Gene Transfer of Endothelial Nitric Oxide Synthase to the Lung of the Mouse In Vivo

Effect on Agonist-Induced and Flow-Mediated Vascular Responses

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Abstract—The effects of transfer of the endothelial nitric oxide synthase (eNOS) gene to the lung were studied in mice. After intratracheal administration of AdCMVβgal, expression of the β-galactosidase reporter gene was detected in pulmonary airway cells, in alveolar cells, and in small pulmonary arteries. Gene expression with AdCMVβgal peaked 1 day after administration and decayed over a 7- to 14-day period, whereas gene expression after AdRSVβgal transfection peaked on day 5 and was sustained over a 21- to 28-day period. One day after administration of AdCMVeNOS, eNOS protein levels were increased, and there was a small reduction in mean pulmonary arterial pressure and pulmonary vascular resistance. The pressure-flow relationship in the pulmonary vascular bed was shifted to the right in animals transfected with eNOS, and pulmonary vasodepressor responses to bradykinin and the type V cGMP-selective phosphodiesterase inhibitor zaprinast were enhanced, whereas systemic responses were not altered. Pulmonary vasopressor responses to endothelin-1 (ET-1), angiotensin II, and ventilatory hypoxia were reduced significantly in animals transfected with the eNOS gene, whereas pressor responses to norepinephrine and U46619 were not changed. Systemic pressor responses to ET-1 and angiotensin II were similar in eNOS-transfected mice and in control mice. Intratracheal administration of AdRSVeNOS attenuated the increase in pulmonary arterial pressure in mice exposed to the fibrogenic anticancer agent bleomycin. These data suggest that transfer of the eNOS gene in vivo can selectively reduce pulmonary vascular resistance and pulmonary pressor responses to ET-1, angiotensin II, and hypoxia; enhance pulmonary depressor responses; and attenuate pulmonary hypertension induced by bleomycin. Moreover, these data suggest that in vivo gene transfer may be a useful therapeutic intervention for the treatment of pulmonary hypertensive disorders. (Circ Res. 1999;84:1422-1432.)

Key Words: gene transfer ■ in vivo ■ pulmonary vascular bed ■ mouse ■ nitric oxide

Low-baseline vascular resistance in the lung is maintained by endothelium-dependent and -independent mechanisms.1–3 Nitric oxide (NO) is released locally and plays an important role in maintaining pulmonary vascular resistance (PVR) at low levels.1–3 NO is formed from the enzymatic conversion of L-arginine by NO synthase (NOS), which exists in 3 isoforms, endothelial (eNOS; constitutive or NOS III), neural (NOS I), and inducible (iNOS, NOS II).4–6 The constitutive forms of the enzyme, eNOS and neural NOS, are regulated by calcium and calmodulin.4–6 Although iNOS has been shown to be constitutively expressed and present in cells exposed to cytokines, expression is upregulated in pathophysiological conditions.4–6 NO binds to the ferrous heme moiety of soluble guanylate cyclase in vascular smooth muscle and platelets to increase intracellular cGMP levels, inhibit platelet aggregation, and relax vascular smooth muscle.1–7 NO is believed to play an important role in maintaining PVR at low physiological levels,1–3,8–10 but the role of eNOS in the pathogenesis of pulmonary hypertension is controversial.11–18 Although eNOS activity has been reported to be reduced in patients with chronic pulmonary hypertension,11–13 it has also been reported that pulmonary vascular eNOS levels are increased in patients with primary pulmonary hypertension, as well as in experimental models of pulmonary hypertension.14–16 The role of eNOS in the development of pulmonary hypertension has also been investigated in genetic models, and it has been shown that targeted deletion of the eNOS gene results in increases in right ventricular pressure and pulmonary arterial pressure and in right ventricular hypertrophy in mice after chronic hypoxic exposure.8,9 It is generally believed that pathophysiological conditions that reduce NO production result in the development of pulmonary hypertension.8 Inhaled NO has been used in the management of pulmonary hypertension, and aerosolized type V phosphodiesterase...
inhibitors selectively reduce pulmonary arterial pressure by increasing cGMP levels in the lung.17–20 Gene transfer to the pulmonary vascular bed has been used to deliver viral vectors to the pulmonary artery and to pulmonary airway epithelium.21–28 Adenoviral transfer of the eNOS gene to the pulmonary vascular bed of the rat reduces the pulmonary vasoconstrictor response to hypoxia.22 Thus, transfer of the eNOS gene to the lung may constitute a new method for increasing pulmonary NO levels without altering systemic arterial pressure.

The present study was undertaken to investigate the effects of adenoviral gene transfer of eNOS to the pulmonary vascular bed in the intact-chest mouse using a model that we have recently developed. Our goal was to determine effects of eNOS-derived NO on the pulmonary vascular bed in normal mice and in mice with bleomycin-induced pulmonary hypertension.

Materials and Methods

Adenovirus Vectors

Replication-deficient recombinant adenoviruses serotype 5—encoding nuclear-targeted β-galactosidase (AdCMVβgal) and eNOS (AdCMVeNOS), both driven by a cytomegalovirus (CMV) promoter, and AdRSVβgal and AdRSVeNOS, driven by a Rous sarcoma virus (RSV) promoter, were prepared as previously described.27 Recombinant adenoviruses were plaque purified, and virus titer was determined by plaque assay on cells in culture. After purification, the adenoviral transfer of the eNOS gene to the pulmonary vascular bed in normal mice and in mice with bleomycin-induced pulmonary hypertension.

