Aldosterone, Through Novel Signaling Proteins, Is a Fundamental Molecular Bridge Between the Genetic Defect and the Cardiac Phenotype of Hypertrophic Cardiomyopathy

Natalia Tsybouleva, MD; Lianfeng Zhang, PhD; Suetnee Chen, MS; Rajnikant Patel, MD; Silvia Lutucuta, MD; Shintaro Nemoto, MD; Gilberto DeFreitas, BS; Mark Entman, MD; Blase A. Carabello, MD; Robert Roberts, MD; A.J. Marian, MD

Background—Human hypertrophic cardiomyopathy (HCM), the most common cause of sudden cardiac death in the young, is characterized by cardiac hypertrophy, myocyte disarray, and interstitial fibrosis. The genetic basis of HCM is largely known; however, the molecular mediators of cardiac phenotypes are unknown.

Methods and Results—We show myocardial aldosterone and aldosterone synthase mRNA levels were elevated by 4- to 6-fold in humans with HCM, whereas cAMP levels were normal. Aldosterone provoked expression of hypertrophic markers (NPPA, NPPB, and ACTA1) in rat cardiac myocytes by phosphorylation of protein kinase D (PKD) and expression of collagens (COL1A1, COLIA2, and COL3A1) and transforming growth factor-β1 in rat cardiac fibroblasts by upregulation of phosphoinositide 3-kinase (PI3K)-p100. Inhibition of PKD and PI3K-p110β abrogated the hypertrophic and profibrotic effects, respectively, as did the mineralocorticoid receptor (MR) antagonist spironolactone. Spironolactone reversed interstitial fibrosis, attenuated myocyte disarray by 50%, and improved diastolic function in the cardiac troponin T (cTnT)-Q92 transgenic mouse model of human HCM. Myocyte disarray was associated with increased levels of phosphorylated β-catenin (serine 38) and reduced β-catenin–N-cadherin complexing in the heart of cTnT-Q92 mice. Concordantly, distribution of N-cadherin, predominantly localized to cell membrane in normal myocardium, was diffuse in disarrayed myocardium. Spironolactone restored β-catenin–N-cadherin complexing and cellular distribution of N-cadherin and reduced myocyte disarray in 2 independent randomized studies.

Conclusions—The results implicate aldosterone as a major link between sarcomeric mutations and cardiac phenotype in HCM and, if confirmed in additional models, signal the need for clinical studies to determine the potential beneficial effects of MR blockade in human HCM. (Circulation. 2004;109:1284-1291.)

Key Words: cardiomyopathy ▪ myocytes ▪ hypertrophy
tension of 11-β-hydroxysteroid dehydrogenase-2, which increases aldosterone occupancy of the MR, leads to cardiac hypertrophy, fibrosis, and heart failure.\textsuperscript{12} Furthermore, clinical studies in humans with heart failure, which show that blockade of MR reduces cardiovascular mortality and morbidity, emphasize the potential clinical utility of blockade of MR in cardiomyopathies.\textsuperscript{13-14} Accordingly, we investigated the role of aldosterone in the pathogenesis of the cardiac phenotype in human HCM, delineated novel molecular mechanisms for the hypertrophic and profibrotic effects of aldosterone, and determined the effects of blockade of MR on cardiac phenotype in a transgenic mouse model of human HCM mutation.

**Methods**

**Serum and Myocardial Aldosterone Levels in Humans With HCM**

Blood was collected in the morning in sitting position from 11 patients with HCM and 8 healthy asymptomatic individuals. Aldosterone levels were determined with an enzyme competitive immunoassay, whereby aldosterone competes with horseradish peroxidase–conjugated aldosterone for the limited number of binding sites on antialdosterone serum-coated microwell plates, per instruction of the manufacturer (Diagnostic Biochem Canada Inc).

**Myocardial cAMP Levels**

The myocardial level of cAMP, which is known to regulate expression of the aldosterone synthase (CYP11B2) gene,\textsuperscript{15} was measured by a nonacetylation enzyme immunoassay, as described by the manufacturer (Amersham Biosciences).

