin biotin method with a monoclonal antibody against human smooth muscle actin (DAKO A/S) or a polyclonal antibody against mouse iNOS (Santa Cruz), respectively. Muscleization of peripheral arterioles was determined in the lung sections of 7 to 8 animals from each group as described previously. All pulmonary arterioles with the external diameter of <100 µm were counted and classified by circumferential staining with actin as nonmuscular, partially muscular (<75%), or completely muscular arterioles (>75%). For each animal, at least 10 arterioles from each lung were characterized. Furthermore, the wall thickness of the media was measured along the shortest curvature for all the muscularized vessels with an external diameter of 15 to 80 µm, 80 to 150 µm, and >150 µm, respectively. Approximately 50 vessels per animal were measured. All morphometric measurements were performed by 2 independent researchers operating in a blinded manner. Interobserver difference in the measurements was <5%.

**Statistical Analysis**

All values are expressed as mean±SEM. Two-way ANOVA was used to compare the differences among groups, and Bonferroni’s test was used for post hoc analysis. A value of *P*<0.05 was considered statistically significant.

**Results**

**Protein Analysis of eNOS and Measurements of cGMP Levels in the Lung**

In normoxia, eNOS protein levels and Ca²⁺-dependent NOS activity in the lung of eNOS-Tg mice were significantly higher than those in WT mice (Figure 1A and B). After 3-week hypoxic exposure, eNOS protein levels were slightly increased in both WT and eNOS-Tg mice. On the other hand, Ca²⁺-dependent NOS activity was not significantly changed by chronic hypoxia in both genotypes, though it showed a tendency to increase in WT mice (Figure 1B).

Because hypoxic exposure is reported to upregulate the iNOS gene in pulmonary artery endothelial cells and SMC, immunohistochemistry for iNOS was performed and Ca²⁺-independent NOS activity in the cytosolic fraction of the lung homogenates was measured. The immunostaining of iNOS was, however, not detected in either group exposed to hypoxia for 3 weeks. Ca²⁺-independent NOS activity in the lung was also very low at the basal levels and showed minimal changes by chronic hypoxia in both groups (data not shown).

Pulmonary cGMP contents in eNOS-Tg mice were 3 times as high as those in WT mice under normoxic conditions (Figure 1C). No significant increases were observed in either genotype exposed to hypoxia compared with those exposed to normoxia.
Both eNOS-Tg mice and WT mice showed similar hematocrit values under normoxic conditions (Tg, 0.41±0.01 versus WT, 0.39±0.02). Chronic hypoxic exposure resulted in a 1.5-fold increase in hematocrit values in both groups, and there was no difference between the 2 groups (Tg, 0.61±0.01 versus WT, 0.58±0.02).

Right Ventricular Hypertrophy Caused by Chronic Hypoxia

To examine the extent of chronic hypoxia–induced right ventricular hypertrophy, the right ventricle–to–left ventricle plus septum weight ratio (RV/LV+S) and right ventricle–to–body weight ratio (RV/BW) were measured. There were no significant differences in RV weight (RVW) and these parameters between eNOS-Tg and WT mice under normoxic conditions (RVW: Tg, n=28; 22.1±0.8 mg versus WT, n=30; 22.6±0.8 mg, NS). After 3 weeks of hypoxia, RVW and these parameters were increased in both groups; however, the degree of right ventricular hypertrophy was significantly more attenuated in eNOS-Tg mice than in WT mice (RVW: Tg, n=28; 25.6±1.1 mg versus WT, n=30; 28.3±1.1 mg, P<0.05).

Morphometric Analysis

Figure 3A shows the distribution of vessel types in both conditions. After 3 weeks of hypoxia, the distributions of peripheral muscular arterioles were increased in both groups. The reduction of nonmuscular arterioles in NOS-Tg mice was significantly smaller than in WT mice and the number of muscular arterioles in eNOS-Tg mice did not increase as much as in WT mice. The ratio of the number of muscular to nonmuscular arterioles shows that chronic hypoxia–induced neomuscularization was significantly attenuated in eNOS-Tg mice. L-NAME administration caused a 1.9-fold increase in muscularized arterioles in normoxic WT mice and a 1.8-fold increase in normoxic eNOS-Tg mice. Similarly, L-NAME caused a 1.3-fold increase in muscularized arterioles in hypoxic WT mice and a 1.5-fold increase in hypoxic eNOS-Tg mice compared with the untreated hypoxic mouse. Consequently, the difference in distribution of muscularized arterioles between hypoxic WT mice and hypoxic eNOS-Tg mice disappeared by L-NAME treatment.

As shown in Figure 4, the wall thickness of pulmonary vessels with external diameters of 80 to 150 μm and >150 μm were increased similarly by chronic hypoxia in the two genotypes. However, medial wall thickness of pulmonary vessels with the smallest external diameter was significantly reduced in eNOS-Tg mice than that in WT mice. In additional experiments, medial wall thickness in small pulmonary arteries was measured in both groups of mice treated with L-NAME. L-NAME induced increases in wall thickening to a similar extent in WT mice (+10% increases from that in nontreated normoxic WT mice) and eNOS-Tg mice (+12% increases from that in nontreated normoxic eNOS-Tg mice). Furthermore, L-NAME treatment canceled the difference in hypoxia-induced wall thickening between WT and eNOS-Tg mice.

