Cardiac hypertrophy is an adaptive condition frequently associated with impairment of contractility, often leading to heart failure.1,2 The mechanisms involved in this transition are not known; however, one mechanism appears to be impaired signaling through myocardial β-adrenergic receptors (βARs).3 We recently demonstrated that dysfunctional βAR signaling associated with pressure overload ventricular hypertrophy is caused by enhanced expression and activity of the βAR kinase (βARK1), which phosphorylates agonist-occupied βARs leading to desensitization.4 βARK1, a member of the G protein–coupled receptor kinase (GRK) family, appears to be a critical modulator of in vivo myocardial function. We have shown that increased expression of βARK1 in the hearts of transgenic mice leads to blunting of βAR inotropic responses,5 whereas selective decreases of βARK1 activity6 or expression7 in the heart result in enhanced myocardial performance. Moreover, adding to the importance of βARK1 are the findings that this GRK is increased in several cardiovascular disorders besides hypertrophy, such as myocardial ischemia,7 hypertension,8 and heart failure.9

Increased sympathetic nervous system (SNS) activity is a feature of all the above-mentioned conditions including hypertrophy in which βARK1 is increased.10,11 The sympathetic catecholamines norepinephrine and epinephrine can interact with both α1- and βARs present on the sarcolemmal membranes of cardiomyocytes leading to the activation of differential intracellular signaling pathways.12,13 Stimulation of either βARs or α1ARs can lead to myocardial hypertrophic responses.14

The object of the present study was to test whether cardiac adrenergic activation is responsible for the increase in βARK1 levels during hypertrophy and to investigate the relative role of cardiac α1- and βARs. Cardiac hypertrophy was induced in mice by chronic administration of the β-agonist isoproterenol (ISO) or the α1-agonist phenylephrine (PE). We also studied a transgenic model of direct myocardial α1AR-induced cardiac hypertrophy15 and agonist-treated cultured adult rat ventricular myocytes.

Methods

Animals, Study Design, and Pump Implantation

C57/B16 mice weighing 25 to 30 g were used. The institutional animal usage committee at Duke University Medical Center approved all animal procedures. Miniosmotic pumps (Alzet model 2002) were implanted in mice anesthetized with ketamine (10 mg/kg) and xylazine (0.5 mg/kg).16 Pumps were filled with ISO, PE, or vehicle (0.02% ascorbic acid) and were set to deliver ISO at 30 mg · kg⁻¹ · d⁻¹ and PE at 100 mg · kg⁻¹ · d⁻¹ for 14 days each. After
treatment, animals were weighed and anesthetized, and their hearts were excised, rinsed, and blotted dry. The isolated hearts were then weighed and frozen in liquid nitrogen and stored at −70°C until studied. The heart weight-to-body weight ratio was then calculated (milligrams/gram).

**Ventricular Myocyte Cross-Sectional Area**

After treatment, 3 animals from each group were anesthetized as above, and the hearts were perfusion fixed. A plastic cannula was placed in the aortic root, and the hearts were perfused at the constant pressure of 70 mm Hg through the coronary circulation with ice-cold PBS for 30 minutes, and then fixed through the same route with buffered 1% formaldehyde solution for 30 minutes. Hearts were then cut along a midsagittal plane, paraffin embedded, and sectioned. Sections were then labeled with fluorescein-conjugated wheat germ agglutinin as described.15 Video micrographs of sections were taken by use of systematic sampling, and cross-sectional areas of myocytes were measured by use of NIH-Image 1.63 software. The cross-sectional areas of 100 left ventricular (LV) free wall myocytes were determined from each heart studied.

**βAR Radioligand Binding**

Myocardial membranes were prepared by homogenization of excised hearts in ice-cold lysis buffer [50 mmol/L Hepes (pH 7.3), 150 mmol/L KCl, 5 mmol/L EDTA] as we described previously.5,16 Final membranes were resuspended at a concentration of 2 to 3 mg/mL in ice-cold βAR binding buffer [75 mmol/L Tris-Cl (pH 7.4), 12.5 mmol/L MgCl2, 2 mmol/L EDTA] and binding was performed with the βAR ligand [125I]cyanopindolol as described.5,16 All assays were performed in triplicate, and receptor density (fmol/mg) was normalized to milligrams of membrane protein.

