Aerosol Gene Transfer With Inducible Nitric Oxide Synthase Reduces Hypoxic Pulmonary Hypertension and Pulmonary Vascular Remodeling in Rats

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Background—Nitric oxide (NO) is a potent vasodilator with an important role in the regulation of pulmonary vascular tone. The effects of NO synthase (NOS) gene transfer on pulmonary vascular remodeling associated with hypoxic pulmonary hypertension are unknown.

Methods and Results—We aerosolized 3×10⁹ pfu of an adenoviral vector containing inducible NOS gene (AdNOS2), constitutive NOS3 gene (AdNOS3), or no transgene (AdRR5) into rat lungs. Exhaled NO levels, monitored with chemiluminescence, were higher in AdNOS2-infected rats than in AdNOS3- and AdRR5-infected rats (at 3 days, 33±6 ppb, n=9, versus 17±4, n=9, and 6±2 ppb, n=3, P<0.05 for both). Exposure to FIO₂ 0.10 for 7 days increased pulmonary artery pressure from 19±4 mm Hg (baseline) to 27±1 and 26±2 mm Hg in AdNOS3- and AdRR5-infected rats, respectively, but only to 21±1 mm Hg in AdNOS2-infected animals (P<0.05). After 7 days of hypoxia, total pulmonary resistance in AdRR5- and AdNOS3-infected rats was significantly higher than in AdNOS2-infected animals (0.41±0.05 and 0.39±0.07 versus 0.35±0.03 mm Hg · mL⁻¹ · min⁻¹, respectively, P<0.05). Right ventricular hypertrophy was reduced in AdNOS2-infected rats [right ventricular/(left ventricular+septal) weight, 0.19±0.10 versus 0.28±0.10 and 0.32±0.10 in AdRR5- and AdNOS3-infected rats, respectively, P<0.05]. The percentage of muscularized precapillary pulmonary resistance vessels was also significantly decreased (18±4% versus 25±8% and 30±5% in AdRR5- and AdNOS3-infected rats, P<0.05).

Conclusions—Aerosol NOS2 gene transfer increases pulmonary NO production and significantly reduces hypoxic pulmonary hypertension and pulmonary vascular remodeling. Aerosol NOS2 gene transfer may be a promising strategy to target pulmonary vascular disorders. (Circulation. 2000;102:2880-2885.)

Key Words: nitric oxide synthase ■ hypertension, pulmonary ■ genes ■ vasculature ■ remodeling

Pulmonary hypertension (PHT) is characterized by changes in the pulmonary vascular wall, including abnormal vasoconstriction, enhanced adhesion of circulating platelets and leukocytes, excessive growth and proliferation of vascular smooth muscle cells, and increased extracellular matrix production.¹² Pulmonary endothelium normally releases vasoactive substances that control and modulate vessel tone and architecture.³⁴ One of these substances is nitric oxide (NO),⁴ which is synthesized from L-arginine by a class of enzymes termed NO synthases (NOS). The constitutive NOSs were initially isolated from brain (NOS1) and endothelial cells (NOS3) and are regulated by calcium and calmodulin. In the vessel wall, NO diffuses from the endothelium to underlying smooth muscle cells (SMCs), where it activates soluble guanylate cyclase, leads to elevated cGMP levels, and activates a cGMP-dependent protein kinase. The end result is vasorelaxation, as well as inhibition of migration, proliferation, and matrix production of SMCs.⁹ The inducible enzyme (NOS2) is calcium- and calmodulin-independent and is typically expressed on stimulation by bacterial endotoxin or cytokines in polymorphonuclear cells, SMCs, and macrophages.

