Enhanced Contractility and Decreased β-Adrenergic Receptor Kinase-1 in Mice Lacking Endogenous Norepinephrine and Epinephrine

Myeong-Chan Cho, MD; Madhu Rao, BA; Walter J. Koch, PhD; Steven A. Thomas, MD, PhD; Richard D. Palmiter, PhD; Howard A. Rockman, MD

Background—Elevated circulating norepinephrine (NE) has been implicated in causing the profound β-adrenergic receptor (βAR) downregulation and receptor uncoupling that are characteristic of end-stage human dilated cardiomyopathy, a process mediated in part by increased levels of β-adrenergic receptor kinase (βARK1). To explore whether chronic sustained NE stimulation is a primary stimulus that promotes deterioration in cardiac signaling, we characterized a gene-targeted mouse in which activation of the sympathetic nervous system cannot lead to an elevation in plasma NE and epinephrine.

Methods and Results—Gene-targeted mice that lack dopamine β-hydroxylase (dbh−/−), the enzyme needed to convert dopamine to NE, were created by homologous recombination. In vivo contractile response to the βAR agonist dobutamine, measured by a high-fidelity left ventricular micromanometer, was enhanced in mice lacking the dbh gene. In unloaded adult myocytes isolated from dbh−/− mice, basal contractility was significantly increased compared with control cells. Furthermore, the increase in βAR responsiveness and enhanced cellular contractility were associated with a significant reduction in activity and protein level of βARK1 and increased high-affinity agonist binding without changes in βAR density or G-protein levels.

Conclusions—Mice that lack the ability to generate NE or epinephrine show increased contractility associated primarily with a decrease in the level of βARK1 protein and kinase activity. This animal model will be valuable in testing whether NE is required for the pathogenesis of heart failure through mating strategies that cross the dbh−/− mouse into genetically engineered models of heart failure. (Circulation. 1999;99:2702-2707.)

Key Words: contractility • catecholamines • heart failure • receptors, adrenergic, beta

One of the most important mechanisms for rapidly regulating β-adrenergic receptor (βAR) function is agonist-stimulated receptor phosphorylation by G protein–coupled receptor kinases (GRKs), resulting in decreased sensitivity to subsequent catecholamine stimulation.1 βAR kinase (βARK1) is a member of this family of GRKs that phosphorylate and regulate a wide variety of receptors that couple to heterotrimeric G proteins.1,2

Activation of the sympathetic nervous system is considered one of the cardinal pathophysiological abnormalities in patients with heart failure and frequently precedes the development of overt symptoms.4 Plasma norepinephrine (NE) and renin activity are increased in patients with heart failure and are known prognostic factors for survival.3 Elevated circulating NE and epinephrine have been implicated in contributing to the profound βAR downregulation and receptor uncoupling that are characteristic of end-stage human dilated cardiomyopathy,5 resulting in subsensitivity to β-agonist stimulation,6 a process likely mediated by βARK1.7 Importantly, myocardial βARK1 mRNA and activity are elevated in human heart failure.6,8 It has been postulated that long-term stimulation of myocardial βARs may adversely affect cardiomyocyte viability, possibly through cAMP-mediated Ca2⁺ overload of the cell.10 In this regard, sustained sympathetic activity may both phosphorylate and downregulate phospholamban and act to enhance sarcoplasmic reticulum Ca2⁺ pump activity in the attempt to maintain contractile and relaxation processes.11 This has led to the hypothesis that chronic sustained sympathetic drive is an important pathological element in the progressive deterioration of the failing heart,12 a concept that is supported by data showing a beneficial outcome in heart failure patients treated with βAR antagonists13 and a reduction in sympathetic outflow with ACE inhibitors.14

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To further explore the role of chronic sustained adrenergic stimulation in the pathogenesis of the failing heart, a model system in which activation of the sympathetic nervous system cannot lead to an elevation in plasma NE would be of value. In this regard, gene-targeted mice that lack dopamine β-hydroxylase (dbh−/−), the enzyme needed to convert dopamine to NE, were created by homologous recombination.\(^{15,16}\) Adult dbh−/− mice have virtually no endogenous NE or epinephrine.\(^{15-17}\) The purpose of the present study was to characterize the cardiovascular phenotype and βAR signaling pathway in rescued adult dbh−/− mice that are devoid of endogenous NE and epinephrine.

