In Vivo Gene Transfer of the O²-Sensitive Potassium Channel Kv1.5 Reduces Pulmonary Hypertension and Restores Hypoxic Pulmonary Vasoconstriction in Chronically Hypoxic Rats

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Background—Alveolar hypoxia acutely elicits pulmonary vasoconstriction (HPV). Chronic hypoxia (CH), despite attenuating HPV, causes pulmonary hypertension (CH-PHT). HPV results, in part, from inhibition of O²-sensitive, voltage-gated potassium channels (Kv) in pulmonary artery smooth muscle cells (PASMCs). CH decreases Kv channel current/expression and depolarizes and causes Ca²⁺ overload in PASMCs. We hypothesize that Kv gene transfer would normalize the pulmonary circulation (restore HPV and reduce CH-PHT), despite ongoing hypoxia.

Methods and Results—Adult male Sprague-Dawley rats were exposed to normoxia or CH for 3 to 4 weeks and then nebulized orotracheally with saline or adenovirus (Ad5) carrying genes for the reporter, green fluorescent protein reporter human Kv1.5 (cloned from normal PA). HPV was assessed in isolated lungs. Hemodynamics, including Fick and thermodilution cardiac output, were measured in vivo 3 and 14 days after gene therapy by use of micromanometer-tipped catheters. Transgene expression, measured by quantitative RT-PCR, was confined to the lung, persisted for 2 to 3 weeks, and did not alter endogenous Kv1.5 levels. Ad5-Kv1.5 caused no mortality or morbidity, except for sporadic, mild elevation of liver transaminases. Ad5-Kv1.5 restored the O²-sensitive K⁺ current of PASMCs, normalized HPV, and reduced pulmonary vascular resistance. Pulmonary vascular resistance decreased at day 2 because of increased cardiac output, and remained reduced at day 14, at which time there was concomitant regression of right ventricular hypertrophy and PA medial hypertrophy.

Conclusions—Kv1.5 is an important O²-sensitive channel and potential therapeutic target in PHT. Kv1.5 gene therapy restores HPV and improves PHT. This is, to the best of our knowledge, the first example of K⁺ channel gene therapy for a vascular disease. (Circulation. 2003;107:2037-2044.)

Key Words: catheterization ■ hypoxia ■ cardiac output ■ oxygen ■ gene therapy

Exposure to hypoxia for 1 to 2 weeks elicits chronic hypoxic pulmonary hypertension (CH-PHT). Rodent CH-PHT is a relevant model for PHT that occurs in humans living at high altitude and in patients with chronic lung diseases. Paradoxically, acute hypoxic pulmonary vasoconstriction (HPV) is blunted in CH-PHT, whereas the response to other vasoconstrictors is preserved or enhanced.¹⁻³ The loss of HPV and development of CH-PHT are associated with decreased expression and function of O²- and 4-aminopyridine (4-AP)–sensitive, voltage-gated K⁺ channels (Kv1.5 and Kv2.1).² CH also selectively decreases function and expression of these channels in cultured pulmonary artery smooth muscle cells (PASMCs).⁴

Hypoxic vasoconstriction is intrinsic to and uniquely manifested in the pulmonary circulation.⁵ Hypoxia constricts small pulmonary arteries (PAs) supplying hypoxic alveoli, thereby shunting perfusion to better-ventilated lobes. This matching of perfusion to ventilation optimizes systemic oxygenation.⁶ Although significantly modulated by endothelial mediators (enhanced by endothelin and thromboxane, reduced by nitric oxide and vasodilator prostaglandins), the fundamental mechanism of HPV is intrinsic to the PASMC.⁷ Channel inhibition, whether by 4-AP or hypoxia, decreases whole-cell K⁺ current (I钾) and depolarizes PASMCs. This increases the opening of L-type, voltage-dependent Ca²⁺ channels, raising cytosolic Ca²⁺ and activating the contractile apparatus.⁸⁻⁹ Several O²-sensitive Kv channels seem to be involved in initiating HPV, including Kv1.5,¹⁰⁻¹² Kv2.1/9.3,¹¹,¹³ and possibly Kv1.2 and Kv3.1b.¹⁴,¹⁵ Decreased expression and activity of Kv1.5 occurs in various forms of

