Endothelial nitric oxide synthase gene transfer enhances dilation of newborn piglet pulmonary arteries

JUDY L. ASCHNER,1 NORA KOVACS,1 JAMES V. PERCIACCANTE,1 JORGE P. FIGUEROA,2,3 NISHADI THRIKAWALA,3 GREGORY S. ROBINS,3 AND DAVID W. BUSJIA3
Departments of 1Pediatrics, 2Obstetrics and Gynecology, and 3Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157-1081

Aschner, Judy L., Nora Kovacs, James V. Perciaccante, Jorge P. Figueroa, Nishadi Thrikawala, Gregory S. Robins, and David W. Busija. Endothelial nitric oxide synthase gene transfer enhances dilation of newborn piglet pulmonary arteries. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H371–H379, 1999.—We determined the expression and functional correlate of in vitro transfection with a recombinant adenoviral vector encoding the gene for bovine endothelial nitric oxide synthase (AdCMVeNOS) or Escherichia coli β-galactosidase (AdCMVLacZ) in pulmonary endothelial cells (EC), vascular smooth muscle cells (VSMC), and pulmonary arteries (PA) from newborn piglets. AdCMVeNOS and AdCMVeLacZ vectors, grown in 293-cell monolayers, were purified by double-cesium gradient ultracentrifugation. Cell cultures and PA were incubated with increasing vector titers for 30 or 60 min, followed by incubation in fresh medium for 18 h at 37°C. LacZ expression was assessed by histochemical staining; eNOS expression was evaluated by Western blot analysis. Functional eNOS expression was determined by measurement of cGMP and quantification of the relaxation response to bradykinin (BK). In PA, LacZ transgene expression was preferentially localized to the adventitia and endothelium. Increased eNOS protein expression was observed in EC and VSMC transfected with AdCMVeNOS. Functional studies revealed increased cGMP abundance in cultured cells and enhanced relaxation to BK in AdCMVeNOS-transfected PA. These studies demonstrate that gene transfer with AdCMVeNOS results in functional expression and altered vasoactive responses in the neonatal pulmonary vasculature. Gene transfer with replication-deficient adenovirus vectors is a useful tool for the study of targeted genes in vascular biology.

adeno virus; endothelium; nitric oxide; persistent pulmonary hypertension of the newborn

RELEASE OF endothelium-derived nitric oxide (NO) provides a local mechanism for regulating vascular tone and reactivity (35, 40). The endothelium releases NO on conversion of L-arginine to L-citrulline by type III NO synthase (eNOS) under basal conditions and on activation by physiological and pharmacological stimuli. Endogenous NO production has been shown to play an important role in the circulatory transition from fetal to neonatal life (1, 10, 25, 34, 38). Decreased production of endogenous NO is thought to contribute to the failure of postnatal circulatory adaptation, resulting in the syndrome of persistent pulmonary hypertension of the newborn (PPHN) (1, 8, 10, 34). Investigations of pulmonary vascular responses in animal models of PPHN have demonstrated attenuated NO-mediated relaxation in vivo (20) and in vitro (35) and decreased pulmonary eNOS gene expression (25, 34, 41). Therapeutic replacement of NO at least partially reverses the elevation in pulmonary vascular resistance and improves oxygenation in newborn animals with experimental pulmonary hypertension (6, 20, 42, 43) and in some human infants with PPHN (14, 15, 27, 28). However, not all infants respond favorably to inhaled NO. Infants with parenchymal lung disease or structural vascular abnormalities are less likely to respond favorably. Novel approaches for the treatment of PPHN and other pulmonary disorders in the newborn are needed.

Gene transfer is a promising therapy for many genetic and acquired diseases of the lung (36). Adenovirus-mediated gene transfer provides a tool for insertion of foreign genes into proliferating and nonproliferating cells and tissues in vivo or in vitro (19, 31). Because they can infect both replicating and terminally differentiated cells, replication-deficient adenoviral vectors are well suited for facilitating efficient gene transfer into vascular endothelium. They do not integrate into the host genome and therefore pose little risk of insertional mutations (5), and they have not been associated with persistent infections or malignancies in humans (31). Replication-deficient adenoviral vectors can be grown to high titers and engineered to accommodate large DNA inserts (5, 31). Adenoviral vectors have been reported to infect a wide range of mammalian cells and to express recombinant genes in intact and injured systemic vessels from adult animals (31). However, heterogeneity has been reported in the efficiency of expression among systemic vessels from different anatomic sites (39). Few studies have investigated gene delivery to the pulmonary circulation, a vascular bed with biologic characteristics distinct from those of the systemic vasculature (29, 30). In adult rats, in vivo pulmonary gene transfer using a percutaneous catheterization approach was either not successful (30) or resulted in low-efficiency gene transfer primarily into nonvascular cells (29). In contrast, successful adenovirus-mediated gene transfer of the human placental alkaline phosphatase gene was achieved in mature pigs via percutaneous right heart catheterization and pulmonary artery occlusion (22). Functional expression of a biologically active gene product was not investigated in these in vivo studies.