Methods

Experimental Animals

Details of the gene-targeting strategy used to disrupt the dopamine β-hydroxylase gene have been published elsewhere.\(^{15}\) Mice used in the present study were 4 to 6 months of age and were of either sex. Mutant offspring were generated from homozygous males mated to heterozygous females. Heterozygous dbh+/− mice have normal levels of NE and epinephrine and were therefore used as controls.\(^{16,17}\) In contrast, homozygous dbh−/− mice have serum and tissue NE levels that are below the level of detection.\(^{16,17}\) Homozygous dbh−/− embryos were rescued from embryonic lethality with the administration of L-threo-3,4-dihydroxyphenylserine (L-DOPS) in the maternal drinking water from Day 9.5 until birth.\(^{15,16}\) L-DOPS is a synthetic amino acid that is converted to NE by aromatic l-amino acid decarboxylase, which is present in all adrenergic cells, bypassing the requirement for DBH.

Experiments were performed in an additional group of genetically altered mice that were heterozygous for activation of the βARK1 gene (βARK1+/−).\(^{18}\) βARK1+/− homozygous animals contain a 50% reduction in myocardial levels of βARK1 protein and GRK activity, which results in increased contractile function compared with wild-type animals.\(^{19}\)

Animals in this study were handled according to the animal welfare regulations of the University of North Carolina at Chapel Hill and University of Washington, and the protocol was approved by the animal subjects committees of these institutions.

Physiological Evaluation

Hemodynamic evaluation was performed as previously described.\(^{20-22}\) Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) given intraperitoneally. After endotracheal intubation, mice were connected to a rodent ventilator. The left carotid artery was cannulated with a flame-stretched PE-50 catheter connected to a modified P-50 Statham transducer. A 1.4F high-fidelity micromanometer catheter (Millar Instruments) was inserted into the right carotid and advanced retrogradely into the left ventricle (LV). Hemodynamic measurements were recorded before and after bilateral vagotomy and after 2-minute infusion of isoproterenol (10−4 to 10−6 mol/L) in 250 μL of binding buffer (50 mmol/L HEPES [pH 7.3], 5 mmol/L MgCl2, and 0.1 mmol/L ascorbic acid).\(^{23}\) Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) given intraperitoneally. After endotracheal intubation, mice were connected to a rodent ventilator. The left carotid artery was cannulated with a flame-stretched PE-50 catheter connected to a modified P-50 Statham transducer. A 1.4F high-fidelity micromanometer catheter (Millar Instruments) was inserted into the right carotid and advanced retrogradely into the left ventricle (LV). Hemodynamic measurements were recorded before and after bilateral vagotomy and after 2-minute infusion of incremental doses of isoproterenol.

Myocyte Isolation and Contractile Function

Hearts from separate control and dbh−/− mice were isolated and used to isolate adult myocytes, and contractile function was assessed as previously described.\(^{20}\) Myocyte cell edges were enhanced and processed with a video edge motion-detection system (Crescent Electronics) at a sampling rate of 240 Hz and recorded in digitized form at 400 Hz.

GRK Activity by Rhodopsin Phosphorylation

 Supernatants of cytosolic extracts were prepared as previously described.\(^{20}\) Concentrated (Centricron, Amicon Inc) cytosolic extract (300 μg of protein) was incubated with rhodopsin-enriched rod outer segments in 25 μL of lysis buffer with 10 mmol/L MgCl2 and 0.1 mmol/L ATP containing [γ-32P]ATP. The reactions were incubated in white light for 15 minutes, quenched with 300 μL of ice-cold lysis buffer, and then centrifuged. Sedimented proteins were electrophoresed through SDS–12% polyacrylamide gels, and phosphorylated rhodopsin was visualized by autoradiography and quantified with a phosphorimager (Molecular Dynamics).

Immunoblotting

Pelleted membranes (from above) were resuspended in 50 mmol/L HEPES buffer (pH 7.3) containing 5 mmol/L MgCl2 and electrophoresed on SDS–10% polyacrylamide gels and transferred to nitrocellulose. The ~39-kDa gas protein and 2 forms of stimulatory G (Gs) protein (45 and 53 kDa) were visualized with 1:1000 dilution of polyclonal antibody (1-20 and K-20, respectively; Santa Cruz Bio-technology), and detection of anti-rabbit IgG conjugated with horse-radish peroxidase was performed by enhanced chemiluminescence (ECL; Amersham). Immunoblots were scanned and quantified with an imaging densitometer (Bio-Rad).