PHT is associated with changes in pulmonary NO/cGMP signaling. Impaired pulmonary vasoreactivity to endothelium-dependent vasodilators and changes in NOS expression and NO production are reported in a variety of animal models of PHT and in human PHT.⁷⁻⁹ The role of NO in pulmonary vascular changes associated with PHT has been extensively studied in animal models of chronic hypoxia.¹⁰⁻¹² It remains controversial whether NO production or NOS3 expression is
increased or decreased during chronic hypoxia and to what extent these changes modulate pulmonary vascular remodeling.\textsuperscript{5,11,13} Impaired availability of bioactive NO during prolonged hypoxia may contribute to the architectural remodeling,\textsuperscript{14} as has been observed in NOS3-deficient mice exposed to chronic hypoxia.\textsuperscript{15,16} However, increased mRNA and protein levels of the different NOS isoforms have been reported in hypoxic rats,\textsuperscript{17,18} in which increased NOS3 expression correlated temporally with the onset of increased muscularity in small resistance vessels.\textsuperscript{19} The apparent discrepancy with regard to the role of NO in vascular remodeling may be caused by differences in experimental models, differences in NOS cofactor availability, in the natural history of PHT, hypoxic modulation of NOS enzyme activity, or in pulmonary vascular responsiveness to NO.

NOS3 immunoreactivity in remodeled pulmonary arteries from patients with PHT is either decreased or increased, reflecting potential temporal or spatial differences in the natural history of the disease.\textsuperscript{20–22} Reduced endothelial NO production has been observed in children with congenital heart disease\textsuperscript{23} and in adults with primary PHT,\textsuperscript{24} spurring interest in therapeutic strategies aimed at increasing NO availability in the lung. In newborns with persistent PHT and in patients with congenital heart disease and with preoperative high left-to-right shunt, inhaled NO is an effective therapy compared with extracorporeal membrane oxygenation.\textsuperscript{25–27} The short duration of action of inhaled NO and the requirements for continuous uninterrupted administration may limit the therapeutic utility of these strategies.

Recently, gene-based strategies have been developed to increase pulmonary NO production. Highly efficient pulmonary NOS3 gene transfer with first-generation adenoviral vectors significantly reduced acute hypoxic pulmonary vasoconstriction,\textsuperscript{28,29} but its effect on pulmonary vascular remodeling is unknown. We hypothesized that a single aerosol NOS2 or NOS3 gene transfer would result in sustained, high-level NO production in rat lungs, mitigate the development of hypoxic PHT, and reduce pulmonary vascular remodeling. Sustained increase in exhaled NO levels for 8 days after single aerosol delivery of the NOS2 gene mitigated hypoxic PHT and hypoxia-induced pulmonary vascular remodeling.

Methods

Recombinant Adenoviruses

Recombinant adenoviral vectors carrying a 3.7-kilobase pair EcoRI/BamHI fragment of the human NOS3 cDNA or no transgene (AdRR5) were generated and amplified as described previously.\textsuperscript{28,30} To construct a NOS2 adenovirus vector, a 3.9-Kb Smal restriction fragment of the murine inducible NOS2 cDNA\textsuperscript{31} comprising the entire protein coding region was cloned by blunt-end ligation between the strong enhancer/promoter of the cytomegalovirus (CMV) immediate early gene and the SV40 splice/polyadenylation signal in the bacterial plasmid pACCMVpLpA(−), as described previously.\textsuperscript{30}

Nitrite Measurements In Vitro

NO is oxidized to nitrite (NO$_2^-$) and to a lesser extent nitrate (NO$_3^-$) in culture media. HeLa cells were plated in a 6-well dish (3×10$^4$ cells/well) in DMEM supplemented with 2% FBS (GIBCO BRL), 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were infected with AdNOS2, AdNOS3, or AdRR5 at a multiplicity of infection of 100 for 4 hours in the presence or absence of N-nitro-L-arginine (L-NAME, 50 μmol/L). After 3 days, medium was removed and replaced with DMEM for 60 minutes. Cells infected with NOS3 were stimulated for 10 minutes with a calcium ionophore (2 μmol/L, A23187, Sigma). Nitrite concentration in conditioned medium was measured with a chemiluminescence analyzer (Sievers NOA 280).\textsuperscript{32}