In Vivo Gene Delivery to the Pulmonary Vascular Bed

CD-1 mice (Harlan-Sprague Dawley, Indianapolis, IN) weighing 22 to 30 g were anesthetized with thiopentobarbital (85 to 95 μg/g IP) and ketamine (3 μg/g IP) and placed in a supine position on a thermoregulated surgical table. Body temperature was monitored continuously with a rectal temperature probe (Yellow Springs Instruments) and was maintained at 37°C with a water-jacketed heating blanket. Using a sterile technique, the trachea was approached via a midline neck incision and isolated by blunt dissection. Using a 27-gauge needle attached to a microtiter syringe, 50 μL of vehicle (3% sucrose) was instilled into the trachea. Micrometer cryostat sections were mounted on poly-L-lysine–coated slides and counterstained with eosin Y. Lung sections were examined for positive staining of β-galactosidase (blue nuclei) by light microscopy.

Expression of β-Galactosidase and eNOS

One day after administration of AdCMVβgal and 19 days after administration of AdRSVβgal, heparin (1000 U/kg IV) was injected, and the animals were killed with pentobarbital. The pulmonary vascular bed was perfused through the right ventricle with PBS at 80 mm Hg, and the lungs were removed. Expression of β-galactosidase was evaluated by measurement of β-galactosidase activity in tissue samples and by histochemical staining.

For determination of β-galactosidase activity (Galacto-Light Plus, Tropix), the lobes of the lungs were excised and homogenized in a solution of Tris-HCl (250 mmol/L, pH 7.4), EDTA (10 mmol/L), and EGTA (10 mmol/L) at 4°C. The supernatant was incubated in NADPH [1,2-dioxetane-3,2'-tricyclo-[3.3.1.13,7]-decan]-4-yl]-phenyl-β-galactopyranoside. Light emission was measured with a luminometer (Luminoscan RS, Labsystems) and calibrated with a standard curve generated with the use of purified Escherichia coli β-galactosidase. Protein concentrations of the samples were determined (DC Protein Assay, Bio-Rad Laboratories), and normalized β-galactosidase activity was expressed as milliunits β-galactosidase per milligram protein.28 In a separate series of experiments, the time course of β-galactosidase activity was determined. Lungs of mice were evaluated 1, 3, 5, 7, 14, and 21 days after transfection with AdCMVβgal and 1, 3, 5, 7, 14, 21, and 28 days after administration of AdRSVβgal.

For histochemical analysis of β-galactosidase localization, the transfected animals were killed and 2% paraformaldehyde/0.2% glutaraldehyde in PBS was perfused through the right ventricle and the trachea for 10 minutes. The lung lobes were cut in 2-mm sagittal sections, incubated in X-Gal stain (in PBS: 20 mmol/L K F e [CN] 4 -3H 2 O, 20 mmol/L K Fe [CN] 6 , 3H 2 O, 20 mmol/L MgCl 2 , and 1 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal; Sigma] in DMSO) for 2 hours at 24°C, rinsed in PBS, and postfixed in 7% buffered formalin for 6 hours. The sections were then placed in 20% sucrose for 12 hours, overlaid with OCT compound embedding medium (Sakura Finetek, Inc), and frozen in liquid nitrogen. Seven-micrometer cryostat sections were mounted on poly-L-lysine–coated slides and counterstained with cosin Y. Lung sections were examined for positive staining of β-galactosidase (blue nuclei) by light microscopy.

Expression of eNOS in mouse lung was assessed 1 day after transfection. Animals were killed, and the lungs were excised and processed immediately or quick-frozen in liquid nitrogen. To extract total protein, lungs were homogenized (tissue homogenizer; Polytron, Brinkmann Instruments) in ice-cold buffer (in mmol/L, HEPES [pH 7.9], MgCl 2 , 1.5, EDTA 0.2, DTT 0.5, and phenylmethylsulfonyl fluoride 0.5, and glycercol 26%) with NaCl (300 mmol/L final) and incubated on ice for 30 minutes. After centrifugation twice at 15 000g at 4°C for 20 minutes, the supernatant was mixed with an equal volume of 2% SDS/1% β-mercaptoethanol and fractionated using 8% SDSPAGE (70 μg/lane). Proteins were then transferred to a nitrocellulose membrane (Hybond enhanced chemiluminescencence; Amersham Life Sciences) by semidry electroblotting for 1 hour. The membranes were blocked for 1 hour at room temperature with Blotto-Tween (5% nonfat dry milk and 0.1% Tween-20) and incubated with a primary monoclonal mouse anti-eNOS IgG antibody (Santa Cruz Biotechnology). Bound antibody was detected with labeled rabbit anti-mouse IgG secondary antibody (Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence.

For immunohistochemical localization of eNOS and iNOS in lungs of control mice and in lungs of mice transfected with AdCMVeNOS, the mice were killed and the lungs were sectioned as described above. The lungs segments were overlaid with OCT compound and frozen in liquid nitrogen. Seven-micrometer cryostat sections were mounted on slides, washed twice with PBS, blocked with normal rabbit serum for 20 minutes, and stained using a commercial assay system (Santa Cruz Biotechnology). Briefly, the sections were incubated for 2 hours in a primary monoclonal mouse anti-eNOS IgG antibody (Santa Cruz), followed by a 30-minute incubation in biotinylated secondary antibody. The sections were then incubated for 30 minutes with horseradish peroxidase–streptavidin, followed by development with horseradish peroxidase–substrate mixture. The sections were then rinsed and counterstained with hematoxylin and mounted.