Evidence exists for differences in efficiency of adenovirus-mediated gene transfer in newborn versus adult systemic blood vessels and skeletal muscle (2, 21). No
studies have been published that examine the susceptibility of the newborn pulmonary circulation to adenovirus-mediated gene transfer. The objective of our study was to determine whether ex vivo gene transfer with a replication-deficient recombinant adenovirus encoding the Escherichia coli β-galactosidase (LacZ) reporter gene (AdCMVeLacZ) or the bovine eNOS gene (AdCMVeNOS), driven by the cytomegalovirus promoter, can be functionally expressed in cultured pulmonary endothelial cells (EC), vascular smooth muscle cells (VSMC), and pulmonary blood vessels isolated from newborn piglets.

METHODS

Adenoviral vector preparation. AdCMVeNOS was kindly provided by Drs. Alex Chen and Zvonimir Katusic (Mayo Clinic, Rochester, MN). The construction of this vector has been described in detail by Chen et al. (7). AdCMVeLacZ was a gift from Dr. James Wilson (University of Pennsylvania, Philadelphia, PA). Viral vectors were prepared by infecting a confluent monolayer of 293 cells, human embryonic kidney carcinoma cells transformed with the left end of human adenovirus type 5 DNA (7). Concentrated viral stocks (5.9 × 10^12 particles/ml for AdCMVeLacZ; 5.0 × 10^12 particles/ml for AdCMVeNOS) were purified by double-cesium-gradient ultracentrifugation and dialysis for 4 h at 4°C against (in mM) 10 Tris, 1.0 MgCl2, and 1.0 HEPES containing 10% glycerol. Aliquots of 20 µl were stored at 80°C until use. Viral titer was determined by the optical density at 260 nm (OD260), where 1 OD260 unit = 10^12 virus particles (36). The viral titer in functional plaque-forming units (pfu) has been previously found to be 1–10% of the particle concentration as estimated by plaque assay using 293 cells (9). Virus dose is expressed as particles per milliliter with particle number determined by OD260. With the use of this definition the multiplicity of infection (MOI) is higher than in studies that express particle number as a functional unit (pfu) (36).

Isolation of newborn piglet pulmonary arteries. All animal procedures conformed with the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1985] and were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine. Newborn piglets <7 days of age were killed with an overdose (75 mg/kg ip) of pentobarbital sodium. Under sterile conditions, heart and lungs were removed en block. Third- to fourth-generation pulmonary arteries (PA) were steriley dissected from surrounding parenchymal lung tissue. Under a dissecting microscope, vessels were cleaned of excess connective tissue, and vascular rings 3–5 mm in length were cut and prepared for adenoviral vector transfection and vasoreactivity studies.

Cell culture. Pulmonary microvascular smooth muscle cells were isolated under sterile conditions from newborn piglets. Pulmonary resistance vessels with diameters of 100–300 µm were isolated from peripheral lung parenchyma under a dissecting microscope and placed in 1 ml of warm collagenase dissociation buffer consisting of 0.3% type II collagenase, 0.4% Polypep, and 0.05% trypsin. The vessels were incubated on an orbital shaker at 37°C incubator for 2 h and then centrifuged for 5 min at 1,500 rpm. The supernatant was discarded, and 1 ml of 0.05% trypsin was added. The cell digest was placed back on the orbital shaker for 8 min and then centrifuged at 1,500 rpm for 5 min and the excess trypsin removed. The pellet was resuspended in 10 ml of warmed DMEM and centrifuged for 5 min at 1,500 rpm. This wash procedure was repeated two more times. The cells were resuspended and incubated in DMEM containing 10% FCS, 1% penicillin-streptomycin, 0.1% gentamicin reagent solution, 1% L-glutamine, and 1% nonessential amino acid solution. After 24 h, the medium was changed to medium 199 (M199) with 10% FCS, 1% L-glutamine, and 1% penicillin-streptomycin. Pulmonary microvascular smooth muscle cells were maintained in a 5% CO2 incubator at 37°C, passaged at weekly intervals, and used before the sixth passage. Identity of the cells was confirmed by positive α-actin smooth muscle staining.