**Isolation of Neonatal Rat Cardiac Myocytes and Fibroblasts**

Neonatal rat cardiac myocytes (NRCMs) and fibroblasts (CF) were isolated from the ventricular tissue of 1- to 2-day-old neonatal rats by collagenase digestion and selective enrichment by differential centrifugation through a discontinuous Percoll gradient.\textsuperscript{16}

**Aldosterone Treatment**

Cultured NRCMs and CFs were treated with 25 or 50 nmol/L aldosterone (Sigma-Aldrich Corporation), angiotensin II at a concentration of 10\textsuperscript{-7} mol/L as a positive control, or the MR antagonist spironolactone (100 nmol/L) at several time points. For in vivo experiments, aldosterone was dissolved in DMSO, diluted in 0.9% NaCl to 1 µmol/L concentration, and injected intraperitoneally at 150 µL per mouse. Mice were killed at different time points after injection, and cardiac ventricular tissues were collected for the experiments.

**cTnT-Q92 Transgenic Mice**

The Animal Subjects Committee of Baylor College of Medicine approved the experiments. The cardiac troponin T (cTnT)-Q92 transgenic mouse model has been published previously.\textsuperscript{9,17,18}

**Reverse Transcription–Polymerase Chain Reaction**

Expression levels of the molecular marker of cardiac hypertrophy, namely, NPPA, NPPB, ACTA1, ATP2A2, encoding atrial natriuretic peptide, brain natriuretic peptide, skeletal α-actin, and sarcomplasmic reticulum calcium ATPase 2, respectively, and COL1A1, COL1A2, and COL3A1, encoding procollagen COL1α1, COL1α2, and COL3α1, respectively, were determined with specific Taqman probes and oligonucleotide primers in a 7900HT SDS unit (Applied Biosystems, Inc) and were normalized to that of GAPDH. Experiments were performed in triplicate at 3 time points of 12, 24, and 48 hours after treatment and were repeated at least 3 times.

After identification of protein kinase D (PKD) as a mediator of the hypertrophic effect of aldosterone, reverse transcription–polymerase chain reaction (RT-PCR) experiments were repeated in the presence of 100 and 500 nmol/L doses of a classic protein kinase C/PKD inhibitor, Go6976 (Calbiochem). Similarly, after identification of phosphoinositide 3-kinase (PI3K)-p110 and as a mediator of the profibrotic effects of aldosterone, RT-PCR experiments were repeated in the presence of 50 and 100 nmol/L of wortmannin (Calbiochem), a classic PI3K inhibitor.

**Immunoblotting**

The list of antibodies used in immunoblotting is available on request. Each set of the experiments was repeated from 3 to 5 times, and expression levels of the proteins were quantified by spot densitometry as described previously.\textsuperscript{19} The membranes were probed with specific antibodies to detect levels of total signaling proteins, tubulin or sarcomeric actin, as controls.

**Randomized Placebo-Controlled Spironolactone Therapy**

We performed 2 independent (main and replication) placebo-controlled, randomized studies. The replication study was designed to test the findings of the primary study assuming a priori null hypothesis of no effect. Age- and gender-matched adult mutant cTnT-Q92 mice were randomized to treatment with either a placebo or spironolactone, and a third group of age- and gender-matched nontransgenic mice were included as controls (n=13 per group in the main study and n=10 in the replication studies). All mice underwent 2D and Doppler echocardiography before and after treatment. Spironolactone was dissolved in olive oil at a concentration of 10 mg/mL and injected subcutaneously at a dose of 50 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} per mouse, a dose shown to reduce myocardial fibrosis in a variety of experimental models.\textsuperscript{19-22} The placebo group was injected with olive oil alone. After 10 weeks of therapy, mice were killed for morphometric and molecular phenotyping.

**M-Mode, 2D, and Doppler Echocardiography**

Transsthoracic echocardiography was performed, before and after completion of spironolactone therapy, with an Acuson Sequoia Cardiac System equipped with a 15-MHz linear transducer (Acuson Co).

**Morphometric Analyses**

All quantification was performed in a random order by an examiner without knowledge of the experimental groups. The percentage of myocardium that showed disarray and the collagen volume fraction (CVF) were quantified in 240 fields (×400) per mouse, as described previously.\textsuperscript{9,10} Interobserver and intraobserver variabilities of calculating the extent of myocyte disarray and fibrosis were determined in 312 fields.