For determination of eNOS enzyme activity (Calbiochem-Novabiochem Corp), l-arginine to l-citrulline conversion was assayed in pulmonary extracts. Briefly, lung samples were homogenized in a solution of Trits-HCl (250 mmol/L, pH 7.4), EDTA 10 mmol/L, and EGTA (10 mmol/L) and centrifuged at 12 000 rpm for 10 minutes at +4°C. The supernatant was incubated in NADPH (10 mmol/L), l-[3H]arginine (1 μCi/μL), CaCl 2 (6 mmol/L), Tris-
Measurement of Tissue and Plasma cGMP Levels

One day after instillation of vehicle, AdCMVβgal, or AdCMVεNOS, blood was withdrawn and the plasma removed. The lungs were then perfused with PBS, quick-frozen in liquid nitrogen, and stored at −70°C until determination of cGMP levels. Plasma samples were added to ethanol, vortexed, and incubated at room temperature for 5 minutes. The samples were then centrifuged at 2500 g for 10 minutes, and supernatant was collected, dried by vacuum centrifugation, and resuspended in phosphate buffer. Whole lung tissue was homogenized in 1 mL ice-cold 6% trichloroacetic acid, pH 4.0. Each sample was then centrifuged at 1500 g for 10 minutes at 4°C, the supernatant was transferred to a 10-mL test tube, and the trichloroacetic acid was extracted with H2O-saturated diethyl ether. The samples were assayed for cGMP using an enzyme immunoassay kit (Cayman Chemical). Lung cGMP levels are expressed as pmol cGMP per mg of protein, and plasma levels are expressed as pmol/mL.

Measurement of Pulmonary Vascular Responses and Cardiac Output

One day after administration of vehicle or adenovirus, the mice were anesthetized with thiopentobarbital (85 to 95 μg/g IP) and ketamine (3 μg/g IP) and placed on a thermoregulated surgical table. The catheter was cannulated (PE 90 tubing) for the administration of agonists and tachometer (model 7P44A, Grass Instruments). The left jugular vein was cannulated (PE 10 tubing) to monitor the pressure waveform. A trachea was cannulated (PE 10 tubing) to maintain a patent airway, and the animals breathed room air enriched with 95% O2/5% CO2. A femoral artery was cannulated (polyethylene [PE] 10 tubing heated 0.15N NaOH in 0.9% NaCl at a concentration of 3 mg/mL and U46619 (1.0 μg/mL) was dissolved in 100% ethanol and diluted with 0.9% NaCl. The type

Drugs

Endothelin-1 (ET-1; Peptide Research Laboratories, Tulane University School of Medicine), angiotensin II, bradykinin, norepinephrine, and bleomycin (Sigma) were dissolved in 0.9% NaCl. U46619 (9.11-dideoxy-11a,9α-epoxymethano-prostaglandin F2α) (Upjohn) was dissolved in 100% ethanol and diluted with 0.9% NaCl. The type V eNOS-selective phosphodiesterase inhibitor zaprinast (2-[3-(2-pyridyl)-5-azapurin-6-one; Rhone-Poulenc) was dissolved in 0.15N NaOH in 0.9% NaCl at a concentration of 3 mg/mL, and diluted in 0.9% NaCl. The vehicles for the agonists used in this study did not alter vascular pressures or responses to the agonists. The stock solutions were stored in a freezer in 1-mL opaque tubes, and working solutions were prepared daily and kept on crushed ice during the course of the experiment.

Statistics

The data are expressed as mean±SE and were analyzed using ANOVA followed by the Newman-Keuls post hoc test to determine statistical significance in multiple testing between groups or a Student t test. P<0.05 was used as the criterion for statistical significance.
Results

Gene Transfer to the Pulmonary Vascular Bed

One day after AdCMVβgal transduction, β-galactosidase protein was expressed diffusely in airway epithelial cells in proximal bronchi and distal bronchioles, alveolar lining cells, and adventitial cells in medium- and resistance-sized (100- to 300-μm) pulmonary arteries (Figure 1). β-Galactosidase staining was not observed in lungs from mice treated with vehicle or with AdCMVeNOS (Figure 1).

β-Galactosidase activity was minimal in lungs from mice treated with vehicle or AdCMVeNOS (0.14 ± 0.07 mU/mg protein). In lungs from mice treated with AdCMVβgal, β-galactosidase activity was 23 ± 5.1 mU/mg protein (P < 0.05) 1 day after transfection. β-Galactosidase activity was similar in all lung lobes from mice treated with AdCMVβgal.

The time course of the expression of β-galactosidase activity was determined in the lungs of mice 1, 3, 5, 7, 14, and 21 days after transfection with AdCMVβgal and 1, 3, 5, 7, 14, 21, and 28 days after transfection with AdRSVβgal. In mice treated with AdCMVβgal, β-galactosidase activity reached peak expression 1 day after transfection and decreased gradually over a 7- to 14-day duration (Figure 1). Fourteen days after transfection, β-galactosidase activity was not significantly different when compared with baseline activity (Figure 1). The T_{1/2} of β-galactosidase activity (defined as the number of days required for the activity to return to one-half peak expression) was ≈ 5 to 7 days after transfection (Figure 1).