Bovine lung microvascular EC were obtained from VEC Technologies (Rensselaer, NY) at the tenth population doubling. These cells were identified as endothelial in origin by the characteristic cobblestone monolayer morphology and homogeneous positive incorporation of acetylated low-density lipoprotein. EC were cultured in DMEM plus 10% FCS, 0.1% gentamicin reagent solution, and 1% nonessential amino acid solution and supplemented weekly with 1% L-glutamine. EC were passaged a maximum of six times.

Type II collagenase, Polypep, nonessential amino acid solution, and BSA were obtained from Sigma Chemical (St. Louis, MO). All other cell culture reagents were purchased from Gibco BRL (Life Technologies, Gaithersburg, MD).

Transfection protocol. All aliquots of adenovirus were thawed on ice and diluted with DMEM plus 0.1% BSA to the desired titer. Confluent pulmonary EC and VSMC cultures were incubated with adenoviral vector at titers of 3 × 10^10–3 × 10^11 particles/ml. Freshly isolated third- to fourth-generation newborn piglet PA rings were incubated with vector at 3 × 10^10–3 × 10^11 particles/ml. In all cases, the transfection buffer was DMEM plus 0.1% BSA (37°C) and the viral transfection time was 30 or 60 min. Cell cultures and vessels were then transferred to fresh DMEM plus 0.1% BSA and incubated for 18 h in a 37°C CO2 incubator before being studied. EC and PA designated as controls were exposed to the transfection buffer in the absence of virus and similarly incubated for 18 h at 37°C in DMEM plus 0.1% BSA.

Histochemical staining for LacZ. To determine whether the transfection protocol resulted in recombinant protein expression, histochemical staining for LacZ expression was performed in cultured cells and isolated PA rings. Cells were fixed for 5 min, and pulmonary arteries for 15 min, in 1.25% glutaraldehyde (Sigma Chemical) before being stained with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal reagent; ICN, Aurora, OH). Stained vessels were embedded in paraffin and cut into 4-mm sections for localization of LacZ expression to a specific layer of the vessel wall. The specificity of the histochemical staining for LacZ was examined by comparable staining of control vessels and vessels transected with AdCMVeNOS.

Immunoblot analysis. Cell extracts were homogenized in Tris-buffer containing 50 mM Tris, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 150 mM NaCl, 1% Triton X, 0.1% SDS, 1% sodium deoxycholate, 12 mM 2-mercaptoethanol, 2 µM leupeptin, 1 µM pepstatin, and 1 mM phenylmethylsulfonfyl fluoride. The crude homogenate was centrifuged at 2,000 g, and protein concentration was measured with the bicinchoninic acid (BCA) method using BSA as the standard (Pierce, Rockford, IL). Protein aliquots were mixed 1:4 in loading buffer, separated in 8% Tricine gels (Novex, San Diego, CA), and blotted onto polyvinylidene difluoride membranes (Immobilon; Millipore, Marlborough, MA) by semidry electroblotting. Blots were blocked overnight at 4°C with 6% dry nonfat milk, rinsed with Tris-buffered saline-0.05% Tween.
20, and incubated for 2 h at room temperature with a primary monoclonal antibody against mouse eNOS at a 1:750 dilution (Transduction Laboratories; Lexington, KY) followed by incubation for 1 h with a horseradish peroxidase-conjugated secondary antibody at 1:2,500 dilution. A positive reaction was identified with enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) and relative intensity normalized by loading of equal protein amounts and by intensity of the standard preparation (a lysate of bovine EC) in each gel.