Adenovirus-Mediated Gene Transfer to Rat Lungs

Male Wistar rats (weight, 300 to 350 g) were anesthetized with pentobarbital (50 mg/kg IP), intubated with a polyethylene tube (PE-240 tubing; 1.67-mm ID), and mechanically ventilated (60 cycles/min; tidal volume, 2.5 mL). A small silastic catheter was introduced in the trachea distal to the endotracheal tube. Viral solution (300 μL containing 3×10$^9$ plaque forming units AdNOS2, AdNOS3, or AdRR5) was aerosolized over 60 minutes into rat lungs during the inspiratory phase of the ventilatory cycle, as previously described.\textsuperscript{28}

Exhaled NO and Pulmonary cGMP Levels

At 3, 5, 7, and 10 days after gene transfer, rats were reanesthetized and mechanically ventilated as described above. During room air ventilation, exhaled air was collected in an impermeable 500-mL plastic bag. NO levels in exhaled air and room air were measured with chemiluminescence.

To measure pulmonary cGMP levels, animals were killed 3 days after NOS gene transfer, and the lungs were perfused through the pulmonary artery with NaCl 0.9% and frozen in liquid nitrogen. Pulmonary cGMP levels in tissue extracts were determined with a commercially available cGMP assay (Amersham).

Immunohistochemistry of Transduced Rat Lungs

Three days after aerosolization, the lungs of 3 animals from each group were prepared for immunohistochemistry as described.\textsuperscript{28} Seven-micron sections were incubated overnight with either anti-NOS3 or anti-NOS2 antibody followed by incubation for 1 hour with rabbit anti-mouse IgG peroxidase conjugate. Antibody binding was visualized with 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical Co) in 0.1 mol/L Tris buffer, pH 7.2, containing 0.01% H$_2$O$_2$. Sections were counterstained with Harris hematoxylin.

Hemodynamic Measurements After Acute and Chronic Hypoxia

Rats were aerosolized with AdNOS3, AdNOS2, and AdRR5 and allowed to recover for 24 hours. In a first series of experiments, rats were reanesthetized and mechanically ventilated after 3 days with a normoxic and a hypoxic gas mixture (FiO$_2$, 0.10 for 30 minutes) while pulmonary artery pressure (PAP) was monitored continuously. In a second protocol, adenovirus-infected rats were maintained at an FiO$_2$ of 0.10 in a sealed Plexiglas chamber for 7 days. After 7 days, pulmonary hemodynamics were measured after 1-hour equilibration in room air.

To measure PAP, a silastic catheter (0.30 mm ID, 0.64 mm OD) was introduced via the right jugular vein through the right atrium and right ventricle into the pulmonary artery. To measure systemic arterial pressure, a 2F catheter was placed in the left carotid artery. Both catheters were connected to a pressure transducer (model AA 016; Baxter) and a display oscilloscope (Press Ampl. 863, Siemens). The position of the pulmonary artery catheter was verified by the characteristic pressure tracing on the oscilloscope and was confirmed by autopsy. Cardiac output was measured by the thermodilution technique.\textsuperscript{33} Total pulmonary vascular resistance (mm Hg · min$^{-1}$ · L$^{-1}$) was computed by dividing PAP by cardiac output.

Histological and Morphometric Analysis

The heart and lungs were removed en bloc and the airways perfused through the trachea with 4% formaldehyde. The pulmonary vasculature was simultaneously perfused with physiological saline solution at a perfusion pressure of 100 cm H$_2$O. After fixation of the
lungs, the right ventricular free wall was dissected from the left ventricle and septum. Both ventricles were dried at 40°C. Sections (5 μm) from paraffin-embedded lungs were stained for elastin with resorcin-fuchsin solution. For each animal, 8 sections (from different segments of both lungs) were analyzed by an observer blinded to the treatment. Pulmonary vascular remodeling was measured by means of the method described by Roberts et al.14 Briefly, pulmonary vessels were classified according to wall structure and indexed to the respective airways as either preacinar or intra-acinar. SMCs were identified in the vessel wall between the internal and external elastic laminae. If >75% of the circumference of a blood vessel was encircled by 2 elastic laminae, the vessel was considered a muscular vessel (group 1); if <75% of the vessel circumference had distinguishable elastic laminae, the vessel was categorized as partially muscular (group 2). Vessels with only 1 visible elastic lamina were classified as nonmuscular (group 3), including acinar arteries and veins. For each section, 15 to 20 vessels were counted.