In a separate series of experiments, the duration of expression of β-galactosidase activity was determined in mice transfected with AdRSVβgal. In mice treated with AdRSVβgal, β-galactosidase activity reached peak expression 5 days after transfection, remained elevated for 14 to 21 days, and gradually decreased to ≈ 30% to 50% of maximum at 28 days after transfection (Figure 1). The T_{1/2} for AdRSVβgal was ≈ 21 days after transfection (Figure 1). Peak levels of β-galactosidase activity were similar in mice treated with AdCMVβgal or AdRSVβgal (Figure 1).

Immunohistochemistry of mouse lung 1 day after AdCMVeNOS transfection demonstrated eNOS protein in the majority of the airway epithelial cells, in the alveolar cells lining the lung parenchyma, and in smooth muscle cells of resistance-sized arteries (100 to 300 μm; Figure 2). The distribution of staining for eNOS in nonendothelial cells was similar to that of β-galactosidase staining in mice treated with AdCMVβgal (Figure 2). Immunohistochemical analysis of lung 1 day after transfection with AdCMVeNOS, shows no identifiable increase in iNOS expression (data not shown).

Western immunoblot analysis of whole lung tissue using an eNOS-specific antibody detected abundant levels of eNOS (135-kDa) protein in mice transfected with AdCMVeNOS (Figure 2, lane 2), and levels were greater than in lungs of mice transfected with AdCMVβgal (Figure 2, lane 1). Levels of α-actin protein (42 kDa) were similar in lungs harvested from mice transfected with AdCMVβgal or AdCMVeNOS (Figure 2). eNOS protein levels were similar in mice treated with vehicle or AdCMVβgal (data not shown).

In experiments to determine the activity of the eNOS transgene, the conversion of L-[3H]arginine to L-[3H]citrulline was determined in samples of lungs of mice treated with vehicle, with AdCMVβgal, and with AdCMVeNOS. Calcium-dependent L-[3H]arginine conversion levels in mice 1 day after treatment with vehicle (n = 5) or AdCMVβgal (n = 6) were 13.4 ± 3.2 and 11.9 ± 4.0 pmol/mg protein per hour, respectively. Calcium-dependent L-[3H]arginine conversion to L-[3H]citrulline was 22.1 ± 3.6 pmol/mg protein per hour in lung samples from mice 1 day after transfection with AdCMVβgal (Figure 2).
AdCMVeNOS (n=6; P<0.05 when compared with AdCMVβgal). In experiments to determine the role of calcium-independent conversion of L-arginine, an indicator of iNOS activity, the conversion of L-[3H]arginine to L-[3H]citrulline was studied. Calcium-independent conversion levels of L-[3H]arginine to L-[3H]citrulline in mouse lung 1 day after treatment with vehicle (n=5) or AdCMVβgal (n=6) were 3.3±1.4 and 4.4±1.2 pmol/mg protein per hour, respectively. In mice treated with AdCMVeNOS (n=6), L-[3H]arginine→L-[3H]citrulline conversion was 5.1±1.7 pmol/mg protein per hour (P>0.05 when compared with AdCMVβgal).

Lung and plasma cGMP concentrations were measured 1 day after intratracheal administration of vehicle, AdCMVβgal, or AdCMVeNOS (Table 1). Lung and plasma cGMP concentrations were similar in mice treated with vehicle or with AdCMVβgal (Table 1). cGMP concentrations were significantly increased in whole lung tissue and plasma from mice transfected with AdCMVeNOS when compared with cGMP levels in control or AdCMVβgal-treated mice (P<0.05; Table 1).

Effect of AdCMVeNOS on Baseline Hemodynamics and Pressure-Flow Relationship

One day after transfection with AdCMVβgal or AdCMVeNOS, mice did not show overt signs of infection (fever, tachypnea, or tachycardia). Baseline cardiovascular parameters were measured in animals 1 day after intratracheal administration of AdCMVβgal and AdCMVeNOS, and the data are summarized in Table 2. Baseline cardiovascular parameters were similar in animals receiving vehicle or AdCMVβgal (data not shown). Systemic arterial pressure, cardiac output, total peripheral resistance, right atrial pressure, and pulmonary arterial wedge pressure were similar in animals receiving vehicle or AdCMVeNOS (Table 2). Mean pulmonary arterial pressure and PVR were significantly reduced in animals transfected with AdCMVeNOS (Table 2).

The effect of AdCMVeNOS transfection on the pressure-flow relationship in the pulmonary vascular bed was examined in the intact-chest mouse (Figure 3). Pulmonary arterial pressure rises as pulmonary blood flow is increased by raising body temperature. Pulmonary arterial pressure rises more rapidly at flow rates of 5 to 8 mL/min and more slowly as higher pulmonary blood flow rates are reached (Figure 3). The pressure-flow curves were similar in mice treated with vehicle or AdCMVβgal (data not shown). At the lowest flow rate studied (5 mL/min), pulmonary arterial pressure was similar in animals treated with AdCMVβgal and AdCMVeNOS (Figure 3). However, as the level of blood flow increased, the pressure-flow curve in mice treated with AdCMVeNOS was shifted to the right, which indicates a decrease in PVR at higher levels of blood flow (Figure 3).