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PHT, including CH-PHT. Mice lacking Kv1.5 also have depressed HPV. Furthermore, anorexigenic drugs, which have precipitated outbreaks of human PHT, inhibit K⁺ channels, including Kv1.5. We hypothesized that restoration of Kv1.5 by use of gene transfer, achieved by nebulization of an adenoviral vector, would reduce CH-PHT and restore HPV.

**Methods**

The protocol was approved by the Animal Health Care Committee of the University of Alberta, Edmonton.

**Adenovirus Vector**

A 2.1-kb cDNA fragment of Kv1.5 was obtained by reverse transcription of mRNA derived from the proximal PA of a cardiac transplant donor. A recombinant, replication-deficient serotype 5 adenovirus carrying genes encoding green fluorescent protein (GFP) and Kv1.5 was prepared as described previously. The resulting virus was isolated, precipitated, and concentrated to a final viral titer of 10⁹ pfu/mL.

**CH-PHT**

CH-PHT was induced by gradually acclimating adult male Sprague-Dawley rats (Biosciences Lab Animal Services Center, University of Alberta, Edmonton, Canada) to a 10% O₂ environment, over a period of 2 weeks, in a normobaric hypoxic chamber (Reming Bioinstruments) as described previously.

**In Vivo Gene Delivery to the Lung**

Rats were randomized into the following groups: Normoxic-saline (N-S), CH saline (CH-S), CH-Ad5-GFP, and CH-Ad5-GFP-Kv1.5 gene. Rats were anesthetized (ketamine 75 mg/kg and xylazine 10 mg/kg) and intubated orotracheally with PE-240 tubing (Intramedic) during tracheal transillumination. While breathing spontaneously, rats were nebulized with 100 L of sterile saline, Ad5-GFP, or Ad5-GFP-Kv1.5 by use of an intratracheal microspray device placed in the distal endotracheal tube (MicroSprayer, Penn-Century Inc). This dose was based on preliminary experiments showing that it increased Kv1.5 expression without undue toxicity. Rats recovered from anesthesia for ~24 hours before return to the chamber. Experiments were performed 3 or 14 days after nebulization. Total sample size was 100 rats: day 3 hemodynamics and isolated lungs (n=10×4 groups); day 14 hemodynamics (n=7×3 groups); immunoblotting, reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry, and confocal imaging (n=3×4 groups); and patch-clamping (n=6×4 groups). Time course and organ specificity of transgene expression was measured in normoxic rats at 0, 2, 7, 14, 21, and 28 days (n=3 per period).

**Hemodynamic Measurements**

PA pressure (PAP) was measured in closed-chest rats with a 1.4F, micromanometer-tipped catheter (Millar Instruments) delivered by a customized introducer-sheath system without radiological guidance (Figure 1) as described previously. The mean PAP was determined by electronic averaging over 1 minute. Left ventricular end-diastolic pressure (LVEDP) was measured via retrograde cannulation of the LV and systemic blood pressure via the right common carotid artery. Cardiac output (CO) was measured by use of the Fick method for day 3 studies and by both the Fick and thermodilution techniques for day 14 experiments. The techniques correlated well (Figure 2). Pulmonary vascular resistance (PVR) was calculated as PAH - LVEDP/CO. The ratio of right ventricular (RV)/LV septum weight was used as a measure of RV hypertrophy (RVH). Systemic vascular resistance (SVR) was calculated as mean systemic blood pressure - right atrial pressure/CO. Medial hypertrophy of resistance pulmonary arteries was measured as described previously.