Vasoreactivity protocol. Bradykinin (BK), sodium nitroprusside (SNP), ACh, N^o-nitro-L-arginine (L-NNA), and 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin F_2α (U-46619) were purchased from Sigma Chemical (St. Louis, MO). BK and SNP were prepared as aqueous solutions, U-46619 was dissolved in ethanol, and L-NNA was prepared in double-distilled H_2O with dropwise addition of HCl (1 M) until dissolved. Precautions were taken to protect the SNP solution from light. The vehicle had no effect on the final pH of the bathing solution or on vascular reactivity of PA ring preparations. All concentrations of the drugs used were expressed as final molar concentrations in the organ bath.

Vascular rings were mounted between two parallel, triangular 28-gauge stainless steel wires (Arista Surgical, New York, NY) and placed in individual 16-ml tissue baths (Radiotni Glass, Monrovia, CA) filled with physiological bicarbonate solution (PBS) maintained at 37°C and bubbled with a gas mixture of 21% O_2-5% CO_2-balance N_2. The composition of the PBS (in mM) was 130 NaCl, 4.7 KCl, 1.17 MgSO_4-7H_2O, 1.18 KH_2PO_4, 14.9 NaHCO_3, 5.5 dextrose, 0.03 NaCa_2EDTA, and 1.6 CaCl_2-2H_2O. Each ring was connected to a model FT03 force transducer (Grass Instruments, Quincy, MA) for the measurement of isometric tension. Recordings were made on a personal computer data-acquisition program written for this purpose. Vascular rings were stretched and equilibrated for 60 min at a passive force of 1.5 g. This resting tension was previously determined to be optimal on the basis of length-tension curves to a depolarizing dose of KCl. After a 1-h equilibration period at optimal resting tension, the ability of the smooth muscle to contract was assessed by the addition of 50 mM KCl, and the viability of the endothelium was assessed by addition of ACh (0.1 mM). After another recovery period, the vessels were constricted with 0.1 µM U-46619. The ability of constricted PA rings to relax to both endothelium-dependent and -independent vasodilator stimuli was assessed sequentially in all vessels by the addition of increasing concentrations of BK (1–1,000 nM) followed by 0.01 mM SNP. To some vessels, L-NNA (0.1 mM) was added to the bath after maximal constriction to U-46619 and 15 min before the addition of BK, to inhibit NO. To avoid the confounding effect of tachyphylaxis, separate PA rings were used to compare the BK-induced relaxation response in the presence and absence of L-NNA. Vessels were excluded from the final analysis if they met one or more of the following criteria: 1) they failed to constrict by at least 100 mg to 50 mM KCl; 2) they failed to relax by at least 100 mg to 0.01 mM SNP; or 3) they constricted rather than dilated to BK, demonstrating a loss of viable endothelium.

cGMP enzyme immunoassay. Pulmonary microvascular EC grown to confluence in Costar six-well culture dishes (Corning, Corning, NY) were incubated with control medium (DMEM plus 0.1% BSA) or medium containing various titers of AdCMVeLaCZ or AdCMVeNOS at 37°C for 30 or 60 min as described in Transfection protocol. The transfection buffer was removed, EC cultures were washed with fresh buffer, and 1 ml of DMEM containing newborn pulmonary VSMC (3 × 10^6 cells/ml) plus 0.1% BSA was layered on top of the transfection EC monolayer. The combined cocultures were plated in the CO_2 incubator at 37°C. After 18 h, the medium was removed and the cocultures were washed with NaHCO_3-based buffer containing (in mM) 115 NaCl, 25 NaHCO_3, 2.5 CaCl_2, 4.5 KCl, 1.2 Mg SO_4, 1.2 KH_2PO_4, and 10 dextrose and equilibrated for 10 min with room air-5% CO_2 (pH 7.4). The cocultures were then incubated for 1 h at 37°C in 1 ml of the same buffer containing 0.1 mM 3-isobutylyl-L-methylxanthine in the presence or absence of L-NNA (0.1 mM) and/or BK (0.1 µM). Total cellular-plus-excreted cGMP was extracted by the addition of 0.1 N HCl for 20 min at 4°C. cGMP was measured by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). The developed chromogen absorbance was measured at 405 nm using a VMAX 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA). cGMP concentration was calculated from a four-parameter fit, linear regression curve of standards (0.23–30 pmol/ml). The protein content of each cell-culture well was determined by the BCA protein assay (Pierce), and the cGMP content of each well was calculated as picomoles per milligram of protein. The results from each well were normalized to the average control value (in pmol/mg protein) of each experiment to facilitate comparison between studies performed on different days.

