In Vivo Cardiac Gene Transfer of Kv4.3 Abrogates the Hypertrophic Response in Rats After Aortic Stenosis

Djamel Lebeche, PhD*; Roger Kaprielian, PhD*; Federica del Monte, MD, PhD; Gordon Tomaselli, MD; Judith K. Gwathmey, VMD, PhD; Arnold Schwartz, MD; Roger J. Hajjar, MD

Background—Prolongation of the action potential duration (APD) and decreased transient outward K⁺ current (Iₒ) have been consistently observed in cardiac hypertrophy. The relation between electrical remodeling and cardiac hypertrophy in vivo is unknown.

Methods and Results—We studied rat hearts subjected to pressure overload by surgical ascending aortic stenosis (AS) and simultaneously infected these hearts with an adenovirus carrying either the Kv4.3 gene (Ad.Kv4.3) or the β-galactosidase gene (Ad.β-gal). Iₒ density was reduced and APD50 was prolonged (P<0.05) in AS rats compared with sham rats. Kv4.2 and Kv4.3 expressions were decreased by 58% and 51%, respectively (P<0.05). AS rats infected with Ad.β-gal developed cardiac hypertrophy compared with sham rats, as assessed by cellular capacitance and heart weight–body weight ratio. Associated with the development of cardiac hypertrophy, the expression of calcineurin and its downstream transcription factor nuclear factor of activated T cells (NFAT) c1 was persistently increased by 47% and 36%, respectively (P<0.05) in AS myocytes infected with Ad.β-gal compared with sham myocytes. In vivo gene transfer of Kv4.3 in AS rats was shown to increase Kv4.3 expression, increase Iₒ density, and shorten APD50 by 1.6-fold, 5.3-fold, and 3.6-fold, respectively (P<0.05). Furthermore, AS rats infected with Ad.Kv4.3 showed significant reductions in calcineurin and NFAT expression. (P<0.05).

Conclusions—Downregulation of Iₒ, APD prolongation, and cardiac hypertrophy occur early after AS, and in vivo gene transfer of Kv4.3 can restore these electrical parameters and abrogate the hypertrophic response via the calcineurin pathway. (Circulation. 2004;110:3435-3443.)

Key Words: hypertrophy ■ gene therapy ■ ion channels ■ potassium ■ stenosis

In response to excess hemodynamic loading and neurohormonal activation, the myocardium undergoes adaptive hypertrophic growth to temporarily augment cardiac function. Cardiac hypertrophy is associated with an increased cardiovascular morbidity and mortality,1 which is in part due to electrical remodeling. A central and consistent electrophysiological change in cardiac hypertrophy is usually manifested by prolongation of the action potential duration (APD), which increases the propensity to develop lethal cardiac arrhythmias.2 At the cellular level, APD prolongation is attributed to a reduction of a hyperpolarizing current provided by the calcium-independent transient outward K⁺ channel (Iₒ).3–6 With concomitant reductions in Kv4.2 and Kv4.3 expression,5,7,8 A reduction in Iₒ density and APD prolongation represent early electrical remodeling events in the diseased myocardium,9 pointing toward a potential role in disease initiation and progression.

Although considerable progress has been made in understanding the molecular pathogenesis of hypertrophy, the role of electrical remodeling associated with hypertrophy remains limited. Furthermore, the relation between Iₒ density, APD prolongation, and cardiac hypertrophy in vivo is unknown. Reduction of Iₒ density and prolongation of the APD can regulate calcium entry via the voltage-sensitive calcium channels and augment calcium transient ([Ca²⁺]i) amplitude in cardiac myocytes,4,10,11 which may help to support contraction of the compromised myocardium but may also harm the myocardium by increasing the propensity to develop arrhythmias3,12 and by activating hypertrophic signaling pathways. Activation of the calcineurin pathway and onset of cardiac hypertrophy have also been reported in neonatal rat ventricular myocytes with reduced Iₒ by dominant-negative inhibition and pharmacological blockade of Kv4.2-based Iₒ channels. In addition, abnormal calcium handling in heart failure seems to be correlated with calcineurin activation.13 In the present study, we wanted to determine whether in vivo adenoviral gene transfer of Kv4.3-based Iₒ channels can

Received June 29, 2004; revision received August 10, 2004; accepted August 17, 2004.
From the Cardiovascular Research Center (D.L., R.K., F.d.M., R.J.H.), Massachusetts General Hospital, Harvard Medical School, Charlestown, Mass; the Institute of Molecular Pharmacology and Biophysics (D.L., A.S.), University of Cincinnati Medical Center, Cincinnati, Ohio; the Johns Hopkins University School of Medicine (G.T.), Baltimore, Md; and Boston University School of Medicine (J.K.G.), Boston, Mass.
*The first 2 authors contributed equally to this work.
Correspondence to Roger J. Hajjar, MD, Cardiovascular Research Center, Massachusetts General Hospital, 149 13th St, CNY-4, Charlestown, MA 02129. E-mail hajjar@cvcrg.mgh.harvard.edu
© 2004 American Heart Association, Inc.
Circulation is available at http://www.circulationaha.org
DOI: 10.1161/01.CIR.0000148176.33730.3F
increase $I_{\text{so}}$ density, shorten the APD, and modulate the signaling pathways that regulate the hypertrophic response.