**Thermodilution**

A theromistor (ADInstruments) was calibrated at 0°C and 37°C by use of a digital thermometer (VWR Canlab) and inserted retrogradely into the aorta. Saline (1 mL at 0°C) was injected into the RV via the PA catheter. The thermodilution curve was recorded by an analog-digital converter CO pod by use of Chart 4.2 (ADInstruments). CO was computed by use of the following equation:

\[
CO(\text{mL/min}) = \frac{c \times (T_{\text{blood}} - T_{\text{injectate}}) \times V_{\text{injectate}}}{f \Delta T dt}
\]

where c is a constant (1.0) describing the relative heat capacities of blood and injectate, Tblood is the baseline blood temperature, Tinjectate and Vinjectate are the temperature and volume of the injectate, and fΔTdt represents the area under the thermodilution curve. The curve was integrated from 1 second before to 9 seconds after the initial deflection.
Fick Method

Anesthetized, intubated rats were ventilated with 40% O₂ (Inspiratory ASV rodent ventilator, Harvard Apparatus). Tidal volume (Vₜ) and ventilation rate (V₉) were weight-adjusted. The ventilator exhaust was connected via 3-way stopcock to a collection bag, and inspired and expired gas samples (I₂O₂ and E₂O₂) were collected over 1 minute. Arterial and mixed venous blood samples (PaO₂ and PvO₂) were withdrawn from aortic and PA catheters, respectively. Arterial and mixed venous saturation (SaO₂ and SvO₂) and hemoglobin (Hgb, g/dL) were calculated from these samples. Gas and blood samples were analyzed by use of a Bayer-288 Blood Gas Analyzer. CO was calculated by use of the Fick equation:

\[
CO \text{ (mL/min)} = \frac{100 \times Vₜ \times (I₂O₂ - E₂O₂)}{P_{atm}} \times Hgb \times O₂ \text{ binding factor}
\]

P_{atm} is barometric pressure. The constant 100 matches the denominator (in dL) to the numerator (in mL). The value 1.34 is the Hgb-O₂ binding factor at standard temperature and pressure. The O₂ solubility coefficient is 0.003 mL · mm Hg⁻¹ · dL⁻¹.

Blood Work

After hemodynamic measurements, 1 to 2 mL of blood was collected to assess possible Ad5 toxicity (Table).

Isolated Lung Perfusion

The isolated perfused lung model was performed as described previously. Flow of Krebs solution containing 4% BSA was maintained constant at 0.04 mL · g⁻¹ · min⁻¹ by a roller pump. Therefore, changes in PAP reflected solely changes in PVR. The lungs were ventilated with either normoxic or hypoxic humidified gas (20% or 2.5% O₂, respectively, plus 5% CO₂, balance N₂). Normoxic and hypoxic pH, PCO₂, and PO₂ were 7.33 ± 0.01, 31 ± 2.5 mm Hg, and 134 ± 3.7 mm Hg and 7.37 ± 0.02, 31 ± 1.6 mm Hg, and 42 ± 2.1 mm Hg, respectively. The perfusate contained inhibitors of nitric oxide synthase (N⁴-nitro-L-arginine methyl ester, 10⁻⁶ mol/L) and cyclooxygenase (meclofenamate, 10⁻⁵ mol/L). Lungs were exposed to 3 cycles of normoxia (10 minutes), angiotensin II (10⁻⁶ mol/L, bolus into PA line followed by 8 minutes of equilibration), and hypoxia (6 minutes).

Immunoblotting

Immunoblots were performed and analyzed on homogenized lungs as described previously by use of Kv.1.5 and Kv.2.1 channel (Alomone) and GFP antibodies (200 L diluted 1:1000, Clontech Laboratories). Signal intensity of the immunoreactive Kv bands was normalized to the expression of α-smooth muscle actin.

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time PCR was performed as described previously, by use of species-specific K⁺ channel primers. Copy number was expressed as 2^{ΔΔCt} (Ct is cycle time). The 2^{ΔΔCt} normalizes expression to a housekeeping gene (GAPDH) and a calibrator (the sample expressing the lowest amount of the gene of interest).