Effect of AdCMVeNOS on Agonist-Induced Vasodepressor and Pressor Responses

The influence of AdCMVeNOS transfection on pulmonary and systemic responses to bradykinin and zaprinast was investigated (Figure 4). When pulmonary arterial pressure was increased to a high level with U46619 or ET-1 (23 to 27 mm Hg), intravenous injections of bradykinin and zaprinast induced dose-related decreases in pulmonary arterial and systemic arterial pressures (Figure 4). Decreases in pulmonary and systemic arterial pressures were similar in vehicle control and AdCMVβgal-treated animals (data not shown). In mice treated with AdCMVeNOS, the decrease in pulmonary arterial pressure in response to intravenous injection of the 1 µg/kg dose of bradykinin and to all doses of zaprinast was

Table 1: Influence of AdCMVβgal and AdCMVeNOS on Lung and Plasma Concentration of cGMP in the Mouse

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung, pmol/mg Protein</th>
<th>Plasma, pmol/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle)</td>
<td>0.110±0.04</td>
<td>7.3±0.9</td>
</tr>
<tr>
<td>AdCMVβgal (n=10)</td>
<td>0.162±0.06</td>
<td>8.1±1.1</td>
</tr>
<tr>
<td>AdCMVeNOS (n=12)</td>
<td>0.327±0.09*</td>
<td>12.6±2.4*</td>
</tr>
</tbody>
</table>

n indicates number of experiments.

*P<0.05 vs control.
significantly greater than in mice treated with AdCMVβgal (Figure 4). In contrast to results in the pulmonary vascular bed, decreases in systemic arterial pressure in response to bradykinin and zaprinast were not altered in animals treated with AdCMVεNOS (Figure 4). Additional studies showed that decreases in pulmonary and systemic arterial pressure in response to intravenous injections of calcitonin gene-related peptide and vasoactive intestinal peptide, which are endothelium-independent vasodilators, were similar to responses obtained in mice treated with AdCMVβgal (data not shown).

The influence of AdCMVεNOS transfection on pulmonary and systemic pressor responses to angiotensin II, ET-1, norepinephrine, and U46619 was investigated (Figures 5 and 6). Intravenous injections of angiotensin II, norepinephrine, and U46619 induced dose-related increases in pulmonary arterial and systemic arterial pressure (Figure 5). Under normal low-tone conditions, ET-1 induced dose-related increases in pulmonary arterial pressure and biphasic changes in systemic arterial pressure characterized by an initial depressor response followed by a secondary pressor response. Increases in pulmonary and systemic arterial pressure in response to angiotensin II, ET-1, norepinephrine, and U46619 were similar in vehicle control and AdCMVβgal-treated animals (data not shown).

In mice treated with AdCMVεNOS, increases in pulmonary arterial pressure in response to intravenous injections of angiotensin II and ET-1 were reduced when compared with responses in mice treated with AdCMVβgal (Figure 5). In contrast to pulmonary pressor responses to angiotensin II and ET-1, which were reduced in animals administered AdCMVεNOS, the increases in systemic arterial pressure in response to the 2 agonists were not altered (Figure 5). In contrast to the attenuated pulmonary pressor responses to angiotensin II and ET-1 (Figure 6), increases in systemic arterial pressure (data not shown) in response to norepinephrine and U46619 were similar in mice treated with AdCMVβgal or AdCMVεNOS.

In a separate set of experiments, the time course of the alteration of pulmonary vasodepressor responses to bradykinin and pulmonary pressor responses to ET-1 was determined in mice treated with AdCMVεNOS or AdRSVeNOS (Figure 7). Pulmonary vascular responses to bradykinin and ET-1 were evaluated in mice 1, 3, 5, 7, or 14 days after transfection with AdCMVεNOS (Figure 7). In mice treated with AdCMVεNOS, the maximum augmentation of pulmonary vasodepressor response to bradykinin was observed 1 day after transfection, and the responses to bradykinin were significantly different from control responses 7 days after transfection (Figure 1). The augmented decrease in pulmonary arterial pressure in response to bradykinin returned to baseline response level 7 to 14 days after transfection with AdCMVεNOS (Figure 7). The maximum

### TABLE 2. Baseline Hemodynamic Measurements in Mice Transfected With AdCMVβgal or AdCMVεNOS

<table>
<thead>
<tr>
<th></th>
<th>AdCMVβgal</th>
<th>AdCMVεNOS</th>
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<tbody>
<tr>
<td><strong>Aortic pressures</strong></td>
<td></td>
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</tr>
<tr>
<td>Systolic pressure, mm Hg</td>
<td>92±8</td>
<td>90±10</td>
</tr>
<tr>
<td>Diastolic pressure, mm Hg</td>
<td>83±8</td>
<td>86±9</td>
</tr>
<tr>
<td>Mean systemic arterial pressure, mm Hg</td>
<td>86±7</td>
<td>88±10</td>
</tr>
<tr>
<td>Mean right atrial pressure, mm Hg</td>
<td>4.2±0.4</td>
<td>4.0±0.8</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>310±13</td>
<td>319±11</td>
</tr>
<tr>
<td>Cardiac output, mL/min</td>
<td>10.4±0.5</td>
<td>10.1±0.8</td>
</tr>
<tr>
<td><strong>Total peripheral resistance, mm Hg · ml⁻¹ · min⁻¹</strong></td>
<td>8.2±0.5</td>
<td>8.4±0.8</td>
</tr>
<tr>
<td><strong>Pulmonary pressures</strong></td>
<td></td>
<td></td>
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<tr>
<td>Systolic pulmonary arterial pressure, mm Hg</td>
<td>14.5±0.9</td>
<td>13.8±0.8</td>
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<tr>
<td>Diastolic pulmonary arterial pressure, mm Hg</td>
<td>9.1±0.6</td>
<td>8.9±0.7</td>
</tr>
<tr>
<td>Mean pulmonary arterial pressure, mm Hg</td>
<td>12.8±0.8</td>
<td>11.2±0.7*</td>
</tr>
<tr>
<td>Mean pulmonary arterial wedge pressure, mm Hg</td>
<td>4.6±0.7</td>
<td>4.7±0.8</td>
</tr>
<tr>
<td>Pulmonary vascular resistance, mm Hg · ml⁻¹ · min⁻¹</td>
<td>0.78±0.04</td>
<td>0.68±0.03*</td>
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</table>

n=9 to 12.