**Methods**

**Adenovirus Preparation**

Recombinant adenoviruses were generated with the E1-deleted pAdEasy-1 adenoviral plasmid (kindly provided by Dr Bert Vogelstein, John Hopkins University, Baltimore, Md) and the shuttle vector pAdTrack containing the cDNA for enhanced green fluorescent protein (GFP). An adenoviral vector encoding Kv4.3 (Ad.Kv4.3) was constructed by using the human Kv4.3 homologue (hKv4.3.1 cDNA-short splice variant). The Ad.Kv4.3 contained 4 copies of cardiac tissue–specific promoter, namely, the 250-bp fragment of the myosin light chain-2v gene, which we have shown to confer cardiac expression specificity. Ad.$\beta$-gal contained cytogomavir–driven expression cassettes for $\beta$-galactosidase and GFP.

**In Vivo Adenoviral Gene Transfer and Induction of Ascending Aortic Stenosis (AS)**

The method of adenoviral delivery was previously described by our group. After baseline echocardiographic measurements, animals were randomly assigned to sham, control, and experiment groups. The method of adenoviral in vivo delivery was performed as previously described. In brief, male Sprague-Dawley rats (body weight, 250 to 300 g) were anesthetized with ketamine HCl (60 mg/kg IP) and xylazine (10 mg/kg IP) and placed on a ventilator. A 22-gauge catheter containing 300 μL of a saline solution or an adenovirus solution containing $1.5 \times 10^{10}$ plaque-forming units of either AdLacZ or Ad.Kv4.3 was advanced from the apex of the left ventricle to the aortic root. A tourniquet was placed around the aorta and the pulmonary artery at a site distal to the tip of the catheter, and the solution was injected. The tourniquet was held in place for 40 seconds while the heart pumped against a closed system (isovolumetrically) and then released. The rats were then subjected to supravalvular aortic banding with a 0.58-mm (internal diameter) tantalum clip as previously described. Sham-operated animals were used as controls.

**Echocardiography**

Animals were lightly anesthetized with ketamine HCl (50 mg/kg IP) and xylazine (10 mg/kg IP), shaved, and placed in a left lateral position. Transthoracic 2-dimensional and M-mode echocardiographic images were obtained at the midapical level with a fully digitized Vingmed System Five (GE Vingmed Ultrasound) with a 10-MHz pediatric transducer. Images were recorded on a magneto-optical disk and subsequently analyzed with EchoPac software (version 6.0, GE Vingmed Ultrasound). Anterolateral and anteroseptal wall thicknesses and left ventricular diameters in end-diastole (LVDD) and end-systole (LVDS) were measured offline from M-mode recordings, according to the American Society for Echocardiography leading-edge method. Fractional shortening (in percent) was calculated as $\left(100 \times (LVDD-LVDS)/LVDD\right)$. All measurements were averaged over 3 consecutive cycles and were performed by an investigator blinded to the experimental protocol.

**Isolation of Ventricular Myocytes**

On day 8, left ventricular myocytes were isolated for electrophysiological studies and protein analysis as previously described. Hereafter, myocytes derived from sham-operated rats will be referred to as sham myocytes, whereas myocytes derived from rats subjected to aortic stenosis will be referred to as AS myocytes.

**Single-Cell Electrophysiology**

Current densities and action potentials (APs) were recorded by the whole-cell patch-clamp technique with an Axopatch 200B amplifier and a headstage CV 203 BU coupled to a Digidata 1320 personal computer interface (Axon Instruments). Cell capacitance was estimated by integrating the area of the capacitance transients after a 5-mV step from a holding potential of −70 mV. The measured currents were divided by the cell capacitance to normalize currents for cell size.

To measure $I_{\text{so}}$ densities and APs, myocytes were superfused with a modified Tyrode’s solution containing (in mmol/L) NaCl 140, MgCl2 1, HEPES 10, CaCl2 1, and D-glucose 10, with pH adjusted to 7.4 by addition of NaOH. CdCl2 (0.3 mmol/L) was routinely added to block $I_{\text{CaL}}$ when $K^+$ currents were being recorded. Patch pipettes were pulled, fire-polished to final tip resistances of 2 to 4 MΩ, and then filled with pipette solution containing (in mmol/L) potassium aspartate 130, KCl 20, HEPES 10, MgCl2 1, NaCl 5, EGTA 10, and MgATP 5, with pH adjusted to 7.2 by addition of Trizma base. Junction potentials (−8.2 mV) after rupture were corrected for AP recordings only. $I_{\text{so}}$ was measured by depolarizing the myocyte membrane to +70 mV for 500 ms from a resting potential of −80 mV. Tip potentials were zeroed before formation of the membrane pipette seal in the Tyrode’s solution. Mean seal resistance averaged 12.5±0.9 GΩ, and pipette resistance averaged 2.7±0.1 MΩ. Voltage-clamp experiments were performed with an interpulse interval of 5 seconds. APs were initiated by short depolarizing current pulses (2 to 3 ms, 500 to 800 pA) at 0.2 Hz. In