Immunohistochemistry

Immunohistochemistry for Kv.1.5 was performed on paraffin-embedded, formaldehyde-fixed lungs counterstained with hematoxylin.

Confocal Microscopy

Confocal imaging of GFP was obtained by use of an LSM-510 confocal microscope (Zeiss) at excitation 488 nm and detection 505 to 530 nm.
At 14 days after infection (Figure 2), Hemodynamics

Hematology

At 3 days after infection (Figure 1), CH significantly increased mean PAP and PVR, and this was unaffected by the Ad5-GFP control virus. Cardiac index (CI) was significantly greater in the CH-Ad5-Kv1.5 group than the other CH groups, and consequently, PVR index was reduced to normoxic levels. SVR tended to be lower in the Ad5-Kv1.5 group, but this was not statistically significant. RVH occurred in all CH groups, and regression was not evident 3 days after Ad5-Kv1.5.

Results

Hemodynamics

At 3 days after infection (Figure 1), CH significantly increased mean PAP and PVR, and this was unaffected by the Ad5-GFP control virus. Cardiac index (CI) was significantly greater in the CH-Ad5-Kv1.5 group than the other CH groups, and consequently, PVR index was reduced to normoxic levels. SVR tended to be lower in the Ad5-Kv1.5 group, but this was not statistically significant. RVH occurred in all CH groups, and regression was not evident 3 days after Ad5-Kv1.5.


Regression of Hypertrophy and Vascular Remodeling

There was partial regression of RVH in the CH-Ad5-Kv1.5 group at day 14. Likewise, there was a reduction in the medial hypertrophy in small PAs in the CH-Ad5-Kv1.5 versus the CH-S group.

Isolated Perfused Lungs

At 3 days after infection (Figure 3), HPV was reduced in the CH-Ad5-GFP and CH-S groups compared with the N-S group and was restored to normal levels by Kv1.5 gene transfer. The ΔA-II response was also enhanced in the CH-Ad5-Kv1.5 group.

With regard to Kv1.5 and GFP expression, transgene expression was evident 2 days after nebulization and persisted for >14 days (Figure 2C). GFP protein expression was noted in the resistance PAs, veins, and airways of CH-Ad5-Kv1.5 rats (Figure 4), and Kv1.5 expression was enhanced (Figure 5A). GFP was detected only in the CH-Ad5-Kv1.5 and CH-Ad5-GFP groups (Figure 4, A and B). Rat Kv1.5 was most abundant in the lung compared with other organs (Figure 6A). Quantitative real-time PCR performed on individual, isolated resistance PAs demonstrated that CH significantly decreased mRNA for endogenous rat Kv1.5 but not Kir2.1 or large-conductance calcium-sensitive K^+ (BKCa) channels (Figure 6D). Kv1.5 protein expression was reduced in CH-S versus N-S. Human Kv1.5 expression was restricted to the lung (Figure 6B) and did not alter expression of rat Kv1.5 (Figure 2C) or other Kv channels (eg, Kv2.1, Figure 4C). Unlike endogenous Kv1.5, which decreased in CH (Figure 6D), human Kv1.5 expression tended to increase (Figure 6E).

Electrophysiology

Hypoxia and the Kv blocker, 4-AP, both reversibly inhibited identical portions of I_K in N-S PASMCs (Figure 7). CH significantly decreased current density because of loss of this O_2-sensitive current. CH-Ad5-Kv1.5 treatment increased normoxic current density beyond control levels and restored the hypoxia- and 4-AP-sensitive I_K. There was no effect of the Ad5-GFP on I_K. The increase in current density occurred at all voltages, including those near the resting membrane potential.