Statistical analysis. All results are graphically depicted as means ± SE. General linear modeling with repeated measures was performed to determine the effect of different treatment groups on the BK-induced relaxation response. One-way ANOVA was used to compare constriction responses to KCl and U-46619 in PA and cGMP production in EC cultures among the different treatment groups. Post hoc comparisons among groups were made using Tukey’s honestly significant difference test. All analyses were conducted using SPSS Advanced Statistics (version 7.5; SPSS, Chicago, IL) for Windows version 7.0. For all tests, a two-tailed value of P < 0.05 was regarded as statistically significant.

RESULTS

In newborn piglet PA, transfection with AdCMVeLaCZ resulted in intense blue staining indicative of expression of recombinant LacZ protein in the vessel wall (Fig. 1). No blue staining was seen in nontransfected control vessels or eNOS-transfected vessels fixed and similarly stained with X-Gal reagent. Transfection was dependent on viral titer and transfection time, with more intense staining at 3 × 10^11 versus 3 × 10^10 particles/ml and after 60 min versus 30 min of incubation. Examination of paraffin-embedded and sectioned vessels demonstrates that LacZ expression was localized primarily to the adventitia and the endothelial layers (Fig. 2A). This staining pattern was a consistent finding in all vessels. There was some scattered staining in the smooth muscle layer of most vessels (Fig. 2A), although the identity of the positively stained cells in this layer is not known. LacZ transgene expression was homogeneous throughout the endothelial lining (Fig. 2B). In cell culture, no blue staining was observed in nontransfected control cells (Fig. 3A) or eNOS-transfected EC (not shown). At a titer of 3 × 10^10 particles/ml for 30 min, approximately one-third of EC stained blue after incubation in X-Gal reagent (Fig. 3B). We observed nearly 100% efficiency of LacZ staining in confluent monolayers of pulmonary EC at titers of 3 × 10^10 particles/ml for 30 min (Fig. 3C).

In newborn
pulmonary VSMC cultures, <10% of cells stained positively for LacZ, even at titers of $1 \times 10^{11}$ particles/ml for 60 min (not shown).

Western blot analysis revealed increased eNOS protein levels in cultured EC transfected with AdCMVeNOS at $3 \times 10^{10}$ and $1 \times 10^{11}$ particles/ml compared with nontransfected control cells and AdCMVeLacZ-transfected cells (Fig. 4A). These bands migrate with the human eNOS standard at the appropriate molecular mass of 135 kDa. Figure 4B is a Western blot demonstrating eNOS protein expression in VSMC transfected with AdCMVeNOS ($1 \times 10^{11}$ particles/ml for 60 min). No eNOS protein is expressed in VSMC transfected with AdCMVeLacZ or exposed to control transfection buffer. A much longer ECL exposure time was required to visualize the eNOS protein band in transfected VSMC cultures (8 min) compared with that for EC cultures (30 s), as demonstrated by the relative intensities of the 5-µl eNOS standard bands in Fig. 4A (lane 5) compared with those in Fig. 4B (lane 1).

Figures 5 and 6 demonstrate total cGMP produced in transfected and control EC grown together with nontransfected VSMC. We observed similar cGMP levels in LacZ-transfected cultures as observed in nontransfected control cultures (Figs. 5 and 6). A dose-dependent increase in cGMP abundance was seen in cells transfected for 30 min with AdCMVeNOS, which was completely inhibited by 0.1 mM L-NNA (Fig. 5). Transfection with AdCMVeNOS ($3 \times 10^{10}$ particles/ml) produced a marked increase in cGMP under both basal and BK-stimulated conditions, with a greater abundance of cGMP in the basal or unstimulated state after the...
60-min transfection time than after the 30-min transfection time (Fig. 6).

There were no statistically significant differences in the contractile responses to KCl (50 mM) or U-46619 (0.1 µM) among control (exposed to vector diluent only), AdCMVeLacZ-transfected, or AdCMVeNOS-transfected vessels (Fig. 7). Figure 8 demonstrates the relaxation response of PA rings to increasing concentrations of BK, an endothelium-dependent vasodilator. Results are expressed as the percent relaxation from the level of tone induced by 0.1 µM U-46619. PA rings transfected with AdCMVeNOS (3 x 10^{10} particles/ml for 30 min) demonstrated an enhanced relaxation response to BK relative to the response for PA rings exposed to the transfection procedure in the absence of virus (control) or those transfected with AdCMVeLacZ. There were no differences in the relaxation responses to SNP (0.01 µM), an endothelium-independent vasodilator, among control, AdCMVeLacZ-transfected, or AdCMVeNOS-transfected vessels. The values (means ± SE) for the percent relaxation to 0.01 µM SNP among control, AdCMVeLacZ-transfected, and AdCMVeNOS-transfected vessels were 90.8 ± 4.6, 96.6 ± 10.8, and 98.9 ± 9.4%, respectively.