![Figure 1](https://example.com/image1.jpg)  
**Figure 1.** Left ventricular myocytes derived from rat hearts after in vivo adenoviral gene transfer. Rod-shaped left ventricular myocytes isolated 8 days after catheter-based gene transfer of $\beta$-gal gene coexpressing GFP with use of Ad.$\beta$-gal. The left panel shows the intact ventricle with most of the myocytes staining positive with an antibody directed against $\beta$-gal (green) with some counterstaining with Calcein AM (red). The middle panel shows a group of myocytes subjected to catheter-based gene transfer of an adenovirus–driven expression cassette for $\beta$-galactosidase. The right panel shows a single myocyte subjected to catheter-based gene transfer of an adenovirus–driven expression cassette for $\beta$-galactosidase and GFP.  

*Abbreviations are as defined in text.*
Echocardiographic Parameters at Baseline and 7 Days After AS

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=8)</th>
<th>AS+Saline (n=6)</th>
<th>AS+Ad.β-gal (n=7)</th>
<th>AS+Kv4.3 (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Day 7</td>
<td>Baseline</td>
<td>Day 7</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>0.30±0.03</td>
<td>0.45±0.02*</td>
<td>0.44±0.03*</td>
<td>0.34±0.04†</td>
</tr>
<tr>
<td>LVDD, mm</td>
<td>6.10±0.30</td>
<td>5.98±0.31</td>
<td>6.23±0.11</td>
<td>7.12±0.39†</td>
</tr>
<tr>
<td>LVDS, mm</td>
<td>3.00±0.20</td>
<td>3.03±0.30</td>
<td>2.80±0.41</td>
<td>2.79±0.30</td>
</tr>
<tr>
<td>FS, %</td>
<td>48.4±5.10</td>
<td>51.2±8.20</td>
<td>51.5±9.0</td>
<td>59.6±7.8</td>
</tr>
<tr>
<td>AWTD, mm</td>
<td>2.27±0.31</td>
<td>2.24±0.31</td>
<td>2.22±0.35</td>
<td>2.97±0.15†</td>
</tr>
<tr>
<td>PWTD, mm</td>
<td>2.16±0.21</td>
<td>2.18±0.29</td>
<td>2.00±0.10</td>
<td>2.88±0.10†</td>
</tr>
<tr>
<td>Capacitance, pF (GFP-positive)</td>
<td>119±4.3</td>
<td>189±14*</td>
<td>150±15‡</td>
<td></td>
</tr>
</tbody>
</table>

HW, indicates heart weight; BW, body weight; FS, fractional shortening; AWTD, anterior wall thickness at end-diastole; and PWTD, posterior wall thickness at end-diastole. All other abbreviations are as defined in text. Data are presented as mean±SD.

*P<0.01 vs sham; †P<0.01 vs baseline; ‡P<0.05 vs AS+β-gal.

Calcium Handling and Contractility

Measurements of intracellular calcium and cell shortening were performed as described earlier. Myocardial cells were loaded with the Ca\(^{2+}\) indicator fura 2 by incubating the cells in medium containing 2 μmol/L fura 2-AM (Molecular Probes) for 30 minutes. Intracellular calcium concentration was calculated according to the formula \([Ca^{2+}]_i = K_d (R_{max} - R)/(R_{max} - R)B\), where \(R\) is the ratio of fluorescence of the cell at 360 and 380 nm; \(R_{max}\) and \(R_{min}\) represent the ratios of fura 2 fluorescence in the presence of saturating amounts of calcium and effectively “zero calcium,” respectively; \(K_d\) is the dissociation constant of Ca\(^{2+}\) from fura 2; and \(B\) is the ratio of fluorescence of fura 2 at 380 nm in zero Ca\(^{2+}\) and saturating amounts of Ca\(^{2+}\). The myocardial cells were imaged with a CCD video camera (Javelin Electronics) attached to the microscope, and motion along a selected rastor line segment was quantified by a video motion-detector system (Ionoptix).

Western Blot Analysis

Protein samples were prepared from sham and AS myocytes immediately after myocyte isolation. They were matched for protein concentration, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and transferred onto nitrocellulose membranes. Blots were incubated with antibodies against Kv4.2, Kv4.3, and Kv1.4 α-subunits (Chemicon); calcineurin; nuclear factor of activated T cells (NFAT) 3; GFP (all from Santa Cruz); and dual phosphospecific antibodies to extracellular signal-regulated protein kinase 1 and 2 (ERK1/2), p38 (New England Bio Labs), and stress-activated protein kinase (SAPK, Promega) or total antibodies (Santa Cruz) overnight at 4°C. Membranes were incubated with appropriate secondary antibody conjugated to horseradish peroxidase for 2 hours, and bands were visualized by their chemiluminescence. Films from at least 3 independent experiments were scanned, and densities of the immunoreactive bands were evaluated with NIH Image software. The mitogen-activated protein kinase (MAPK) activities were verified by determination of the phosphorylation level of each kinase in myocardial lysates with the use of specific phosphoantibodies. Total protein contents of the corresponding MAPKs were also determined after stripping the phosphoblots to verify protein loading.