Discussion

This study has 4 major findings. First, administration of Kv1.5 to the pulmonary circulation via an aerosol is feasible and effective in eliciting transgene expression in resistance PASMCs. Second, administration of Kv1.5 cloned from human PA reduces PVR in experimental PHT. Third, Kv gene therapy with an O_2-sensitive channel restores HPV and O_2-sensitive I_K in rats with established CH-PHT. Fourth, transgene expression and hemodynamic benefit, although selective to the lung and relatively well tolerated, are maintained for only 2 to 3 weeks. This study supports a central role for Kv1.5 in the mechanism of HPV and is

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*P<0.05, value differs from normoxic control.
†P<0.05, Ad5-Kv1.5 value differs from CH-saline group.

Electrophysiology

PASMCs isolated from fourth-division PAs by enzymatic dispersion were studied at 25°C by use of the whole-cell patch-clamp technique. PASMCs were voltage-clamped at a holding potential of −70 mV. Currents were evoked by 200-ms test pulses from −70 to +70 mV. The pipette solution contained (in mmol/L) KCl 134, KHPO_4 1.2, MgCl_2 1.0, HEPES 5, pH 7.30, Na_2ATP 5, and EGTA 5.

Drugs and Statistics

All drugs were obtained from Sigma-Aldrich Chemical Co unless stated otherwise. Values are expressed as mean±SEM. Intergroup differences were assessed by use of factorial ANOVA. Post hoc analysis was performed by use of a Fisher’s protected least significant difference test. A value of P<0.05 was considered statistically significant.

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consistent with the hypothesis that a K⁺ channel–deficiency state is involved in the pathogenesis of PHT. 20,21

During acute, focal airway hypoxia, HPV is an adaptive response, shunting blood from hypoxic alveoli to nonhypoxic alveoli, hence optimizing systemic oxygenation. However, if exposure to alveolar hypoxia is global and sustained, constriction to acute hypoxia but not angiotensin II is depressed (Figure 3).1,2 Teleologically, this may be advantageous in

Figure 3. Blunted HPV caused by CH is restored by Ad5-Kv1.5. A, Representative traces and mean data (B) from isolated perfused lungs showing that HPV is attenuated by CH and is restored CH-Ad5-Kv1.5, but not in control virus group (CH-Ad5-GFP). All indicates angiotensin II.

Figure 4. Demonstration of effective gene transfer to distal airways and PA. A, Representative confocal microscopy images showing GFP fluorescence in PA and airways. This occurred only in CH-Ad5-Kv1.5 and CH-Ad5-GFP lungs. B, Immunoblots show that GFP is present only in Ad5-treated lungs. C, Representative immunoblot (each lane is 1 lung) showing that Ad5-Kv1.5 increases Kv1.5 expression without reducing Kv2.1 expression. D, Mean data at day 3 showing that CH reduces and Ad5-Kv1.5 greatly augments Kv1.5 expression.
minimizing RV strain under circumstances in which HPV cannot augment O₂ uptake. Thus, although CH-PHT may be initiated by HPV, PHT seems to be sustained by the remodeling of the pulmonary vasculature, characterized by distal extension of smooth muscle and medial hypertrophy of small PAs.²²

The loss of Kv1.5 expression seems to be important to the pathogenesis of various forms of PHT, including CH-PHT.²,¹⁶,²⁰ This concept is now supported by the finding that augmentation of Kv1.5 channel expression by gene transfer reduces PHT (Figures 1 and 2). The reduction in PVR was the result of increased CO rather than a decrease in mean PAP. A “resistance” response (ie, a decrease in PVR of >20% with <20% decrease in PAP), although not prognostically as favorable as a 20% decrease in PAP, is not an uncommon response to an acute vasodilator in human PHT.²¹ We attribute the decrease in PVR and increased CI in Ad5-CH-Kv1.5 rats to improved pulmonary vascular compliance and regression in medial hypertrophy of small PAs (Figure 5, B and C). Although SVR was slightly lower (P=NS) in the Ad5-CH-Kv1.5 versus CH-S rats at day 3, there was no such trend at day 14 (Figures 1 and 2). Indeed, the mean aortic pressure was slightly higher in the Ad5-CH-Kv1.5 versus CH-S rats (88 ± 3 versus 79 ± 1 mm Hg), implying that the increased CI in Ad5-CH-Kv1.5 rats is probably a result of decreased RV afterload rather than systemic vasodilation.
The possibility of systemic leak of nebulized Kv1.5 transgene, which theoretically could have increased CI by enhancing cardiac inotropy or promoting peripheral vasodilation, was excluded by demonstrating the absence of human Kv1.5 in systemic organs (Figure 6B). This report describes and validates a relatively simple method for measuring CO by thermodilution (Figure 2A).