**DISCUSSION**

Gene transfer to vascular tissue is an experimental approach that lends itself to functional investigations of targeted genes under physiological and pathological conditions and to potential manipulation of vascular behavior for therapeutic purposes (23, 37). In this paper, we describe the successful transfer and functional expression of a recombinant eNOS gene in arteries isolated from the newborn lung. Whereas these findings are not surprising in light of previous reports demonstrating increased production of NO in the vessel wall of eNOS-transfected cerebral arteries from mature animals (7), the results were not a foregone conclusion. The pulmonary circulation is distinctly different from the systemic circulation, both functionally and structurally (29). The newborn pulmonary circulation, which is undergoing rapid hemodynamic changes characteristic of the perinatal period, demonstrates unique vasomotor responses that are developmentally regulated at the molecular and biochemical level. Newborns differ from mature animals with regard to immunologic responses, receptor expression, and biosynthetic ma-
The susceptibility of the newborn pulmonary circulation to adenovirus-mediated gene transfer and the functional expression of the transgene cannot be inferred from studies conducted in systemic vessels from mature animals.

In these studies, adenovirus-mediated transfection of newborn PA resulted in viral titer- and exposure time-dependent gene transfer as demonstrated by histochemical staining for LacZ. Protein expression appeared to be preferentially localized to the endothelium and adventitia of the vessels wall. This is similar to the findings of Ooboshi et al. (26) in rabbit common carotid arteries, who observed transgene expression in endothelium and adventitia, but not in smooth muscle, after in vivo gene transfer. The assumption made was that the adenoviral vector could not penetrate the endothelial or adventitial barriers into the smooth muscle layer. This was supported by the finding of in vivo smooth muscle transgene expression 3 days after adenovirus-mediated gene transfer to balloon-injured rat carotid arteries (17, 33). However, we found that VSMC cultures were relatively resistant to transgene expression, even after direct application of vector at a higher titer than that required for efficient gene transfer to EC cultures. Our data suggest an intrinsic difference in susceptibility of EC versus VSMC to the adenoviral vector. Kullo et al. (16) reported successful eNOS transgene expression in cultured porcine coronary artery smooth muscle cells.

**Fig. 5.** Inhibition of cGMP abundance by N\textsuperscript{G}-nitro-L-arginine (L-NNA) in eNOS-transfected cocultures. Transfection of EC with AdCMVeNOS (3 \times 10\textsuperscript{10} and 3 \times 10\textsuperscript{12} particles/ml for 30 min) increased cGMP abundance in EC/VSMC cocultures. EC transfection with AdCMVeLacZ had no effect on cGMP levels. L-NNA (0.1 mM) inhibited increase in cGMP induced by eNOS transfection. *Significantly different from control; \# significantly different from L-NNA (n = 2 studies in triplicate wells).

**Fig. 6.** Increased basal and bradykinin (BK)-stimulated production of cGMP in eNOS-transfected cocultures. Transfection with AdCMVeNOS (3 \times 10\textsuperscript{9} particles/ml) for 30 or 60 min increased both basal and BK-stimulated cGMP production, with greater cGMP abundance after 60-min transfection time than after 30-min transfection time in basal or unstimulated state. There were no differences in basal or BK-stimulated cGMP production between control and LacZ-transfected groups. * Significant difference between control and BK within each treatment group; \# significantly different from basal control and LacZ; \# significantly different from BK-stimulated control and LacZ; \# significantly different from basal control, LacZ, and 30-min eNOS (n = 3 studies performed in duplicate or triplicate wells).