Statistical Analysis

All data are expressed as mean±SEM. Statistical comparisons between groups were made with 1-way ANOVA, followed by a Student-Newman-Keuls post hoc test, with the SPSS program (version 7.0 for Windows, SPSS Inc). Probability values <0.05 were deemed statistically significant.

Results

Adenoviral Gene Transfer In Vivo

Eight days after gene transfer, 20% to 30% of viable myocytes were shown to exhibit green fluorescence, as shown in Figure 1(A, direct light, and B, fluorescent). The green fluorescence of myocytes verified infection with Ad.β-gal coexpressing GFP. GFP expression was also confirmed by Western blotting, as shown in Figure 1C. The fraction of infected cells was determined by counting green myocytes expressing GFP (infected cells) and nongreen myocytes (normal cells) in a given field by light and fluorescence microscopy. The following were the fractions recorded for the different groups: sham+Ad.β-gal, 34±5% (n=6), and aortic banding+Ad.β-gal, 29±4% (n=7). Echocardiography was performed before (baseline) and 7 days after viral injection and aortic banding as shown in the Table. The aortic-banded hearts transduced with Ad.β-gal developed marked left ventricular hypertrophy, characterized by a 20% to 30% increase in the thickness of the posterior wall at end-diastole. Cross sections of harvested hearts were prepared, and dimensional measurements were carried out. The data demonstrated that a pronounced left ventricular hypertrophy developed in the hearts transduced with Ad.β-gal and AS, with significant increases in left ventricular weight–body weight ratio, increases in interventricular septal thickness, increases in anterior wall thickness, and increases in posterior wall thickness. However, hypertrophy was not seen in aortic-banded hearts transduced with Ad.Kv4.3. Taken together, these data suggest that overexpression of Kv4.3 inhibits the development of pressure overload–induced cardiac hypertrophy. However, the data in the Table also show that there is a trend toward left ventricular dimensions in rat hearts with AS+Ad.Kv4.3. This can be explained by the fact that if the ventricle is blocked from developing hypertrophy under pressure-overload conditions, it will increase its left ventricular chamber size to generate the appropriate pressure to overcome the aortic banding. The heart utilizes the Starling curve to increase left ventricular pressure by operating at a higher point on the left ventricular volume axis. For that reason, it was not surprising to see a higher ventricular...
Effect of Kv4.3 Overexpression on $I_{\text{to}}$ Density and APD

$I_{\text{to}}$ is frequently implicated in the electrical remodeling of cardiac hypertrophy and failure. Figure 2A shows raw traces of $I_{\text{to}}$ recorded from sham and AS myocytes infected with Ad.β-gal or Ad.Kv4.3 in response to depolarizing step voltages from −40 to +70 mV from a holding potential of −80 mV. The corresponding mean $I_{\text{to}}$ densities, defined as peak current elicited by the depolarizing voltage step minus the steady-state current remaining at the end of a 500-ms voltage step, are presented in Figure 2B. $I_{\text{to}}$ density was decreased in AS myocytes derived from hearts infected with Ad.β-gal (9.9±1.0 pA/pF, n=18) compared with myocytes derived from sham hearts (17.6±0.80 pA/pF, n=27, P<0.05, at +40 mV). Overexpression of Kv4.3 resulted in high, robust $I_{\text{to}}$ densities (52.3±7.0 pA/pF, n=12), which were significantly larger compared with that in AS myocytes infected with Ad.β-gal or sham myocytes (Figure 2A and 2B).

To investigate the molecular basis for the changes in $I_{\text{to}}$ density, we examined the expression of Kv4.2, Kv4.3, and Kv1.4 α-subunits, which represent candidate voltage-dependent K+ channels encoding $I_{\text{to}}$ in the rat ventricle. Figure 3 shows typical Western blots demonstrating immunoreactive proteins for Kv4.3 (A), Kv4.2 (B), and Kv1.4 (C) in left ventricular myocytes derived from sham and AS hearts infected with Ad. β-gal and Ad.Kv4.3. Consistent with a reduction in $I_{\text{to}}$ density, the expression of Kv4.3 decreased by 50% in AS myocytes infected with Ad.β-gal (77.8±4.2 AU, n=4) compared with sham myocytes (158.8±6.9 AU, n=4 P<0.05; Figure 3A). Similarly, the expression of Kv4.2 decreased by 58% in AS myocytes infected with Ad.β-gal (46.3±9.6 AU, n=4) compared with sham myocytes (110.9±2.3 AU, n=4 P<0.05; Figure 3B). Unlike Kv4.3 and Kv4.2, Kv1.4 protein expression did not differ (6.4%) between AS myocytes infected with Ad.β-gal (143.7±3.2, n=4), and sham myocytes (153.6±3.3, P>0.05) as shown in Figure 3C. Overexpression of Kv4.3 increased significantly in AS myocytes infected with Ad.Kv4.3 (123.4±7.5 AU, n=4) compared with AS myocytes infected with Ad.β-gal (77.8±4.2 AU, n=4 P<0.05; Figure 3A) but had no effect on

Dimension in the AS+Ad.Kv4.3 hearts. It remains to be determined whether long-term infection with Ad.Kv4.3 and long-term inhibition of cardiac hypertrophy would significantly modify left ventricular dimensions.

