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

Zlatko I. Pozeg, MD; Evangelos D. Michelakis, MD; M. Sean McMurtry, MD; Bernard Thébaud, MD, PhD; Xi-Chen Wu, PhD; Jason R.B. Dyck, PhD; Kyoko Hashimoto, BSc; Shaohua Wang, MD; Rohit Moudgil, MSc; Gwyneth Harry, MSc; Richard Sultanian, MSc; Arvind Koshal, MD; Stephen L. Archer, MD

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 (GFP) and 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 (Iₖ) and depolarizes PASMCs. This increases the opening of L-type, voltage-dependent Ca²⁺ channels, raising cytosolic Ca²⁺ and activating the contractile apparatus.⁸⁻¹⁰ Several O₂-sensitive Kv channels seem to be involved in initiating HPV,¹⁰ including Kv1.5,¹¹,¹² 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
PHT, including CH-PHT. Mice lacking Kv1.5 also have depressed HPV. Furthermore, anorexigens, 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 1.5 \times 10^{10} 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 \muL 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 \sim 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=4 groups); day 14 hemodynamics (n=7 groups); immuno blotting, reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry, and confocal imaging (n=4 groups); and patch-clamping (n=6 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 PAP/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 thermostir (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:

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

where c is a constant (1.0) describing the relative heat capacities of blood and injectate, T_{\text{blood}} is the baseline blood temperature, T_{\text{injectate}} and V_{\text{injectate}} are the temperature and volume of the injectate, and \int \Delta T \text{dt} 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% O\textsubscript{2} (Inspira-ASV rodent ventilator, Harvard Apparatus). Tidal volume (V\textsubscript{T}) and ventilation rate (V\textsubscript{R}) were weight-adjusted. The ventilator exhaust was connected via 3-way stopcock to a collection bag, and inspired and expired gas samples (I\textsubscript{O}2 and E\textsubscript{O}2) were collected over 1 minute. Arterial and mixed venous blood samples (Pa\textsubscript{O}2 and Pv\textsubscript{O}2) were withdrawn from aortic and PA catheters, respectively. Arterial and mixed venous saturation (Sa\textsubscript{O}2 and Sv\textsubscript{O}2) 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:

\[
\text{CO (mL/min)} = \frac{100 \times V_T \times (I_{O_2} - E_{O_2})/P_{atm}}{[0.003 \times Pa_0 + 1.34 \times Hgb \times Sa_0] - (0.003 \times Pv_0 + 1.34 \times Hgb \times Sv_0)}
\]

P\textsubscript{atm} is barometric pressure. The constant 100 matches the denominator (in mL) to the numerator (in mL). The value 1.34 is the Hgb-O\textsubscript{2} binding factor at standard temperature and pressure. The O\textsubscript{2}-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% O\textsubscript{2} respectively, plus 5% CO\textsubscript{2} balance N\textsubscript{2}). Normoxic and hypoxic pH, PC\textsubscript{O}2, and PO\textsubscript{2} 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 (\textsuperscript{cG}-nitro-L-arginine methyl ester, 10⁻⁵ mol/L) and cyclooxygenase (meclomenamate, 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 Kv1.5 and Kv2.1 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 -smooth muscle actin.

Quantitative Real-Time Polymerase Chain Reaction
Quantitative real-time PCR was performed as described previously, by use of species-specific K\textsuperscript{+} channel primers. Copy number was expressed as 2⁻ΔCt (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 Kv1.5 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.
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 groups, and consequently, PVR index was reduced to normoxic levels. SVR index was increased in the CH-Ad5-Kv1.5 group, but this was not statistically significant. RVH occurred at all voltages, including those near the resting membrane potential.

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 6C) 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\textsubscript{K} in N-S PASMCs (Figure 7). CH significantly decreased current density because of loss of this O\textsubscript{2}- and 4-AP-sensitive current. CH-Ad5-Kv1.5 treatment increased normoxic current density beyond control levels and restored the hypoxia- and 4-AP-sensitive I\textsubscript{K}. There was no effect of the Ad5-GFP on I\textsubscript{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\textsubscript{2}-sensitive channel restores HPV and O\textsubscript{2}-sensitive I\textsubscript{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
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 O<sub>2</sub> 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.

Figure 5. Human Kv1.5 is overexpressed in CH-Ad5-Kv1.5 PAs. A, Immunohistochemistry for Kv1.5 (brown signal) is increased in both airway and PAs of CH-Ad5-Kv1.5 group versus CH-S group. Control slide (no primary antibody) was blank, indicating lack of nonspecific staining (not shown). B, Increase in medial thickness seen in small alveolar pulmonary arteries in CH-PHT is significantly reduced in CH-Ad5-Kv1.5 group. C, Hematoxylin-eosin staining of small PAs showing partial regression of hypertrophy in CH-Ad5-Kv1.5 group. BR indicates bronchiole.

Figure 6. Localization, quantification, and duration of transgene expression. A, Quantitative real-time PCR demonstrating that endogenous Kv1.5 is most abundant in lung. B, Transgene expression is found only in lung (day 3 after infection). C, Human Kv1.5 mRNA is present only in CH-Ad5-Kv1.5 lungs. D, CH significantly decreased rat Kv1.5, but not Kir2.1 or BK<sub>Ca</sub><sub>2</sub>, mRNA expression. Comparison of relative copy number (2<sup>-<Ct></sup> ) is valid only within a group (eg, N-S versus CH-S), and amount of Kv1.5 expressed, relative to other channels, was not determined. E, Human Kv1.5 mRNA expression is increased by CH.
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 Ik 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 IK 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 element, 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.

Acknowledgments
Drs Michelakis and Archer were supported by the Alberta Heritage Foundation for Medical Research, the Canadian Foundation for Innovation, the Heart and Stroke Foundation of Canada, and the Canadian Institutes of Health Research.
References


In Vivo Gene Transfer of the O$_2$-Sensitive Potassium Channel Kv1.5 Reduces Pulmonary Hypertension and Restores Hypoxic Pulmonary Vasoconstriction in Chronically Hypoxic Rats


_Circulation_. 2003;107:2037-2044; originally published online April 14, 2003;
doi: 10.1161/01.CIR.0000062688.76508.B3

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/107/15/2037

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/