In separate hearts, immunodetection of myocardial levels of βARK1 was performed on cytosolic extracts after immunoprecipitation, as previously described.\(^{20}\) βARK1 was immunoprecipitated from 600 μmol/L clarified cytosol extract with a 1:1000 (1 μL) monoclonal anti-βARK1 (CS/1) antibody and 35 μL of a 50% slurry of protein A-agarose conjugate agitated for 1 hour at 4°C. Immune complexes were washed, resuspended in 40 μL of protein-gel loading buffer, then heated for 3 minutes at 85°C and electrophoresed through SDS–12% polyacrylamide gels. After transfer to nitrocellulose, the ~80-kDa βARK1 protein was visualized with the monoclonal antibody (CS/1) and chemiluminescent detection of anti-mouse IgG conjugated with horseradish peroxidase (ECL; Amersham).

βAR Density and Radioligand Binding

Myocardial sarcolemmal membranes were prepared by homogenization of whole hearts in ice-cold buffer as previously described.\(^{20,21,22}\) Total βAR density was determined by incubation of 25 μg of cardiac sarcolemmal membranes with a saturating concentration (80 pmol/L) of [125I]cyanopindolol and 20 μmol/L alpenolol to define nonspecific binding.\(^{23}\) Typical nonspecific binding is ~40% of the total. Competition-binding isotherms in sarcolemmal membranes were done in triplicate with 18 varying concentrations of isoproterenol (10−13 to 10−6 mol/L) in 250 μL of binding buffer (50 mmol/L HEPES [pH 7.3], 5 mmol/L MgCl2, and 0.1 mmol/L ascorbic acid).\(^{23}\) Assays were conducted at 37°C for 60 minutes and then filtered over GF/C glass fiber filters (Whatman) that were washed and counted in a gamma counter. Competition isotherms were analyzed by nonlinear least squares curve fit (GraphPad Prism).

Statistical Analysis

Data are expressed as mean±SEM. Two-way repeated ANOVA was used to evaluate the hemodynamic measurements under basal conditions and with dobutamine stimulation. When appropriate, post hoc analysis was performed with a Newman-Keuls test. Student’s t test with Bonferroni correction for multiple comparisons was used to assess differences in isolated myocyte mechanics and for analysis of the biochemical data. For all analyses, P<0.05 was considered significant.

Results

Although the dbh−/− mice were smaller than controls, myocardial growth in the adult mouse was proportionate. No significant difference was found between dbh−/− and control mice for LV weight normalized for either body weight or tibia length (Figure 1). This is consistent with our previously reported data showing that adult dbh−/− mice are morphologically normal and eventually grow to 80% (male) and 88% (female) of littermate adult weight.\(^{15}\)

Cardiac catheterization was used to measure in vivo responsiveness to dobutamine in intact anesthetized dbh−/− and control mice. Before vagotomy, LV dP/dtmax in the dbh−/−/
mice was significantly greater than in control animals (Figure 2A). As expected, after bilateral vagotomy, there was a small increase in LV dP/dtmax that was seen in both genotypes (Figure 2A). In response to dobutamine, a significant increase in LV dP/dtmax was seen in dbh−/− mice compared with control mice (Figure 2A). The change of LV dP/dtmax from baseline, ΔLV dP/dtmax, is a measure of βAR responsiveness. As shown in Figure 2B, dbh−/− animals had a marked enhancement of LV dP/dtmax in response to dobutamine infusion, which suggests increased βAR coupling and sensitivity to β-agonist stimulation.

Hemodynamic parameters obtained during catheterization are shown in Figure 3. Before vagotomy, heart rate in dbh−/− mice was identical to that for control animals. After bilateral vagotomy, heart rate increased to a greater extent in dbh−/− mice, indicating higher parasympathetic tone in dbh−/− mice than in control animals (Figure 3A). With dobutamine infusion, the increase in heart rate was significantly less in dbh−/− mice than in control animals, indicating a reduced chronotropic effect of β-agonist stimulation in dbh−/− mice (percent increase from after vagotomy, control 22.5 ± 2.5% versus dbh−/− 11.8 ± 1.2%; P < 0.01). LV systolic pressure and end-diastolic pressures were not statistically different between groups at baseline or with dobutamine stimulation (Figure 3B and 3C). These data demonstrate a marked enhancement of LV contractility and minimal chronotropic effect with βAR stimulation in mice chronically lacking NE and epinephrine. Furthermore, the increased prevagotomy LV dP/dtmax in dbh−/− mice with similar heart rate to controls suggests a state of enhanced contractility. It is interesting that heart rate before vagotomy was identical to that in control mice, which suggests that an elevation in parasympathetic tone may exist in the dbh−/− mice.