**Fig. 7.** Constrictor responses of PA rings to KCl and U-46619. No significant differences were noted in vasoconstrictor responses of PA rings to KCl (50 mM) or U-46619 (0.1 \mu M) among control, AdCMVeLacZ-transfected, or AdCMVeNOS-transfected vessels (control: n = 11 vessels from 8 piglets; LacZ: n = 9 vessels from 7 piglets; eNOS, 30': n = 9 vessels from 8 piglets; eNOS, 60': n = 8 vessels from 5 piglets).

**Fig. 8.** BK-induced relaxation response in PA rings. An enhanced relaxation response to BK was observed in PA transfected with AdCMVeNOS (3 \times 10\textsuperscript{10} particles/ml for 30 min) that was completely abolished in presence of 0.1 mM L-NNA. *Significantly different from control, LacZ, and eNOS; \# significantly different from control and eNOS/L-NNA; * significantly different from LacZ, control, and eNOS/L-NNA (control: n = 11 vessels from 8 piglets; LacZ: n = 9 vessels from 7 piglets; eNOS: n = 9 vessels from 8 piglets; eNOS/L-NNA: n = 8 vessels from 4 piglets).
48 h after transfection with AdCMV eNOS for 1 h at a high MOI. This suggests that smooth muscle cells from different vascular sites (pulmonary vs. coronary) or different age groups (newborn vs. adult) display different susceptibilities to transfection. Alternatively, relative to EC, successful transgene expression in VSMC may require longer (48 h) viral translation and, therefore, longer incubation time (48 h vs. 18 h).

Enhanced protein expression does not necessarily endow the host cell with altered biologic function. The overexpressed gene must not only be incorporated into the host cells but also must be integrated into the host signaling machinery in such a way as to be responsive to stimuli. Therefore, we examined the effect of AdCMV eNOS transfection on agonist-induced vasorelaxation. Compared with AdCMV LacZ-transfected or nontransfected control PA, isolated pulmonary vessels transfected with AdCMV eNOS demonstrated enhanced relaxation in response to BK, an agonist that we have previously shown mediates relaxation in piglet pulmonary arteries by a mechanism that is entirely dependent on endothelium-dependent NO (3). Relaxation to the endothelium-independent vasodilator SNP was unaffected. Furthermore, in control and LacZ-transfected vessels, the relaxation responses to BK were similar, supporting the conclusion that the effect of transfection with AdCMV eNOS was attributable to increased local production of NO and not to nonspecific effects of adenoviral transfection. In our study, we did not find altered vasoconstrictor responses to KCl or U-46619 in eNOS-transfected vessels. This finding is similar to that reported by Ooboshi et al. (26) but differs from the findings of Chen et al. (7). Our failure to find a statistically significant difference in response to constrictor agents may be related to the relatively low viral titers used in our vessel studies (3 × 10^{10} particles/ml or ~10^8 pfu/ml), short viral exposure time (30 min), and short incubation time (18 h), which likely resulted in lower transgene expression, particularly in the VSMC. Nevertheless, the degree of transgene expression resulting from this protocol was sufficient to augment endothelium-dependent relaxation to BK while preserving endothelial integrity.

We also examined the effect of transfection with AdCMV eNOS and AdCMV LacZ on cGMP production from cocultures of EC and VSMC. NO, produced in the endothelium, mediates its effects by activation of soluble guanylate cyclase, resulting in hydrolysis of GTP and formation of cGMP (11, 40). NO can exert its effects in an autocrine or paracrine manner, activating soluble guanylate cyclase in the same or adjacent EC or in the underlying VSMC. We have previously reported that EC and VSMC grown together in coculture produce several-fold more cGMP than the sum of that produced by EC and VSMC grown separately, suggesting cross talk between the two cell types (4). The finding of increased basal and BK-stimulated cGMP in the cocultures containing AdCMV eNOS-transfected EC strongly suggests that expression of recombinant eNOS protein in the EC results in enhanced NO formation, leading to increased production of cGMP in both the transfected EC and nontransfected VSMC. This increase was inhibited by l-NNA, confirming that the enhanced production of cGMP was mediated via the NOS pathway. LacZ and control cocultures produced similar amounts of cGMP, refuting the possibility that the increase in cGMP in AdCMV eNOS-transfected cultures was caused by a nonspecific effect of the virus or adenovirus-mediated activation of inducible NOS.