ET-1 Levels in Lung and Plasma

Because the increased production of ET-1 in the lung and plasma is suggested to be involved in the mechanisms of
pulmonary hypertension, ET-1 levels were measured. Lung ET-1 levels in eNOS-Tg mice were similar to those in WT mice in normoxia (Tg, 0.41 ± 0.08 ng/mg protein versus WT, 0.70 ± 0.16 ng/mg protein, NS). Chronic hypoxia elevated ET-1 levels in the lung of each group. However, there was no difference in the increase of ET-1 levels between eNOS-Tg mice and WT mice (Tg, 1.80 ± 0.20 ng/mg protein versus WT, 2.11 ± 0.34 ng/mg protein, P < NS). Plasma concentra-
In this study, eNOS protein levels and Ca\textsuperscript{2+}-dependent NOS activity in eNOS-Tg mice were significantly higher than those in WT mice in normoxia and 3 weeks after hypoxia. Moreover, cGMP contents in the lung were also higher in eNOS-Tg mice compared with WT mice in both normoxic and chronic hypoxic conditions. Although it has been reported that endothelial dysfunction contributes to various forms of pulmonary hypertension in both humans and animals, WT mice showed a small increase in eNOS protein levels, and NOS activity and cGMP levels in the lung did not decrease after hypoxic exposure. Therefore, hypoxic exposure for 3 weeks did not impair the production of eNOS protein and its activity in the endothelium of pulmonary artery, and it is considered that the increases of NO production and subsequent cGMP production in the lung of eNOS-Tg mice may contribute to the attenuation of the RVSP elevation.

In normoxic conditions, RVSP in eNOS-Tg mice was similar to that in WT mice. This finding is different from a recent study of eNOS-deficient mice showing that mild pulmonary hypertension under normoxic conditions is caused by elevated pulmonary vascular resistance. However, there are redundant vasoactive substances modulating pulmonary circulation, and NO alone is not sufficient to control basal vascular tone. Therefore, the present finding is not contradictory to the study in eNOS-deficient mice.

Chronic hypoxic exposure causes pulmonary vascular remodeling characterized by proliferation and migration of SMC as well as an increased accumulation of extracellular matrix. In the histological analysis, this study demonstrated the increases of medial wall thickness in pulmonary arteries and the increases of neomuscularized arterioles in chronic hypoxia. However, medial wall thickening in pulmonary small vessels was attenuated and the number of neomuscularized arteries was reduced in eNOS-Tg mice. Moreover, NOS inhibition with L-NAME canceled the amelioration of pulmonary vascular structural changes in eNOS-Tg mice. These findings agree with the recent reports in which the administration of the NO precursor L-arginine and chronic NO gas inhalation could ameliorate the vascular structural changes induced by hypoxia. However, our study extended those studies in that overproduction of eNOS-derived NO inhibited pulmonary vascular remodeling.

One possible explanation for the improvement of pulmonary vascular remodeling is the direct action of NO on nearby vascular SMC through cGMP-dependent pathways. cGMP has been proposed to inhibit the mitogenesis and proliferation in cultured rat SMC, and cGMP-dependent protein kinase is also reported to inhibit proliferation of SMC in the pulmonary artery. Furthermore, the reduced mechanical stress in pulmonary vasculature of eNOS-Tg mice was probably involved in the improvement of vascular structural alterations. Because mechanical stress promotes SMC proliferation by inducing Ca\textsuperscript{2+} influx and increases growth factor expression, the reduced RVSP in eNOS-Tg mice might partly participate in the amelioration of pulmonary vascular remodeling.

Several factors are considered to be involved as modulators or mediators of the vascular structural alterations induced by hypoxic pulmonary hypertension. Among those factors, ET-1
is a potent endothelium-derived vasoconstrictor peptide with mitogenic effects on vascular SMC and is suggested to be involved in the development of pulmonary hypertension. Because it is proposed that ET-1 and NO function to regulate each other’s level during hypoxia and NO has been shown to inhibit the expression of ET-1 induced by hypoxia, ET-1 levels were examined in the lung and plasma under both normoxic and hypoxic conditions. However, ET-1 levels and the degree of their increases by hypoxia in the lung and plasma were not significantly different between the two genotypes. In this study, it is difficult to clarify the interaction of NO and ET-1 because of the use of ppET-1 promoter in the transgene of eNOS-Tg mice. However, it seems unlikely that the inhibitory effects of NO in increase in RVSP and pulmonary vascular remodeling are mediated by its action in ET-1 production. As a limitation in this study, we used RVSP as an estimate of pulmonary systolic pressure. Measurements of RVSP without taking transpulmonary pressure into account in the presence of positive-pressure ventilation may not reflect the real pulmonary systolic pressure. However, the comparison of RVSP between two genotypes successfully provide us the beneficial effects of eNOS-derived NO on pulmonary vascular beds.

In conclusion, we demonstrated in eNOS-Tg mice that not only the increases in RVSP but also pulmonary vascular remodeling and right ventricular hypertrophy induced by chronic hypoxia were attenuated by chronic increases of NO production in the endothelium of pulmonary vessels. Recent studies demonstrated the importance of therapeutic strategy toward increasing endothelial NO availability in the treatment of various vascular disorders. At present, there have been no reports investigating whether pharmacological manipulations can prevent or improve pulmonary hypertension and pulmonary vascular remodeling by increasing eNOS activity or protein expression. The present study shows the protective role of endogenous NO in the pathogenesis of chronic hypoxic pulmonary hypertension in vivo and implies that upregulation of eNOS or an increase of endogenous NO production can serve as important means for treatment of pulmonary hypertension.

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