To define myocyte boundary, fresh frozen thin myocardial sections were stained with a rabbit polyclonal anti-laminin antibody (Sigma) at a concentration of 1:50, and myocyte cross-sectional area (CSA) was determined by quantitative indirect immunofluorescence. The secondary antibody was Texas red streptavidin-conjugate goat anti-mouse antibody (concentration of 1:200). Nuclei were stained with Hoechst dye. The mean myocyte CSA was determined in 40 fields per mouse in 5 mice per group (~2500 myocytes for each group).
Statistical Methods
Differences in variables among nontransgenic, placebo, and spironolactone groups were compared by ANOVA, followed by Bartlett’s test for the homogeneity of variances. Variables with unequal SDs, such as CVF, were compared by the nonparametric Kruskal-Wallis test. Differences between final and baseline values in each group were compared by paired t test.

Results
Myocardial Aldosterone and CYP11B2 Levels in Humans
HCM patients comprised 6 males and 5 females with a mean±SD age of 55.6±16.6 years, septal thickness of 2.03±0.49 cm, left ventricular (LV) mass of 332.8±93.8 g, LV ejection fraction of 68.9±5.8%, and LV outflow tract gradient of 54.4±23.8 mm Hg. They were symptomatic for dyspnea, with a mean New York Heart Association functional class of 1.6±0.98. Myocardial tissues were collected during surgical myomectomy or at postmortem examination. The control myocardial tissues were age- and gender-matched normal donor hearts not used for cardiac transplantation. Tissues were frozen in liquid nitrogen and stored at −135°C. Mean serum aldosterone levels were not significantly different between HCM and control subjects (Figure 1A). In contrast, myocardial aldosterone levels were increased by 4.5-fold in HCM (Figure 1B). Concordantly, expression levels of CYP11B2 mRNA in the heart, quantified by RT-PCR, were increased by 6.9-fold (Figure 1C).

Myocardial cAMP Levels
Myocardial cAMP levels were not significantly different between HCM patients and controls (12.1±6.8 versus 8.7±10.8 fmol/mg, respectively; P=0.552).

Hypertrophic and Profibrotic Effects of Aldosterone
Treatment of NRCMs with 2 doses of aldosterone provoked significant expression of NPPA, NPPB, and ACTA1 at 12, 24, and 48 hours (Figure 2) and modestly reduced expression levels of ATP2A2. Similarly, treatment of cultured CFs with 2 doses of aldosterone significantly increased expression levels of COL1A1, COL1A2, and COL3A1 mRNAs at 24 and 48 hours (Figure 3). Spironolactone blocked the hypertrophic and profibrotic effects of aldosterone in cultured myocytes and fibroblasts ( Figures 2 and 3). Concordantly, expression levels of transforming growth factor (TGF)-β1, a major profibrotic factor, were also increased in cultured CFs after 30 and 60 minutes of treatment with aldosterone and were blocked by spironolactone (Figure 4).

Signal Transducers of Hypertrophic and Fibrotic Effects of Aldosterone
Because classic PKCs are implicated in the mediation of aldosterone signaling,23 we investigated activation of PKC isoforms in NRCMs and CFs after treatment with aldosterone. The most notable effect was phosphorylation of PKD, which was serine residue specific and cyclic (Figure 5A). Aldosterone phosphorylated PKD at serine 916, which peaked initially at 60 minutes. Aldosterone had no major effect on levels of serine 744 to 748 phosphorylated PKD or total PKD. In contrast to NRCMs, aldosterone had no significant effect on levels of S916-phospho-PKD or S744–748-phospho-PKD in CFs (Figure 5B). Increased PKD phosphorylation was also confirmed in vivo in explanted hearts after treatment of adult mice with aldosterone, with increased...
expression levels of S916-phospho-PKD in cardiac myocytes (Figure 5C) but not in fibroblasts (data not shown). Other notable changes were increased levels of phosphorylated p42/44 extracellular signal-regulated kinases and levels of calcineurin in NRCMs after treatment with aldosterone, in accordance with published data.24,25

In the absence of a discernible effect of aldosterone on PKD activation in CFs, and given that the PI3K pathway is implicated in interstitial fibrosis,26 we determined changes in expression levels of the PI3K components after treatment of CFs with aldosterone, in accordance with published data.24,25

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To determine whether phosphorylation of PKD was responsible for the hypertrophic response to aldosterone, cultured NRCMs were treated with 2 different concentrations of Go6976, an inhibitor of classic PKC/PKD, and expression levels of markers of cardiac hypertrophy were determined by RT-PCR. Treatment of cultured NRCMs with Go6976 completely abrogated the hypertrophic response to aldosterone (Figure 6). Similarly, treatment of cultured CFs with 2 concentrations of wortmannin, a PI3K inhibitor, abolished induction of expression of procollagen mRNAs by aldosterone (Figure 6).