**Figure 2.** In vivo expression of Ad.Kv4.3 increased $I_{\text{to}}$ density in left ventricular myocytes subjected to AS. A, Representative whole-cell $I_{\text{to}}$ currents recorded from −40 to +70 mV in sham-operated, Ad.β-gal–infected, or Ad.Kv4.3–infected cells. Electrophysiological recordings were performed only on isolated myocytes displaying green fluorescence. B, Summary data of peak current-voltage relations of $I_{\text{to}}$. $I_{\text{to}}$ current amplitudes were normalized to cell capacitance in each cell (pA/pF). As expected, Kv4.3 overexpression generated robust increase in $I_{\text{to}}$ current and density. Shown are means (symbols) and SE (bars). *Ad.Kv4.3 vs Ad.β-gal, P<0.05; †sham vs Ad.Kv4.3, P<0.05. Abbreviations are as defined in text.

**Figure 3.** Effect of in vivo adenoviral gene transfer of Kv4.3 on candidate Kv α-subunits known to encode $I_{\text{to}}$. Cell lysates were matched for protein concentration, separated by SDS-PAGE, and then transferred to nitrocellulose membranes. Blots were incubated with corresponding antibodies against Kv4.3 (A), Kv4.2 (B), and Kv1.4 (C), followed by detection with enhanced chemiluminescence. Data are expressed as mean±SEM. *Ad.Kv4.3 vs Ad.β-gal, P<0.05; †sham vs Ad.β-gal, P<0.05; ‡sham vs Ad.Kv4.3, P<0.05. Abbreviations are as defined in text.
the expression of Kv4.2 (Figure 3B) and Kv1.4 (Figure 3C), which is consistent with our in vitro recordings (data not shown). These data demonstrate that the reduction in \(I_o\) density mirrors the reduction in Kv4.2 and Kv4.3 expression shown). These data demonstrate that the reduction in the expression of robust \(I_o\) is associated with increases in peak calcium and cell shortening when compared with sham (Figure 5A and 5B). Overexpression of Kv4.3 attenuated the enhanced contractile function seen with AS + Ad.\(\beta\)-gal hearts (Figure 5A and 5B).

**Effect of Kv4.3 Overexpression on Calcium Handling and Contractility**

Central to our hypothesis was that the hypertrophic response was associated with increased intracellular calcium. Elevations in intracellular calcium as a result of APD prolongation may provide an important stimulus for the activation of stress pathways, including calcineurin and MAPKs, and induction of hypertrophy. Figure 5 shows recordings from representative cardiomyocytes isolated from sham and aortic-banded hearts infected with Ad.\(\beta\)-gal or Ad.Kv4.3. AS was associated with increases in peak calcium and cell shortening when compared with sham (Figure 5A and 5B). Overexpression of Kv4.3 attenuated the enhanced contractile function seen with AS + Ad.\(\beta\)-gal hearts (Figure 5A and 5B).

**Effect of Kv4.3 Overexpression on Hypertrophy and Activation of MAPK Pathways**

AS was associated with global and cellular hypertrophy on day 8 as shown in the Table. AS rats infected with Ad.\(\beta\)-gal developed hypertrophy compared with sham-operated rats, as assessed by the significant increase in myocyte capacitance (119 ± 4.3 pF, \(n = 30\), versus 189 ± 14 pF; \(n = 30\); \(P < 0.05\)) and heart weight–body weight ratio (0.30 ± 0.03 mg/g, \(n = 6\),
Ip38 and whether overexpression of Kv4.3-based analysis confirmed the equivalent expression of each of the ERK1/2, SAPK/JNK, and p38 pathways. In vivo overexpression of Kv4.3 was increased by 1.97-fold in AS myocytes infected with Ad.Kv4.3 (Figure 6A). Similarly, the activity of SAPK/JNK was increased by 2.29-fold in AS myocytes infected with Ad.Kv4.3 (Figure 6B). The phosphorylation of p38 was also increased by 2.29-fold in AS myocytes infected with Ad.Kv4.3 (172.9 ± 6.8 AU, n = 4) compared with sham-operated hearts (131.8 ± 6.7 AU, n = 4 P < 0.05; Figure 7A). Similarly, NFATc1 expression was also increased in AS myocytes derived from hearts infected with Ad.β-gal (168.2 ± 10.3 AU, n = 4) compared with sham-operated hearts (120.6 ± 4.0 AU, n = 4 P < 0.05; Figure 7B). Overexpression of Kv4.3 on MAPK activity.