Failure of the gene therapy to lower PAP may relate to the restoration of HPV (normally suppressed by CH) or the use of a simplistic channel replacement strategy, which did not address downregulation of other ion channels, such as Kv2.1. Dichloroacetate, a metabolic modulator that ameliorates CH-PHT and that lowered PAP, acts by increasing Kv2.1 expression and function.16 Furthermore, other pathogenetic abnormalities that promote PHT undoubtedly persist, including activation of hypoxia-inducible factor (HIF). It is likely that activation of HIF-1α continues to drive the residual RVH and polycythemia observed in the CH-Ad5-Kv1.5 rats23 (Table).

Kv1.5 is important to the mechanism of HPV, and its loss, whether by administration of an anti-Kv1.5 antibody11 or by Kv1.5 gene deletion,12 reduces O₂-sensitive \( I_K \) and HPV. Kv1.5 expression in PAs is also selectively downregulated in both human pulmonary arterial hypertension24 and experimental PHT.2,25 In CH, PASMCs are depolarized, and their \( I_K \) is less sensitive to 4-AP and hypoxia,26 as seen in Figure 7. These physiological and electrophysiological abnormalities are associated with, and presumably result from, downregulation of Kv1.5 and Kv2.1.2,4

Kv1.5 expression is regulated at both the transcriptional and translational levels. Kv1.5 mRNA and protein have very rapid turnover.27 Depolarization itself reduces Kv1.5 expression in some cells within 8 hours.28 The rapid loss of lung Kv1.5 expression temporally parallels the suppression of HPV, which occurs within 2 days of CH.2,3 CH could decrease Kv1.5 expression by decreasing Kv1.5 gene transcription, destabilizing Kv1.5 mRNA, or accelerating turnover of Kv1.5 protein. The fact that CH suppresses genomic but not episomally driven Kv1.5 expression (Figure 6E) suggests that the control of endogenous Kv1.5 gene is transcriptionally regulated. We speculate that CH may activate Kv1.5 repressor elements, a dinucleotide repetitive DNA sequence, forming a cell-specific silencer.29 When activated, this K⁺ channel repressor element inhibits transcription of the Kv1.5 gene.29 Perhaps Kv1.5 repressor element, which is redox sensitive and is located 5’ to the Kv1.5 gene, is activated by CH.

Limitations
The limited duration of transgene expression is typical of adenovirus. Ad5 is nonreplicant, does not incorporate into the host genome, and despite modification, ultimately is eliminated by the host. However, the nebulization route of infection is simple, effective, and well tolerated, as reported previously in mice.30 The current vector did not cause mortality, overt lung toxicity, or inflammation, nor did it alter renal function or hematological parameters (Table). There was an increase in aspartate aminotransferase in several rats, without alteration of bilirubin or alkaline phosphatase levels (Table).

In conclusion, Kv1.5 gene transfer via airway nebulization in vivo is feasible and effective in restoring HPV and ameliorating PHT in the CH-PHT model. Future vectors will use cell-type–specific promoters to target endothelium versus SMCs (eg, SM-22), rather than the promiscuous cytomegalovirus promoter, and will also include genes for other O₂-sensitive K⁺ channels.

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References


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