Cardiac Phenotype in Adult cTnT-Q92 Transgenic Mice
Cardiac phenotype in the cTnT-Q92 mice was as published previously.9,17 Heart weight/body weight ratio was smaller by 15% in a total of 33 mutant mice analyzed in the present study compared with nontransgenic littermates; myocyte disarray constituted ~20% of the myocardium, and CVF was increased by 3-fold. The intraobserver and interobserver variabilities in determining CVF were 1.25% and 1.8%, and for disarray, they were 2.3% and 3.6%, respectively.

Myocardial levels of aldosterone were 0.11 ± 0.02 and 0.08 ± 0.04 pg/g of LV tissue in cTnT-Q92 and nontransgenic mice, respectively (P=0.17). Serum levels of aldosterone
were not significantly different between cTnT-Q92 and nontransgenic mice.

**Effects of Spironolactone on Cardiac Phenotype in cTnT-Q92 Transgenic Mice**

The mean age, male/female ratio, heart rate, and body weight were not significantly different among the nontransgenic, placebo, and spironolactone groups in the main and replication studies (data not shown). Results of the main study, shown in the Table, were notable for a smaller heart weight/body weight ratio in the spironolactone group. The extent of myocyte disarray, determined in a total of 3960 fields per group, was reduced by 50% in the spironolactone versus the placebo group. Moreover, CVF was reduced to normal levels in the spironolactone group. Myocyte CSA was not significantly different between spironolactone and placebo groups in the spironolactone group. Myocyte CSA was not significantly different between cTnT-Q92 and nontransgenic mice. Confocal microscopy showed predominant localization of CVF (6.7 ± 4.9 versus 1.4% ± 0.4, P = 0.001). The results in the replication study, scored in a twice larger number of myocardial sections, were concordant with the results of the main randomized study and showed a 50% reduction in the extent of myocyte disarray in the spironolactone versus the placebo group (8.3 ± 2.9% versus 17.5 ± 9.1%, respectively, P < 0.001) and complete normalization of CVF (6.7 ± 4.9 versus 1.4% ± 0.4, P = 0.001).

**Disrupted Localization and Complexing of N-Cadherin–β-Catenin in Disarrayed Myocardium**

We tested the integrity of cadherin-mediated myocyte-myocyte adhesion at the adherens junction (AJ), a potential mechanism for myocyte disarray, by determining expression levels and intracellular distribution of N-cadherin and phosphorylated-β-catenin (serine 37) in the heart of cTnT-Q92 mice. Confocal microscopy showed predominant localization of N-cadherin to myocyte membranes in nontransgenic hearts, whereas its distribution was diffuse and less restricted to AJ in disarrayed myocardium (Figure 7A). Treatment with spironolactone partially restored the distribution of N-cadherin to AJ, in accordance with the reduction in myocyte disarray. Furthermore, levels of S37-phospho-β-catenin were increased by ~2-fold, whereas levels of N-cadherin were unchanged (Figure 7B). Immunoprecipitation experiments (Figure 7C) showed reduced affinity of S37-phospho-β-catenin for N-cadherin, which provides further evidence for destabilized AJ in disarrayed areas. Treatment with spironolactone normalized levels of S37-phospho-β-catenin and β-catenin/cadherin complex formation.

**Discussion**

We explored the molecular mechanisms that link the genetic defect to the cardiac phenotype in HCM, with a particular focus on myocyte disarray and interstitial fibrosis. We provide several lines of evidence to implicate aldosterone in the pathogenesis of cardiac hypertrophy, fibrosis, and disarray in HCM, including elevated myocardial aldosterone and aldosterone synthase mRNA levels in patients with HCM. Aldosterone provoked expression of cardiac hypertrophic and profibrotic responses of aldosterone and Go6976 (100 or 500 nmol/L, respectively) and W100 denote fibroblasts treated with aldosterone and wortmannin 50 and 100 nmol/L, respectively. *P < 0.05 vs C; †P < 0.05 vs aldosterone.