Statistical Analysis
All values are given as mean ± SD. An unpaired Student’s t test was used to compare groups. To isolate differences between multiple groups, ANOVA was performed with Fisher’s protected least-squares differences post hoc test for multiple comparisons. Significance in all cases was defined as P<0.05.

Results
Nitrite Production in Adenovirus-Infected HeLa Cells
Nitrite levels were significantly higher in medium from AdNOS3- and AdNOS2-infected cells than in medium from AdRR5-infected cells. Nitrite levels were significantly higher in medium from AdNOS2-infected than in conditioned medium from AdNOS3-infected cells. L-NAME reduced nitrite production to baseline in AdNOS3-infected cells but had only a partial inhibitory effect on AdNOS2-infected cells (Table).

Expression and Biological Activity of Recombinant AdNOS2 and AdNOS3 in Rat Lungs
To assess the efficacy and distribution of transgene expression in lungs of rats infected with AdNOS2 and AdNOS3, immunohistochemistry with isoform-specific antibodies was performed. NOS2 and NOS3 immunoreactivity was detected in bronchial epithelial cells and alveolar lining cells. There was no detectable endogenous NOS2 immunoreactivity in rat lungs infected with control AdRR5 virus, indicating that adenovirus infection per se did not result in expression of inducible NOS2 (Figure 1). No signs of pulmonary edema were observed in any group.

To study the biological activity of the expressed transgene products, exhaled NO levels were measured. Three days after gene transfer, exhaled NO levels were higher in AdNOS2-infected rats than in AdNOS3-infected animals, which in turn were significantly greater than in control virus-infected animals (Figure 2). Exhaled NO remained elevated in AdNOS2- and AdNOS3-infected animals for 7 days and returned to

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrite, μmol/L</th>
<th>L-NAME, 50 μmol/L</th>
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<tr>
<td>AdNOS2 (n=3)</td>
<td>7.50±0.16*</td>
<td>-</td>
</tr>
<tr>
<td>AdNOS2 (n=3)</td>
<td>2.36±0.14*</td>
<td>+</td>
</tr>
<tr>
<td>AdNOS3 (n=3)</td>
<td>0.38±0.01†</td>
<td>-</td>
</tr>
<tr>
<td>AdNOS3 (n=3)</td>
<td>0.66±0.03</td>
<td>+</td>
</tr>
<tr>
<td>AdRR5 (n=3)</td>
<td>0.47±0.05</td>
<td>-</td>
</tr>
</tbody>
</table>

*P<0.05 vs all; †P<0.05 vs AdNOS3 + L-NAME and vs AdRR5. Values are mean ± SD.

Figure 1. Histological analysis of transgene expression in rat lungs. Rat lungs were infected with murine NOS2 virus (a), control virus (b), or human NOS3 virus (c). Immunohistochemical staining was performed with murine NOS2 monoclonal antibody (a and b) and monoclonal antibody directed against NOS3 (c). In a and c, diffuse brown coloration of alveolar lining cells confirms presence of recombinant NOS2 and NOS3, respectively. No NOS2 immunoreactivity is observed in rat lungs infected with control virus (b).
baseline after 10 days (data not shown). Baseline levels of NO in room air were comparable at each time point and were equivalent to the levels of NO exhaled by AdRR5-infected animals at 3, 5, 7, or 10 days.

To investigate whether hypoxia modulates NOS enzyme activity, exhaled NO levels were measured 3 days after NOS gene transfer during room air breathing and after breathing 10% O2 for 30 minutes. In both AdNOS3-infected rats (n=9) and AdNOS2-infected rats (n=9), exhaled NO levels decreased by 15% during acute hypoxia (from 17±4 and 33±6 ppb NO in room air to 13±3 and 28±4 ppb NO in hypoxia, respectively).