*P<0.05 vs AdCMVβgal.
attenuation of pulmonary vasopressor responses to ET-1 after transfection with AdCMVeNOS was observed 1 to 3 days after transfection, and this attenuation of the response to ET-1 decayed over a 7- to 14-day period (Figure 7). Increases in pulmonary arterial pressure in response to ET-1 14 days after transfection were not different from control responses (Figure 7).

In a separate set of experiments, pulmonary vascular responses to bradykinin and ET-1 were evaluated in mice 5, 14, or 28 days after transfection with AdRSVeNOS (Figure 7). In mice treated with AdRSVeNOS, the maximum augmentation of the response to bradykinin and the reduction of the pressor response to ET-1 were similar in magnitude when compared with responses in mice treated with AdCMVeNOS (Figure 7). However, the alteration of responses to bradykinin and ET-1 were more prolonged with the RSV vector (Figure 1). The augmentation of the pulmonary vasodepressor response to bradykinin was maintained for 14 days and decreased to ~50% of maximum 28 days after transfection (Figure 7). In a manner similar to the effect on the response to bradykinin, the attenuation of the pressor response to ET-1 after transfection with AdRSVeNOS was maintained for 14 days and decayed from day 14 to day 28 (Figure 7). The increases in pulmonary arterial pressure in response to ET-1 28 days after transfection with AdRSVeNOS were not different from control responses (Figure 7).

**Effect of AdCMVeNOS on the Pulmonary Pressor Response to Ventilatory Hypoxia**

The influence of AdCMVeNOS transfer on increases in pulmonary arterial pressure in response to ventilatory hypoxia was investigated 1 day after administration of vehicle, AdCMV\textsuperscript{βgal}, or AdCMVeNOS (Table 3). Baseline mean pulmonary arterial pressures were 12.4±0.7 mm Hg in vehicle control animals, 12.8±0.8 mm Hg in AdCMV\textsuperscript{βgal}-treated animals, and 11.4±0.6 mm Hg in AdCMVeNOS-treated animals. When mice were ventilated with the hypoxic gas mixture (10% O\textsubscript{2}/90% N\textsubscript{2}), pulmonary arterial pressure increased to a similar level in vehicle control animals and in AdCMV\textsuperscript{βgal}-treated animals. In contrast, the increase in pulmonary arterial pressure in response to ventilation with the hypoxic gas mixture was reduced in mice treated with AdCMVeNOS (Table 3).

**Influence of AdRSVeNOS on Bleomycin-Induced Pulmonary Hypertension**

The influence of AdRSVeNOS, a viral vector with a longer period of expression, on increases in pulmonary arterial
pressure and PVR in response to bleomycin exposure was investigated 2 weeks after administration of vehicle, vehicle plus bleomycin, AdRSVβgal plus bleomycin, or AdCMVeNOS plus bleomycin (Table 4). In mice exposed to bleomycin (4 U/kg), pulmonary arterial pressure and PVR were increased significantly when compared with values obtained in animals treated with vehicle (Table 4) and in AdRSVβgal-treated animals (data not shown). In addition, right atrial pressure was increased in animals exposed to bleomycin (Table 4). Pulmonary arterial wedge pressure was similar in vehicle control mice and in animals treated with bleomycin ($P<0.05$; data not shown). In contrast, the increases in pulmonary arterial pressure and PVR in response to bleomycin exposure were less in mice treated with AdRSVeNOS than after bleomycin alone or bleomycin plus AdRSVβgal (Table 4). Right atrial pressure was also less in mice treated with both bleomycin and AdRSVeNOS than AdRSVβgal and bleomycin (Table 4).

**Discussion**

In this study, a new right-heart catheterization technique was used to demonstrate that adenovirus-mediated transfer of the eNOS gene to the mouse lung produces a small decrease in PVR and modifies pulmonary vascular responses without altering systemic arterial pressure. Immunoblot studies demonstrated increased lung levels of eNOS protein and cGMP in lung after intratracheal administration of AdCMVeNOS. These results suggest that the eNOS transgene is biologically active in the lung of the mouse. The mice showed no signs of respiratory distress after adenovirus-mediated transfer of the eNOS gene to the lung, and systemic vascular resistance and systemic vascular responses to vasoactive agents were not altered. These data suggest that transfer of the eNOS gene and its effects on the pulmonary vascular bed were selective in the mice.