Effect of Kv4.3 on MAPK activity.

We investigated the potential role of calcineurin and its downstream transcription factor NFAT in rats subjected to pressure overload. Figure 7 shows calcineurin- and NFAT-specific Western blots performed on protein extracts of myocytes derived from sham-operated and AS hearts infected with Ad.β-gal or Ad.Kv4.3. Calcineurin expression was significantly increased in AS myocytes derived from hearts infected with Ad.β-gal (172.9 ± 6.8 AU, n = 4) compared with sham-operated hearts (131.8 ± 6.7 AU, n = 4 P < 0.05; Figure 7A). Similarly, NFATc1 expression was also increased in AS myocytes derived from hearts infected with Ad.β-gal (168.2 ± 10.3 AU, n = 4) compared with sham-operated hearts (120.6 ± 4.0 AU, n = 4 P < 0.05; Figure 7B). Overexpression of Kv4.3 on MAPK activity.

Effect of Kv4.3 Overexpression on the Calcineurin Pathway

versus 0.44 ± 0.03 mg/g, n = 10; P < 0.01). Kv4.3 overexpression was associated with an attenuation of cardiac hypertrophy, as observed by a reduction in myocyte capacitance (150 ± 15 pF, n = 28, P < 0.05) and a reduction in heart weight–body weight ratio (0.34 ± 0.04 mg/g, n = 10; P < 0.05). The specificity of the Kv4.3 effect was confirmed by measuring the capacitance in the AS + Ad.Kv4.3 group of GFP-negative myocytes, which had a capacitance comparable to AS + Ad.β-gal GFP-negative cardiomyocytes (221 ± 10 pF, n = 33, versus 227 ± 13 pF, n = 32). The GFP-positive cardiomyocytes isolated from AS + Ad.Kv4.3 cells had a lower capacitance than that of the AS + Ad.β-gal GFP-positive cardiomyocytes (150 ± 15 pF, n = 28, versus 221 ± 10 pF, n = 33; P < 0.05).

We next sought to determine whether short-term AS could activate MAPK pathways, namely, the ERK1/2, SAPK, or c-Jun NH2-terminal kinase (JNK) pathways and p38 and whether overexpression of Kv4.3-based I0 channels could modulate these pathways. The activity of ERK1/2 was increased by 1.97-fold in AS myocytes infected with Ad.β-gal (0.61 ± 0.04 AU, n = 4) compared with sham myocytes (0.31 ± 0.03 AU, n = 4, P < 0.05; Figure 6A).

Similarly, the activity of SAPK/JNK was increased by 2-fold in AS myocytes infected with Ad.β-gal (0.75 ± 0.06 AU, n = 4) compared with sham myocytes (0.37 ± 0.02 AU, n = 4 P < 0.05; Figure 6B). The phosphorylation of p38 was also increased by 2.29-fold in AS myocytes infected with Ad.β-gal (1.21 ± 0.01 AU, n = 4) compared with sham myocytes (0.53 ± 0.01 AU, n = 4 P < 0.05; Figure 6C). The degree of activation of these kinases was similar to uninfected AS myocytes (data not shown), demonstrating that pressure overload leads to sustained phosphorylation of ERK1/2, SAPK/JNK, and p38 pathways. In vivo overexpression of Kv4.3 had no effect on phosphorylation of the 3 MAPKs after AS (Figure 6A–6C). Western blot analysis confirmed the equivalent expression of each of the total kinases in sham and AS myocytes infected with Ad.β-gal and Ad.Kv4.3 (Figure 6 A–C; blots labeled as total).

Figure 6. Effect of in vivo adenoviral gene transfer of Kv4.3 on MAPK activity. Western blotting was performed on cell lysates from sham, Ad.β-gal–infected, and Ad.Kv4.3–infected hearts with phosphospecific antibodies to ERK (A), SAPK (B), and p38 (C). Aortic banding induced activation of 3 MAPK pathways in parallel. Bar graphs are quantifications of MAPK activities. Activity of each MAPK was normalized against its corresponding total protein (total ERKs = ERK1 + ERK2; total SAPK = p54SAPK + p46SAPK; total p38). Data, from 3 experiments, are presented as mean ± SE. †Sham versus Ad.β-gal, P < 0.05; ‡sham vs Ad.Kv4.3, P < 0.05. Abbreviations are as defined in text.