**Adenylyl Cyclase Activity**

Crude myocardial membranes were prepared as described above. Membranes (20 to 30 µg protein) were incubated for 15 minutes at 37°C with [α-32P]ATP under basal conditions or in the presence of 0.1 µmol/L ISO or 10 µmol/L NaF, and cAMP was quantitated by standard methods as described.5,16

**Protein Immunoblotting**

Immunodetection of myocardial levels of βARK1 was performed on detergent-solubilized extracts after immunoprecipitation, as we described previously.5,16 Excised hearts were homogenized and βARK1 was immunoprecipitated from 1 mL of clarified extract (equal protein amounts) with 1:2000 (0.5 µmol/L) monoclonal βARK1 antibody4,16 and 35 µL of a 50% slurry of protein A-agarose conjugate agitated for 1 hour at 4°C. Immune complexes were then washed, electrophoresed through 12% polyacrylamide Tris/glycine gels, and transferred to nitrocellulose.4,16 The 80-kDa βARK1 protein then was visualized with the same monoclonal antibody and chemiluminescence detection (ECL, Amersham). Immunodetection of myocardial levels of GRK5 and Gai was performed in myocardial membranes as described.4,16 GRK5 was visualized with a monoclonal antibody raised to the carboxyl terminus of GRK5,16 and Gai was visualized with a commercially available polyclonal antibody to Gai1/3 (Santa-Cruz). Quantitation of immunoreactive βARK1, GRK5, and Gai was done by scanning the autoradiography film and with use of ImageQuant software (Molecular Dynamics).

**GRK Activity Assay**

Cytosolic myocardial extracts were prepared by homogenization of excised hearts in 2 mL of ice-cold lysis buffer [25 mmol/L Tris-Cl (pH 7.5), 5 mmol/L EDTA, 5 mmol/L EGTA, 0.002 mmol/L leupeptin, 0.003 mmol/L aprotinin, and 1 mmol/L PMSF] as we described previously.4,3,16 Cytosolic protein (100 to 150 µg) was incubated with rhodopsin-enriched rod outer segment (ROS) membranes in lysis buffer with 10 mmol/L MgCl2 and 0.1 mmol/L ATP (containing [γ-32P]ATP). After incubation with white light for 15 minutes at room temperature, the reaction was quenched with ice-cold lysis buffer and centrifuged for 15 minutes at 13 000g. The pelleted material was electrophoresed through 4% to 20% polyacrylamide Tris/glycine gels, and phosphorylated rhodopsin was visualized by autoradiography of dried gels and quantified with a Molecular Dynamics PhosphorImager.4,5,16

**Cultured Adult Rat Ventricular Myocytes**

Sprague Dawley rats were anesthetized and heparinized, and the hearts were explanted and rinsed in cold PBS. Isolated rat hearts were then perfused with Joklik’s modified minimum medium containing hyaluronidase, collagenase, bateric protease, and 0.0125 mmol/L CaCl2 and myocytes cultured as we described previously.17 Myocytes were plated at equal density in M199 in the presence of 10% fetal bovine serum (FBS) on 150-mm tissue culture plates precoated with 20 µg/mL of mouse laminin.17 After 2 hours to allow rod-shaped myocytes to attach to the culture plate, cells were incubated with ISO (10−7 mol/L) or PE (10−7 mol/L) in the presence of 1% FBS. Fresh agonists were added to the medium after 12 hours. After 24 hours, the medium was removed, the cells were rinsed, and βARK1 was immunoprecipitated as described above.

**Statistical Analysis**

Data are expressed as mean±standard error. Statistical comparisons were performed by ANOVA, followed by Bonferroni post hoc analysis.

**Results**

**Heart Weight-to-Body Weight Ratios and Histological Measurements**

Chronic PE or ISO infusion resulted in similar increases in the heart weight-to-body weight ratio compared with the control vehicle–treated animals (Figure 1A). Consistent with the increased cardiac mass and hypertrophy, cross-sectional areas of myocytes from ISO- and PE-treated hearts were increased (Figure 1B).

**βAR Density and Signaling**

The effects of chronic exposure to PE and ISO on cardiac βAR signaling were assessed by measuring myocardial βAR density and functional coupling to membrane adenylyl cyclase activity. As expected, chronic exposure to ISO resulted in a decrease in βAR density in the heart which was ~50% (Table). In contrast, PE did not induce any change in βAR density (Table). Adenylyl cyclase activity in cardiac membranes was impaired in the ISO group both basally and after ISO stimulation (Table). Absolute amounts of cAMP production in PE-treated animals also were decreased (Table), but βAR responsiveness to ISO was significantly greater than in ISO-treated cardiac membranes (Table). This was calculated as the percent increase in adenylyl cyclase activity over baseline induced by ISO. In membranes from PE-treated hearts, ISO induced a 52±3% increase in cyclase activity over the basal value, which was similar to the control response (45±2%). βAR responsiveness was significantly reduced in membranes purified from ISO-treated mice (22±3%, P<0.05 versus control), indicating that chronic exposure to ISO results in βAR desensitization. In all groups, stimulation of adenylyl cyclase activity by NaF was similar (Table), suggesting there were no treatment-induced changes in adenylyl cyclase itself.