**Aerosol NOS2 Gene Transfer Reduces Hypoxic Pulmonary Hypertension and Pulmonary Vascular Remodeling**

In acute hypoxia, PAP increased from 19±4 mm Hg at baseline to 28±2 mm Hg in AdRR5 virus-infected rats (n=8, P<0.05). The hypoxia-induced increase in PAP was reduced equally in AdNOS2-infected (n=7) and AdNOS3-infected (n=8) rats (21±5 and 23±2 mm Hg, respectively).

After breathing at FiO2 0.1 for 7 days, PAP was significantly lower in AdNOS2-infected rats than in AdRR5- or AdNOS3-infected animals (21±1 mm Hg, n=6, versus 26±2 mm Hg, n=4, and 27±1 mm Hg, n=8, respectively, P<0.05, Figure 3, left panel). Total pulmonary vascular resistance was reduced in AdNOS2-infected rats compared with AdRR5- and AdNOS3-infected animals (0.35±0.03 versus 0.39±0.07 and 0.41±0.05 mm Hg · mL⁻¹ · min⁻¹, respectively, P<0.05). The right ventricular/left ventricular+septal ratio was significantly lower in AdNOS2-infected animals than in AdRR5- and AdNOS3-infected rats (Figure 3, right panel). Systemic blood pressure remained unchanged and hematocrit rose to a similar extent after 7 days of hypoxia (60±3%, 60±2%, and 59±4% in AdNOS2-, AdNOS3-, and AdRR5-infected rats).

Morphometric analysis showed a higher percentage of fully muscular vessels in AdRR5- and AdNOS3-infected lungs than in AdNOS2-infected lungs (25±8% and 30±5% versus 18±4%, respectively, P<0.05, Figure 4). In addition, the percentage of only partially muscular vessels was higher in AdNOS2-infected rats than in AdRR5- and AdNOS3-infected animals (38±7% versus 30±4% and 28±5%, respectively, P<0.05). The percentage of nonmuscular vessels was similar in the 3 groups.

**Discussion**

NO/cGMP signal transduction plays an important role in the modulation of pulmonary vascular tone and structure in response to acute and chronic changes in oxygen tension. In this study, single aerosol delivery of adenoviral vectors expressing NOS2 and NOS3 gene in rat lungs resulted in immunoreactive NOS2 and NOS3 localized in bronchial and alveolar epithelial cells. NOS gene transfer increased pulmonary cGMP levels and elevated exhaled NO levels for at least 1 week. Significantly higher NO production was observed in NOS2-aerosolized rats than in NOS3- and control virus-infected rats. The acute hypoxia-induced vasoconstrictor response was significantly and equally reduced in both NOS2- and NOS3-infected rats. In contrast, in rats breathing FiO2 0.10 for 1 week, a single administration of AdNOS2 significantly reduced the rise in PAP, the increase in fractional right ventricular weight, and the degree of pulmonary vascular remodeling. In contrast, single administration of AdRR5 or AdNOS3 did not attenuate pulmonary vascular remodeling. Systemic blood pressure and the degree of hypoxia-induced polycythemia were unaffected by gene transfer. Taken together, increased pulmonary NO production after single aerosol NOS2 gene transfer in chronically hypoxic rats can selectively reduce the development of PHT and...
prevent the architectural changes in pulmonary resistance vessels without systemic side effects.

In rodents, chronic hypoxic PHT is characterized by augmented pulmonary vascular tone and extensive remodeling of pulmonary precapillary resistance vessels. These features are a hallmark of many pulmonary vascular disorders and are modulated by a variety of endothelium-derived vasoreactive mediators (NO, prostanoids, endothelin, C-type natriuretic peptide, and 5′-hydroxy-tryptamine) and by oxygen sensitive ion channels. NO/cGMP signaling is a compensatory response opposing vasoconstriction induced by endothelin and hypoxia in experimental animals and in some forms of human PHT. (1) Impaired release of NO in pulmonary vascular endothelial cells, (2) normal release of NO but impaired responsiveness of the pulmonary vascular target cells, or (3) diffusion barriers for NO caused by extensive architectural remodeling all may contribute to PHT and have initiated interest in therapeutic strategies aimed at increasing NO/cGMP signaling.