**Figure 6.** Inhibition of cardiac hypertrophic and profibrotic responses of aldosterone. Top panels represent relative expression levels of 3 cardiac hypertrophic markers, as described in Figure 2. C indicates control myocytes; AT, angiotensin II; A, aldosterone; and A+G100 and A+G500, myocytes treated with aldosterone and Go6976 100 or 500 nmol/L, respectively. Bottom panels show inhibition of induction of expression of 3 major cardiac collagen genes in cardiac fibroblasts. Abbreviations are as above, except CFs were used and W50 and W100 denote fibroblasts treated with aldosterone and wortmannin 50 and 100 nmol/L, respectively. *P < 0.05 vs C; †P < 0.05 vs aldosterone.
A fundamental molecular link between sarcomeric gene mutations and cardiac phenotypes in HCM. These results, if confirmed in additional studies and for other HCM-causing mutations, in conjunction with the results of recent clinical studies showing the beneficial effects of blockade of MR in heart failure illustrate the need for clinical studies in humans to determine the potential beneficial effects of blockade of MR in HCM.

Aldosterone, discovered 50 years ago, is known primarily for its effects on sodium transport in renal tubules, whereas its direct effects on cardiac structure and function are largely unrecognized. The observed increased myocardial aldosterone levels in patients with HCM is in accordance with the results of recent studies showing extraadrenal gland synthesis of aldosterone, including studies in cardiac myocytes and fibroblasts and the myocardium of patients with heart failure. In the adrenal glands and cultured cells, expression of CYP11B2 is regulated by a diverse array of factors, including angiotensin II, cAMP, 12-lipoxygenase, PKC isoforms, and calmodulin-dependent protein kinases. These factors, alone or in combination, are plausible candidates to affect expression of CYP11B2 in HCM hearts. In the present study, myocardial cAMP levels and lipid peroxide levels, an overall index of oxidative stress, were not significantly elevated (data not shown), perhaps because they were less stable in the stored tissue. The molecular mechanisms that regulate expression of CYP11B2 and production of aldosterone in the heart in general and in HCM in particular remain to be established.

<table>
<thead>
<tr>
<th>Echocardiographic parameters</th>
<th>Nontransgenic Mice</th>
<th>cTnT-Q92 Mice</th>
<th>Spironolactone</th>
<th>Placebo</th>
<th>P&lt;0.001</th>
<th>P&lt;0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart/body weight ratio, g/mg</td>
<td>4.93±0.65</td>
<td>3.94±0.54</td>
<td>3.43±0.27</td>
<td>0.008</td>
<td>&lt;0.001</td>
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<tr>
<td>CVF, %</td>
<td>2.35±0.7</td>
<td>6.41±6.04</td>
<td>2.84±0.91</td>
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<td>0.01</td>
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<tr>
<td>Myocyte disarray, %</td>
<td>4.77±1.6</td>
<td>20.68±7.2</td>
<td>10.23±3.1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>Myocyte CSA, mm²†</td>
<td>499.6±49.3</td>
<td>411.0±26.6</td>
<td>419±27.0</td>
<td>0.617</td>
<td>0.004</td>
<td></td>
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<tr>
<td>LV end-systolic dimension, mm</td>
<td>4.93±0.65</td>
<td>3.94±0.54</td>
<td>3.43±0.27</td>
<td>0.008</td>
<td>&lt;0.001</td>
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<tr>
<td>LV end-diastolic dimension, mm</td>
<td>2.9±0.33</td>
<td>2.3±0.37</td>
<td>2.3±0.32</td>
<td>0.658</td>
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<td>FS, %</td>
<td>30.4±3.8</td>
<td>43.1±7.1</td>
<td>41.3±5.9</td>
<td>0.329</td>
<td>&lt;0.001</td>
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<td>LV ejection fraction, %</td>
<td>64.3±6.2</td>
<td>78.1±6.9</td>
<td>78.0±5.9</td>
<td>0.457</td>
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<tr>
<td>Aortic ejection time, ms</td>
<td>53.1±5.7</td>
<td>65.0±9.8</td>
<td>63.6±6.8</td>
<td>0.709</td>
<td>0.001</td>
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<tr>
<td>E/A ratio</td>
<td>2.93±1.12</td>
<td>1.83±0.60</td>
<td>2.48±1.06</td>
<td>0.079</td>
<td>0.04</td>
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</table>