**Cardiac βARK1, GRK5, and Gai Expression and Activity**

To assess the possible involvement of βARK1 in uncoupled βAR signaling, we examined βARK1 levels in control, ISO-,
and PE-treated hearts. As shown in Figure 2A, bARK1 was significantly elevated in hearts treated with ISO but not PE, which corresponded to enhanced cytosolic GRK activity (Figure 2B). To rule out the involvement of other GRKs in this result, we examined the levels of GRK5, a second GRK expressed in the heart that can act on βARs.18 In contrast to the results with βARK1, no differences in myocardial GRK5 expression were seen in any of the hearts (data not shown).

Because our results with βARK1 suggested differential regulation by ISO- and PE-induced treatment, we examined a second model of α1 AR-mediated myocardial hypertrophy. This is a transgenic mouse with myocardial-targeted overexpression of a constitutively activated mutant (CAM) α1b AR.15 This mouse develops myocardial hypertrophy because of enhanced signaling through the CAM-α1bARs.15 βARK1 content in these CAM-α1b AR hearts was similar to βARK1 content in their nontransgenic littermates (Figure 3) confirming the inability of α1-adrenergic signaling to regulate myocardial βARK1 levels. This also demonstrated that increases in βARK1 expression are not associated with this model of myocardial hypertrophy.

Our results fit with the hypothesis of a specific impairment of βAR signaling induced by chronic activation of cardiac βARs but not α1 ARs. Nevertheless, in PE-treated animals we found a reduction in basal adenylyl cyclase activity (Table). To explore a possible mechanism to explain this alteration, we assessed Gαi level in PE-treated mouse hearts. We found that in PE-treated hearts the content of Gαi was significantly increased by 50% (Figure 5). ISO did not induce this increase (data not shown). Thus, reduced adenylyl cyclase activity in PE-treated hearts may be explained by enhanced Gi levels.

Figure 1. Effects of chronic ISO or PE on cardiac size. A, Bars show the heart weight-to-body weight ratio (milligrams/gram), calculated in control (vehicle), ISO-treated, and PE-treated mice. Both of the catecholamine treatments were able to induce similar myocardial hypertrophy, without affecting body weight (ISO: baseline, 28.4±0.7 g; after treatment, 29.6±0.8 g, NS; PE: baseline, 28.4±0.6 g; after treatment, 29.6±1.1 g, NS). Data are expressed as mean±SEM. *P<0.05 (ANOVA). B, Cross-sectional area of ventricular myocytes were measured in control, ISO-treated, and PE-treated mice. Shown are representative sections of hearts stained with wheat-germ agglutinin (see Methods). Both ISO and PE treatments significantly increased cardiomyocyte cross-sectional area compared with control hearts (Control: 149±27 μm²; ISO: 249±21 μm²; P<0.05 versus Control; PE: 216±23 μm²; P<0.05 versus Control; NS versus ISO; ANOVA). The white bar shown is equal in length to 10 μm.

**βAR Density and Adenylyl Cyclase Activity in Cardiac Membranes Purified from Control, ISO-, and PE-Treated Mice**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISO</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>βAR density (fmol/mg)</td>
<td>36±2</td>
<td>23±1*</td>
<td>39±4</td>
</tr>
<tr>
<td>Adenylyl cyclase activity (pmol·min⁻¹·mg⁻¹)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Basal</td>
<td>42±3</td>
<td>26±3*</td>
<td>21±4*</td>
</tr>
<tr>
<td>ISO (10⁻⁴ mol/L)</td>
<td>61±5</td>
<td>31±5*</td>
<td>32±7*</td>
</tr>
<tr>
<td>NaF (10⁻² mol/L)</td>
<td>347±30</td>
<td>309±42</td>
<td>391±11</td>
</tr>
<tr>
<td>βAR responsiveness (% ISO over basal)</td>
<td>45±2</td>
<td>22±3*</td>
<td>52±3</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SEM of 6–10 individual cardiac membrane preparations done in triplicate.