Gene transfer to blood vessels is usually accomplished by intraluminal delivery with the use of perforated, double-balloon, or gel-coated balloon catheters or by direct injection. For gene transfer to the lung, intraluminal and intratracheal methods have been used. The technique used for intratracheal instillation of AdCMVβgal in the present study resulted in expression in airway cells, in alveolar cells, and in adventitial cells of medium and small arteries. This observation is consistent with a previous report in the rat and suggests that gene transfer to the pulmonary vascular bed by an intratracheal approach may be useful for administering a viral vector in the treatment of pulmonary hypertensive disorders. Although expression of β-galactosidase was observed in adventitial cells and in perivascular cells with less expression in vascular smooth muscle, the present data, together with previous results, suggest that transduction of adventitial cells and perivascular cells with a diffusible lipophilic gene product such as NO may be sufficient to alter vascular function. These data suggest that, although labile, NO may act as a paracrine factor to alter the function of nearby vascular smooth muscle cells. Prominent expression was observed in the pulmonary vascular bed, particularly in 100- to 300-µm arteries, and it is likely that X-Gal staining underestimated the number of cells transduced. Immunohistochemical analysis of lung 1 day after transfection with AdCMVeNOS showed eNOS expression that was similar to β-galactosidase expression in that there was prominent expression in the airway epithelial cells, perivascular cells with some staining in small arteries.

It is possible that adenovirus-mediated gene transfer to the lung induces an inflammatory response and, thus, increases levels of the iNOS in the lung. However, several lines of evidence suggest that an inflammatory response to the adenoviral vector did not account for the findings in the present study. First, there was no increase in cGMP levels in lungs transfected with AdCMVβgal, a vector that is devoid of vascular activity. Second, immunohistochemistry for iNOS showed no change in iNOS expression 1 day after transfec-
tion with AdCMVβgal or AdCMVeNOS. Third, the calcium-independent conversion of L-arginine to L-citrulline, an indicator of iNOS activity, was not increased at a time when calcium-dependent conversion, an indicator of eNOS activity, was increased. Fourth, baseline pressures in the pulmonary and systemic vascular beds were similar in animals treated with vehicle and AdCMVβgal, whereas AdCMVeNOS produced small alterations in mean baseline pressure and in vascular responses in the lung. Fifth, there were no overt signs of an inflammatory response in animals treated with AdCMVβgal or AdCMVeNOS under the conditions of the present experiment. Leukocyte counts in blood samples from animals treated with vehicle (11 200 ± 2100 · μL⁻¹), AdCMVβgal (12 300 ± 3000 · μL⁻¹), or AdCMVeNOS (11 500 ± 2800 · μL⁻¹) were similar (P>0.05). Histological examination of lungs from animals treated with AdCMVβgal and AdCMVeNOS showed a few polymorphonuclear neutrophils, which suggests that a local inflammatory response was present. However, this inflammatory response does not appear to alter pulmonary vascular tone or responses 1 day after transfection.

The observation that the pulmonary arterial pressure-flow relationship was shifted to the right at higher flow rates provides support for the conclusion that the eNOS transgene was active, but the influence of a temperature-induced change in blood viscosity cannot be ruled out. Moreover, the results of the present study, which demonstrated a rightward shift of the pressure-flow curve after eNOS gene transfer, together with the results of a recent study showing a leftward shift of the pressure-flow curve in eNOS-deficient mice,⁸ provide support for the concept that formation of NO by eNOS plays a role in regulating baseline tone and the pressure-flow relationship in the pulmonary vascular bed of the mouse. In addition to contributing to the regulation of baseline tone, release of NO from the endothelium is believed to modulate vasoconstrictor responses and mediate vasodilator responses to several stimuli. In studies in several species, NOS inhibitors have been shown to increase baseline PVR, attenuate endothelium-dependent vasodilator responses, and enhance pressor responses to hypoxia and angiotensin II.¹⁰,²⁹,³³,³⁴ The observations that baseline tone is decreased; pressor responses to hypoxia, ET-1, and angiotensin II are attenuated; and vasodepressor responses to bradykinin are enhanced after adenovirus-mediated transfer of the eNOS gene are consistent with the postulated role of NO in the regulation of the pulmonary vascular bed.¹⁻³,⁸⁻¹⁰ Baseline mean pulmonary arterial pressure of mice treated with AdCMVeNOS was statistically different when compared with mice treated with AdCMVβgal. Because pulmonary arterial pulse pressure varied to a greater extent than did mean pulmonary arterial pressure, the systolic and diastolic pressures were not significantly different. Although the change in mean pulmonary arterial pressure was statistically different in mice treated with AdCMVeNOS, it is unlikely that this small decrease is physiologically significant. However, pulmonary blood flow increases, and the pressure-flow curve is shifted to the right in mice treated with AdCMVeNOS. This observation may suggest that the transgene product may play a more important role under high blood flow conditions.

Although changes in NOS activity are not consistent in several forms of pulmonary hypertension, it is well established that NO has pulmonary vasodilator activity and that treatment with NO is beneficial in some forms of pulmonary hypertension, particularly in pediatric patients.¹⁷⁻²⁰ Results of the present study in the mouse and a recent study in the rat indicate that adenovirus-mediated eNOS gene transfer to the

### Table 3. Influence of AdCMVβgal and AdCMVeNOS on the Increase in Pulmonary Arterial Pressure in Response to Ventilatory Hypoxia (Fraction of Inspired Oxygen, 0.10%) in the Intact-Chest Mouse

<table>
<thead>
<tr>
<th></th>
<th>Control (vehicle) (n=8)</th>
<th>AdCMVβgal (n=7)</th>
<th>AdCMVeNOS (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in Pulmonary Arterial Pressure (in mm Hg) in Response to Hypoxia</td>
<td>4.5 ± 0.8</td>
<td>4.4 ± 0.7</td>
<td>1.7 ± 0.9*</td>
</tr>
<tr>
<td>Percentage Increase in Pulmonary Arterial Pressure in Response to Hypoxia</td>
<td>33.5 ± 9.8</td>
<td>36.9 ± 10.4</td>
<td>14.8 ± 9.5*</td>
</tr>
</tbody>
</table>

n = number of experiments.
*P<0.05 vs control.