Impaired release of NO in hypoxic PHT was suggested by the attenuated relaxation to acetylcholine or adenosine diphosphate and by the significantly enhanced vasopressor responses to endothelin in arterial rings from chronically hypoxic but not from normoxic rats. A similar observation was made in pulmonary arterial rings obtained from explanted lungs of patients with chronic obstructive lung disease. The hypothesis that defective NO release in chronic hypoxia contributed to pulmonary vasomotor dysfunction and hypertension was further supported by the observation that L-arginine but not D-arginine fully restores NO/cGMP levels in preconstricted, isolated conduit pulmonary vessels or to increase pulmonary vascular tone and extensive remodeling of pulmonary precapillary resistance vessels, suggesting preserved reponse to endothelin-dependent relaxation of rat pulmonary artery in vivo. In isolated lungs from chronically hypoxic rats, however, maintained NO-dependent vasomotor responses have been reported that may be caused by differences in experimental design, animal species, or the degree and duration of hypoxia. Recently, it was reported that basal release of NO was maintained in lungs from patients with plexogenic or hypoxic PHT but that stimulated release of NO was significantly impaired. Our observation in rats breathing at $F_{O_2}$ 0.1 for 7 days that only high levels of NO, obtained after NOS2 gene transfer, protected against hypoxia-induced pulmonary hypertension and architectural remodeling suggests a critical level of NO production is necessary to offset hypoxia-induced hypertensive changes.

Impaired responsiveness of the pulmonary target cells to NO or impaired NO diffusion in remodeling pulmonary arteries may also contribute to the hypertensive process. In severely hypertensive rats, NO donors induced vasorelaxation of pulmonary resistance vessels, suggesting preserved responsiveness of pulmonary smooth muscle target cells whereas these vasodilators were unable to relax isolated rings from conduit pulmonary vessels or to increase pulmonary artery tissue cGMP levels. In preconstricted, isolated lungs from hypobaric, chronically hypoxic rats, inhaled NO induced a similar segmental pulmonary vasodilation as in preconstricted lungs from normobaric control animals.

Single aerosol delivery of NOS2 but not NOS3 gene before a 7-day hypoxic exposure prevented the development of pulmonary hypertension and the vascular remodeling associated with breathing 10% $O_2$ for 7 days. The difference between NOS treatment regimens may relate to the different absolute levels of NO production or to differential modulation of recombinant enzyme activity in hypoxia. The latter possibility is, however, unlikely because exhaled NO levels measured during an acute hypoxic challenge were equally and proportionately reduced in NOS2- and NOS3-infected rats.

Our findings suggest that single NOS aerosol gene transfer may be equally effective in preventing PHT as chronic NO inhalation or chronic L-arginine supplementation. All 3 strategies were associated with a significant reduction in architectural remodeling. Aerosol gene transfer had no significant pulmonary toxic side effects, and body weights were not affected by adenovirus infection (data not shown). However, cellular and humoral immune responses against the first-generation adenoviral vectors remain a considerable limitation of this approach and prohibit repeated vector administration. New vector technologies (eg, adeno-associated viruses, lentiviruses) probably will improve the vector efficacy profile with extended gene expression or will allow repetitive gene transfer so that aerosol gene therapy may become a promising therapeutic option for the treatment of hypoxic PHT. Extrapolation of NOS gene transfer to other models of PHT as well as its potential effect in reversing established pulmonary hypertension requires further investigation.

Acknowledgments

This work was supported by grants from the University of Leuven and the Fund for Scientific Research Flanders (to Stefan Janssens) and by the National Institutes of Health (NIH 55377 to Kenneth D. Bloch). Kenneth D. Bloch is an Established Investigator of the American Heart Association. Stefan Janssens is a Clinical Investigator for the Fund for Scientific Research Flanders and holder of a chair financed by Zeneca Inc. The authors thank Veerle Lauryens for technical assistance and Ann Vandenhoeck for help with the artwork.

References

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Circulation. 2000;102:2880-2885
doi: 10.1161/01.CIR.102.23.2880

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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