Figure 7. Effect of in vivo adenoviral gene transfer of Kv4.3 on expression of calcineurin. Cell lysates from sham, Ad.Kv4.3–infected, and Ad.β-gal–infected hearts were subjected to Western blotting as described for calcineurin (A) and NFATc1 (B). Quantification of calcineurin and NFAT expression was determined by densitometric scanning. There were significant decreases in protein expression of both calcineurin and NFAT in hearts transduced with Ad.Kv4.3. Data from at least 3 experiments are shown. *Ad.Kv4.3 vs Ad.β-gal, P < 0.05; †sham vs Ad.β-gal, P < 0.05; ‡sham vs Ad.Kv4.3, P < 0.05. Abbreviations are as defined in text.
of Kv4.3 significantly reduced the expression of calcineurin by \( \approx 69\% \) (53.0 \pm 6.9 AU, \( n = 4, P < 0.05 \)) and of NFAT by \( \approx 61\% \) (66.0 \pm 7.0 AU, \( n = 4, P < 0.05 \)) compared with Ad.\( \beta \)gal. These results indicate that short-term delivery of the Kv4.3-expressing adenovirus is associated with inhibition of calcineurin activity in pressure-overloaded hearts.

**Discussion**

Although changes in many ion channel currents have been reported in cardiac hypertrophy, a reduction in the density of \( I_{\text{Ca}} \) is the most prominent ionic current change resulting in APD prolongation in a process referred to as electrical remodeling.\(^2,7,18\) It is still unclear whether APD prolongation is an adaptive or a maladaptive event of cardiac hypertrophy. Whereas \( I_{\text{Ca}} \) downregulation is frequently associated with APD prolongation regardless of the experimental animal model, it seems to occur early in the disease process,\(^4,18,19\) which may in the short term enhance contractility of the compromised myocardium. It is conceivable that an increase in \([\text{Ca}^{2+}]\)d, modulates cardiac hypertrophy, because \( \text{Ca}^{2+} \) is an essential cofactor in the activation of several hypertrophic signaling pathways, including calcineurin and MAPKs. This is the first study demonstrating that in vivo Kv4.3 gene transfer can reverse \( I_{\text{Ca}} \) downregulation and attenuate the compensatory cardiac hypertrophy of the left ventricle under augmented loading conditions. Our findings demonstrate that short-term AS is associated with a significant reduction of Kv4.2- and Kv4.3-based \( I_{\text{Ca}} \), APD prolongation, and cardiac hypertrophy, which is consistent with previous results.\(^19\) Overexpression of Kv4.3 was able to significantly increase the expression of Kv4.3 \( \alpha \)-subunits, increase \( I_{\text{Ca}} \) density, shorten APD, and attenuate the hypertrophic response to pressure overload.

**Calcineurin**

Calcineurin is a calcium/calmodulin-dependent phosphoprotein phosphatase that can dephosphorylate NFATc, resulting in its nuclear translocation and the transcriptional activation of numerous hypertrophy genes.\(^20\) We report that short-term AS is associated with an increase in the expression of calcineurin and the calcineurin transcription factor NFATc1, which was restored by overexpression of Kv4.3. The role of calcineurin in the development of structural hypertrophy is well established; more recently, APD prolongation was shown to modulate this pathway. Indeed, APD prolongation secondary to reductions in \( I_{\text{Ca}} \) density was shown to influence transsarcolemmal \( \text{Ca}^{2+} \) influx through recruitment of additional \( L \)-type, voltage-dependent \( \text{Ca}^{2+} \) channels, thereby increasing cytosolic \([\text{Ca}^{2+}]_c \), in healthy and diseased myocardium. More recently, a connection between \( I_{\text{Ca}} \) reduction, APD prolongation, \( \text{Ca}^{2+} \) entry, activation of calcineurin, and cellular growth has been demonstrated in cultured neonatal ventricular myocytes infected with adenoviruses harboring dominant-negative constructs for Kv4.2.\(^21\) Activation of calcineurin and cellular hypertrophy were inhibited by coinflection with Ad.CAIN (calcineurin inhibitor).\(^21\) In this regard, enhanced \( \text{Ca}^{2+} \) transients, inotropy, and cardiac hypertrophy have been reported in transgenic mice with selective Kv4.2-based \( I_{\text{Ca}} \) reduction. Indeed, these transgenic mice exhibit significant activation of the calcineurin pathway, which may be prevented by treatment with verapamil and may explain progression of the disease from hypertrophy to failure.\(^22\) Consistent with these results, we also reported the induction of cardiac hypertrophy and disease progression to cardiac failure in transgenic mice overexpressing the \( \alpha 1 \)-subunit of the \( L \)-type \( \text{Ca}^{2+} \) channel.\(^23\) We also reported a hypercontractile phenotype, increased density of \( L \)-type calcium channels, and APD prolongation in 6- to 7-week-old calcineurin-overexpressed transgenic mice.\(^24\) Interestingly, the activation of calcineurin and the onset of cardiac hypertrophy have been shown to be associated with APD prolongation secondary to an increase in \( L \)-type calcium channel density in rats subjected to AS, which was reversed with cyclosporine treatment.\(^25\) Collectively, these studies suggest that activity of calcineurin may be modulated with a cellular profile of increased calcium influx, either by directly increasing the expression of \( L \)-type calcium channels or indirectly by reducing the expression of the \( I_{\text{Ca}} \) channels. Furthermore, activation of calcineurin alone exerts a feedback to increase calcium influx. This bimodal model of transsarcolemmal calcium influx and calcineurin activation may explain the commonality of these transgenic mice in disease progression and the development of heart failure at 3 to 4 months of age.