Functional expression of the eNOS transgene resulting in enhanced NO production may have important clinical implications. Whereas in vivo studies will be necessary to address concerns about cytotoxicity and possible host immune responses to the viral proteins, these data suggest that short-term exposure to viral vector at fairly low titers can result in functional upregulation of NOS gene expression in PA without loss of vasomotor function. At higher titers (>10^{11} pfu/ml), adenovirus-mediated gene transfer in rabbit arteries was reported to cause vascular cell activation, inflammation, and neointimal hyperplasia (24); high-titer adenoviral vector also caused endothelial denudation (32) and smooth muscle cell loss (33) in rat carotid arteries. This may be related to the ability of recombinant adenovirus to become replication competent at a high titer (7) or to a humoral or cellular immune response to the internalization of viral proteins (24, 32).

The application of gene therapy for human pathology will be contingent on the development of improved vectors that will be inert with regard to host immune responses and on the introduction of novel cell- or organ-specific delivery systems. Although luminal administration of vector has been shown to target gene delivery and expression to the endothelium (32), this approach requires temporary interruption of blood flow, a distinct disadvantage under most clinical circumstances. In the context of gene therapy for pulmonary hypertension, inhalation of aerosolized vector holds promise as a future therapeutic strategy. In a report by Janssens et al. (12), aerosolized recombinant adenovirus encoding the eNOS gene resulted in transgene expression in the endothelium of medium and small pulmonary vessels (as well as in basal airway epithelial cells and alveolar lining cells) and significantly attenuated hypoxia-induced pulmonary vasoconstriction in adult rats without effect on the systemic blood pressure. The aerosolized adenovirus was administered via inhalation during mechanical ventilation. Whether this approach will prove efficacious in larger animal models of PPHN in which endogenous eNOS expression and VSMC NO responsiveness is suppressed awaits future investigations. An alternative novel strategy for delivery and distribution of transgene to the pulmonary circulation is the use of surfactant or liquid perfluorocarbons as the delivery vehicle (18). The use of cell type-specific promoters or tissue-specific transcriptional regulatory elements represents an alternative strategy to restrict adenoviral transgene to specific cell lineages or tissues in vivo (13).

Few investigations have addressed the clinical applicability of virally mediated gene transfer for pulmonary hypertensive states; none has specifically done so in
the context of PPHN. The potential utility of virally mediated gene transfer for infants with PPHN will ultimately depend on whether transgene expression of eNOS will prove superior to inhaled NO therapy in terms of distribution, uniformity of response, or toxicity. One potential advantage is that, unlike inhaled NO, which requires continuous administration for sustained effect, a single aerosol dose of AdCMV\textit{eNOS} may be able to ameliorate pulmonary hypertension in many clinical circumstances (12). Although adenovirus-mediated gene transfer results in only transient (1–2 wk) expression of the transgene, a disadvantage for the treatment of chronic diseases, the transient nature of the transfection may be an advantage in the treatment of many pulmonary vascular disorders, such as PPHN and pulmonary hypertension after cardiac surgery. It remains to be determined whether continuous local overexpression of NO over a period of several weeks, which can be achieved by transgene expression with adenoviral vectors, can reverse the smooth muscle cell hypertrophy that can accompany some forms of PPHN and that seems to correlate with a poor response to inhaled NO. Mortality for such infants remains quite high despite referral for therapy with extracorporeal membrane oxygenation (ECMO). Successful treatment of such infants will likely require a combination of modalities, including novel strategies to reverse the pulmonary vascular remodeling often seen at autopsy in infants unable to be successfully decannulated after ECMO.

In summary, these studies demonstrate that adenoviral vectors are effective tools for the transfection and expression of recombinant \textit{LacZ} and eNOS genes in isolated blood vessels and cultured EC from the newborn pulmonary circulation. Transfection with AdCMV\textit{eNOS} results in functional upregulation of NOS activity, resulting in increased cGMP-mediated relaxation responses. These findings suggest that pulmonary vascular resistance in the neonate can be modulated by recombinant eNOS expression in the pulmonary circulation. We conclude that gene transfer with replication-deficient adenoviral vectors is a useful tool for the study of basic mechanisms of vascular biology. We speculate that development of this technology may prove useful as a vehicle for human gene therapy for pediatric pulmonary vascular diseases such as PPHN and pulmonary hypertension after cardiac surgery.

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Address for reprint requests and other correspondence: J. L. Aschner, Dept. of Pediatrics, Wake Forest Univ. School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157-1081 (E-mail: jaschner@wfubmc.edu).

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