### Table 4. Influence of AdRSVeNOS on Increases in Pulmonary Arterial Pressure, Pulmonary Vascular Resistance, and Right Atrial Pressure in Bleomycin-Exposed Mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AdRSVβgal + Bleomycin</th>
<th>AdRSVeNOS + Bleomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean pulmonary arterial pressure, mm Hg</td>
<td>12.7 ± 1.0</td>
<td>28.6 ± 2.5*</td>
<td>17.3 ± 1.6†</td>
</tr>
<tr>
<td>Mean pulmonary arterial wedge pressure, mm Hg</td>
<td>4.6 ± 0.8</td>
<td>4.1 ± 0.9</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>Pulmonary vascular resistance, mm Hg · mL⁻¹ · min</td>
<td>0.78 ± 0.04</td>
<td>2.75 ± 0.14*</td>
<td>1.4 ± 0.03†</td>
</tr>
<tr>
<td>Cardiac output, mL/min</td>
<td>10.2 ± 0.7</td>
<td>8.9 ± 1.0</td>
<td>9.6 ± 0.9</td>
</tr>
<tr>
<td>Mean right atrial pressure, mm Hg</td>
<td>4.2 ± 0.4</td>
<td>7.9 ± 0.8*</td>
<td>5.1 ± 0.5†</td>
</tr>
</tbody>
</table>

n = 9
* P<0.05 vs control; † P<0.05 vs AdRSVβgal + bleomycin–treated group.
lung enhances pulmonary eNOS activity and attenuates vasoconstrictor responses. In addition to showing that baseline vascular resistance is lower and vasodepressor responses to bradykinin are enhanced, the present results show that the vasodepressor response to the type V cGMP phosphodiesterase inhibitor zaprinast are increased. These data are consistent with the concept that the eNOS transgene product is biologically active and suggest that in vivo eNOS gene transfer, together with the administration of a cGMP phosphodiesterase inhibitor, may represent a new strategy for treatment of pulmonary hypertensive disorders. The results of the present study showing that in vivo eNOS transfer attenuates the pressor response to ventilatory hypoxia are consistent with the results of a recent study in the rat. The results of the present study show that eNOS gene transfer attenuates pulmonary pressor responses to angiotensin II and ET-1 but does not alter responses to norepinephrine or to the thromboxane A2 mimic U46619. The explanation for the differential effect of eNOS gene transfer on pressor responses is uncertain but is consistent with the observation that ET-1 and angiotensin II release NO from the endothelium and that NOS inhibitors potentiates pressor responses to angiotensin II. By enhancing vasodilator responses and inhibiting vasoconstrictor responses, it has been postulated that eNOS gene transfer may be beneficial in the treatment of several cardiovascular disorders. It is possible that a compensatory mechanism that is stimulated by the increase in eNOS expression may account for changes in responses to bradykinin, angiotensin II, and ET-1. However, because the time course of the alteration of responses to bradykinin and ET-1 correlate with the expression of β-galactosidase activity, these data may be interpreted to suggest that the overexpression of eNOS accounts for the observed alteration in responses, although a compensatory mechanism cannot be ruled out by the present data.

Intratracheal administration of bleomycin produces pulmonary fibrosis in the mouse. Bleomycin-induced pulmonary fibrosis is associated with pulmonary hypertension in the rat. The results of the present study show that bleomycin-induced pulmonary fibrosis is associated with an increase in pulmonary arterial pressure and PVR in the intact-chest mouse. Treatment with AdRSVeNOS attenuates increases in pulmonary arterial pressure, PVR, and right atrial pressure, which suggests that enhanced expression of eNOS and NO release in the pulmonary vascular bed may attenuate pulmonary hypertension. The results of the present study show that, in addition to attenuating increases in pulmonary arterial pressure in response to acute hypoxia and vasoactive hormones, transfer of the eNOS gene reduces pulmonary arterial pressure in a chronic form of fibrogenic pulmonary hypertension.

In regard to study limitations, Western analysis of whole lung tissue demonstrates increased eNOS protein in lungs transected with AdCMVeNOS. Because histochemical analysis showed some transgene expression in the small arteries, it would be useful to obtain Western blots from small arteries that regulate vascular resistance within the pulmonary vascular bed. However, for technical reasons, we are not able to dissect 100- to 300-μm arteries and obtain sufficient tissue for Western analysis on resistance vessels.

In summary, the results of the present study show that adenovirus-mediated transfer of the eNOS gene increases eNOS protein levels and elevates cGMP levels in the lung of the mouse. Overexpression of eNOS decreases PVR; enhances depressor responses to bradykinin and zaprinast; and attenuates pressor responses to hypoxia, ET-1, and angiotensin II in the lung. Transfer of the eNOS gene shifts the pressure-flow curve to the right without affecting systemic arterial pressure or responses. Moreover, the results of the present study indicate that eNOS gene transfer attenuates the chronic increase in pulmonary arterial pressure in response to exposure to bleomycin. The results of the present study are consistent with the hypothesis that in vivo transfer of the eNOS gene to the lung selectively alters PVR and responses and suggest that adenovirally mediated transfer of the eNOS gene, along with administration of a cGMP phosphodiesterase inhibitor, may represent a new form of therapy for the treatment of pulmonary hypertensive disorders.

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