**Mitogen-Activated Protein Kinases**

The ERKs, JNKs, and p38 MAPKs have all been implicated in the development of cardiac hypertrophy.\(^26\) We report the sustained activation of the 3 MAPK signaling pathways after AS. Activation of these pathways is known to occur as early as 5 minutes\(^27\) and may be observed 8 weeks after AS. A relation between calcium influx via \( L \)-type calcium channels, activation of MAPKs,\(^28\) and alterations in gene expression has already been demonstrated in neuronal cells and mammalian cell lines.\(^29\) We found that overexpression of Kv4.3-based \( I_{\text{Ca}} \) did not alter the activation of these kinases, suggesting that there is no association between \( I_{\text{Ca}} \) density, membrane potential, and MAPK activation. However, it is not clear whether the lack of effect may be due to the fact that <30% of the myocardium was infected in vivo and that a higher infectivity may be required to modulate the activity of these kinases. It is also quite possible that the apparent effect of \( I_{\text{Ca}} \) on MAPKs is inhibited or antagonized by more dominant mechanisms in the physiological setting, where many agonists act in concert to remodel the myocardium. Nevertheless, it remains to be determined whether \( I_{\text{Ca}} \) suppression would have any effect on the activation of MAPKs.

**Contractility**

At 4 mmol/L \( \text{Ca}^{2+} \), there was significantly enhanced contractility in terms of peak intracellular \( \text{Ca}^{2+} \) and percent shortening, whereas no significant changes were observed in the in vivo setting. In early hypertrophy (1 to 2 weeks after aortic constriction), as the ventricle is adapting to the increased load, cardiac myocytes hypertrophy, and with prolonged AP, more calcium moves through the sarcolemma and an in-
creased sarcoplasmic reticulum calcium release is observed. A number of studies have also related this phenomenon of enhanced peak calcium in isolated cells from early hypertrophied hearts.30 Other studies in early hypertrophy have also shown enhanced contractility in rat hearts after short-term pressure overload.31 In general, in vivo measurements have not shown enhanced contractility, even though some studies did show increased fractional shortening.32 Our own previously published studies have shown also that short-term pressure overload did not enhance in vivo contractile function.33 Even though each myocyte forms the functional basis for ventricular function, in our experiments, single cardiomyocytes are unloaded and in this setting, the contractile parameters cannot be extrapolated to the loaded state of the ventricle in the body.

Limitations of This Study
This study has demonstrated that overexpression of Kv4.3-based \( I_{Ks} \) abrogates the hypertrophic response in the short term. It remains to be determined whether long-term infection with Ad.Kv4.3 would significantly modify \( I_{Ks} \) expression and calcineurin levels. We and others4,10,16,23 reported that modulation of early repolarization is an important determinant of L-type calcium channel activity and calcium influx in the myocardiun. It is unknown whether these results may be transferable to the diseased human myocardium. Rodents possess a characteristic triangular short AP because of prominent expression of \( I_{Ks} \), whereas larger mammalian species and humans possess a much smaller \( I_{Ks} \) density and an AP profile with a spike-dome morphology. These data are interesting and may not be extrapolated to heart failure patients because of the presence of comorbid conditions and large differences in AP profile. It is interesting to note that patients with long Q-T syndrome, which has been attributed to mutations of the delayed rectifier (rapid and slow component) \( K^+ \) channel, do not develop ventricular hypertrophy. One possible explanation may be the fact that these channels operate toward the end of cardiac repolarization, during which time calcium channels have already been rendered inactive.

The results in the present study support the hypothesis that cardiac hypertrophy, downregulation of \( I_{Ks} \), and APD prolongation occur early after pressure overload and that in vivo cardiac gene transfer of Kv4.3-based \( I_{Ks} \) cannot increase \( I_{Ks} \) density, shorten APD, and attenuate the hypertrophic responses via a calcineurin pathway.

Acknowledgments
This work was supported in part by grants from the National Institutes of Health: HL 57623, HL 71763 (to R.J.H.), HL 49574 (to J.K.G.), 5 T32 HL07382 (to A.S.), PO1 22619 (to A.S.), HL076659 (to D.L.), and HL 69842 and a Bessone Scholar Award to R.J.H. from the American Federation of Aging Research. R.K. was supported by a research fellowship award from the Heart and Stroke Foundation of Canada.

References


In Vivo Cardiac Gene Transfer of Kv4.3 Abrogates the Hypertrophic Response in Rats After Aortic Stenosis

Djamel Lebeche, Roger Kaprielian, Federica del Monte, Gordon Tomaselli, Judith K. Gwathmey, Arnold Schwartz and Roger J. Hajjar

_Circulation_. 2004;110:3435-3443; originally published online November 22, 2004; doi: 10.1161/01.CIR.0000148176.33730.3F

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 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/110/22/3435

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/