*P<0.05 vs control (vehicle), ANOVA.
Discussion

Chronic treatment of mice with PE and ISO resulted in similar cardiac hypertrophic responses, which were manifest as increased myocardial mass and size of ventricular myocytes. Although myocardial hypertrophy was induced by both treatments, only ISO induced changes in myocardial GRK activity that were due to enhanced levels of \( \beta ARK1 \). Previously, we showed that chronic treatment with ISO can lead to increased expression of \( \beta ARK1 \) in the heart, which in turn impairs \( \beta AR \) signaling\(^{16} \); however, PE-induced hypertrophy is not associated with a similar enhancement of \( \beta ARK1 \) expression, which demonstrates a differential regulation of \( \beta ARK1 \) in response to SNS activity. Thus, \( \beta AR \) but not \( \alpha_1 \)AR stimulation selectively regulates myocardial \( \beta ARK1 \) expression.

Increased expression of \( \beta ARK1 \) can have detrimental effects on myocardial \( \beta AR \) signaling and function, and enhanced \( \beta ARK1 \) actually appears to be an early defect in ventricular hypertrophy induced by pressure overload.\(^4 \) The increased \( \beta ARK1 \) found in pressure overload hypertrophy is likely responsible for impaired \( \beta AR \) contractile responses, because pressure overload in transgenic mice expressing a \( \beta ARK1 \) inhibitor does not lead to impaired in vivo cardiac function.\(^4 \) The mechanisms that underlie increases in myocardial \( \beta ARK1 \) in pressure overload hypertrophy are not clear, but catecholamines resulting from enhanced SNS activity are elevated in hypertrophy. It is likely that the chronic adrenergic activation by endogenous catecholamines can sustain the increase in cardiac \( \beta ARK1 \) through the stimulation of the myocardial ARs. The results of the present study, in fact, indicate a major role of \( \beta AR \)s in \( \beta ARK1 \) upregulation, because the chronic stimulation of \( \alpha_1 \)ARs does not induce any change in cardiac \( \beta ARK1 \) content. The selective increase of \( \beta ARK1 \) only in the presence of ISO-induced hypertrophy, but not in PE-induced hypertrophy, also rules out the possibility that hypertrophy per se increases \( \beta ARK1 \).

The mechanisms of PE- and ISO-induced cardiac hypertrophy are not completely understood. We know that in...
isolated neonatal cardiac myocytes treatment with PE or ISO leads to the activation of different nuclear factors, which results in increased myocardial protein synthesis. However, in vivo PE treatment can induce a temporary increase in blood pressure, thus simulating a model of cardiac hypertrophy caused by pressure overload. Differences in the hemodynamic effects induced by ISO and PE might be responsible for the differential regulation of βARK1 observed, rather than the chronic activation of cardiac β- and α1 ARs. To rule out this possibility, we added two controls to our experimental design.

First, we used a transgenic mouse model of α1-AR-induced myocardial hypertrophy (CAM-α1AR). In this model, cardiac hypertrophy develops because of the direct activation of the cardiac α1AR signaling pathway in the absence of changes in blood pressure. We observed no changes in the cardiac content of βARK1 of these transgenic animals, which suggests that changes are occurring in myocardial adenylyl cyclase signaling activity, however, was reduced in PE-treated animals, which suggests that changes are occurring in myocardial adenylyl cyclase signaling activity. In rat models of hypertension and hypertrophy an increase in the myocardial Gαi content was observed. Thus, we examined Gαi in PE-treated versus control mouse hearts and did find a significant increase, suggesting that this could be responsible for the reduction of cardiac adenylyl cyclase activity seen in this model. We previously found no change in myocardial Gαi expression in pressure overload ventricular hypertrophy demonstrating that Gi and βARK1 can both be differentially regulated depending on the hypertrophy stimulus.

In conclusion, this study shows that in catecholamine-induced cardiac hypertrophy the observed increase in cardiac βARK1 content is due to the chronic activation of βARs but not α1ARs. This may represent the mechanism that triggers the increase in βARK1 content observed in pressure overload hypertrophy as well as in other pathophysiological conditions associated with adrenergic activation, such as heart failure and ischemia. Thus, targeted inhibition of myocardial βARK1 activity may be a novel therapeutic approach for preventing dysfunctional βAR signaling.

Acknowledgments

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β-Adrenergic Receptor Kinase-1 Levels in Catecholamine-Induced Myocardial Hypertrophy: Regulation by β- but not α1-Adrenergic Stimulation
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