†n=5 mice per group.
Aldosterone imparts a diverse array of biological effects comprising genomic (slow) and nongenomic (rapid) components, including but not limited to changes in the intracellular calcium concentration, \( \text{Na}^{+}/\text{H}^{+} \) exchanger, \(^{37}\) and activation of extracellular signal-regulated kinases \( p22/44,^{25} \) phospholipase \( C,^{23} \) and calcineurin. \(^{24}\) The present findings implicate PKD and PI3K-p110\( \delta \) as the molecular mediators of cardiac hypertrophic and profibrotic effects and protein binding control. \(^{19}\) The present data indicate that impaired myocyte-myoityc attachment at AJ because of impaired phospho-\( \beta \)-catenin and N-cadherin complexing is responsible, at least in part, for myocyte disarray in HCM. The extracellular domains of cadherins form the intercellular bonds between adjacent myocytes, and the cytoplasmic domains attach to cytoskeletal actin through \( \beta \)-catenin and other effector proteins. The integrity of AJ is essential for the proper alignment of myocytes. Alternative mechanisms for the observed reduction of myocyte disarray could include normalization of interstitial fibrosis and thus restoration of myocardial architecture. It is also possible that increased extracellular matrix proteins in HCM, by activating the integrin/wnt signaling pathways, affect the integrity of the \( \beta \)-catenin–cadherin–axin complex and thus myocyte morphology and arrangement. \(^{41}\) Accordingly, multiple interdependent mechanisms could be involved in the pathogenesis of myocyte disarray in HCM and its attenuation by spironolactone.

The results of 2 independent, randomized, placebo-controlled studies with spironolactone in cTnT-Q92 mice were concordant and in accordance with the mechanisms delineated for the effects of aldosterone in cell culture studies. Detailed quantification of the extent of myocyte disarray in the primary and replication studies, scored in a total of 11 880 microscopic fields in 23 mice per group, showed a 50% reduction with spironolactone (4.2±1.8 in nontransgenic mice, 19.3±8.0 in the placebo mice, and 9.5±3.0 in the spironolactone group; \( P<0.001 \)), which is beyond the 3% to 4% interindividual and intraindividual variabilities. Reversal of interstitial fibrosis with spironolactone is also in agreement with the results of previous observations in other animal models of cardiovascular disorders. \(^{19–22,29,42}\) As is often the case in humans with cTnT mutations, \(^{43,44}\) the cTnT-Q92 mice do not exhibit cardiac hypertrophy. \(^{17,45}\) Therefore, the impact of spironolactone on potential regression of cardiac hypertrophy in vivo could not be assessed. Treatment with spironolactone further reduced the heart weight/body weight ratio (3.5±0.5 versus 4.0±0.7 in the placebo group, \( P<0.001 \)). Because myocyte CSA did not change, the reduction in heart weight/body weight ratio in the spironolactone group most likely represents the reduction in interstitial fibrosis. Because human patients with the cTnT-Q92 mutation exhibit extensive myocyte disarray, a potential risk factor for SCD, \(^{44}\) the findings raise the possibility of reducing the risk of SCD in HCM through blockade of MR, a hypothesis that awaits testing in humans.

In summary, the present data implicate aldosterone in the pathogenesis of the cardiac phenotype in HCM and identify novel mechanisms for its hypertrophic and profibrotic effects by implicating PKD and PI3K-p110\( \delta \), respectively. Our data also implicate impaired N-cadherin–\( \beta \)-catenin complexing at AJ as a mechanism for myocyte disarray, the pathological hallmark of HCM. Finally, our data establish the reversibility of cardiac phenotypes through blockade of MR. These
findings, in the absence of a specific therapy for human HCM, if confirmed in additional studies, emphasize the need for clinical studies to determine potential salutary effects of blockade of MR in human patients with HCM.

Acknowledgments
This study was supported by grants from the National Heart, Lung, and Blood Institute, Specialized Centers of Research (P50-HL42267-01) and R01-HL68884.

References
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Circulation. 2004;109:1284-1291; originally published online March 1, 2004;
doi: 10.1161/01.CIR.0000121426.43044.2B
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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