To test whether dbh−/− mice have enhanced contractility at a cellular level, cell mechanics were measured in single adult myocytes. Under conditions of constant pacing in unloaded, freshly isolated myocytes, the rate of cell shortening (−dL/dt) and percentage of cell shortening were significantly higher
Contractile Parameters in Adult Myocytes Isolated From Control and dbh−/− Hearts

<table>
<thead>
<tr>
<th></th>
<th>Control (n=7)</th>
<th>dbh−/− (n=6)</th>
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</thead>
<tbody>
<tr>
<td>−dL/dt, μm/s</td>
<td>−294.5±11.4</td>
<td>−392.2±14.1†</td>
</tr>
<tr>
<td>+dL/dt, μm/s</td>
<td>208.3±6.6</td>
<td>218.0±7.2</td>
</tr>
<tr>
<td>L max, μm</td>
<td>115.8±2.3</td>
<td>114.2±2.1</td>
</tr>
<tr>
<td>L min, μm</td>
<td>94.4±2.4</td>
<td>89.3±1.6</td>
</tr>
<tr>
<td>% CS</td>
<td>18.6±0.6</td>
<td>21.9±0.5*</td>
</tr>
</tbody>
</table>

−dL/dt indicates rate of shortening; +dL/dt, rate of relengthening; % CS, % cell shortening (calculated as % change in myocyte length from rest [L max] to minimum length [L min]). Ten to 15 myocytes from each heart were studied. The operator was blinded to the genotype of the animals.

*P<0.001, †P<0.01 dbh−/− vs control, unpaired t test with Bonferroni correction for 5 comparisons.

(33% and 18%, respectively) in dbh−/− cells than in control cells (Table). By eliminating the influence of heart rate and loading conditions on the measurement of contractile function, we demonstrated that myocytes isolated from dbh−/− hearts have enhanced cellular contractility.

The enhanced cardiac contractility and βAR sensitivity in the dbh−/− mice were similar to mice with myocardial expression of a peptide inhibitor of βARK123 and to genetargeted mice with a 50% reduction in βARK1 levels (βARK1−/−). Therefore, we investigated βARK1 expression and activity in these mice. βARK1 is a cytosolic enzyme, and our previous studies in mice demonstrated that GRK activity in those extracts primarily reflects βARK1. Kinase activity of cytosolic extracts from dbh−/− hearts was ≈50% lower than values obtained from control hearts (Figure 4A and 4B). Consistent with the reduction in cytosolic kinase activity, the level of βARK1 protein, as assessed by immunoblotting, was also significantly diminished in the hearts of dbh−/− mice compared with control mice (Figure 4C). These data show that the enhanced βAR responsiveness and cellular contractility in mice lacking NE and epinephrine are associated with a significant reduction in βARK1 activity and protein.

To determine whether altered levels of G proteins contributed to the cardiac phenotype, the levels of Gαs and Gαi protein in cardiac membranes were measured by immunoblotting (Figure 5A and 5B). No difference in Gαs protein levels between control (5.0±0.3 arbitrary units [AU]) and dbh−/− hearts (4.3±0.3 AU) was found by densitometry. Although the higher molecular weight form of Gαi protein was somewhat lower in the dbh−/− hearts, this did not reach statistical significance (control versus dbh−/−: 45-kDa band, 5.6±0.3 versus 5.8±0.4 AU; 53-kDa band, 6.3±0.3 versus 4.6±0.6 AU; P=NS).

To investigate the molecular mechanisms for the increased contractility and enhanced βAR responsiveness, we evaluated receptor-effector coupling in sarcolemmal membranes from hearts of dbh−/− mice and compared it with that of βARK1−/− mice. The total number of βARs in the dbh−/− hearts (n=6) (56.1±4.9 fmol/mg membrane protein) was not significantly different than that in control hearts (n=7) (49.1±6.4 fmol/mg membrane protein). In contrast, the percentage of βARs exhibiting high-affinity binding for isoproterenol was significantly greater in membranes prepared from dbh−/− hearts than in those from controls (Figure 6A). Furthermore, βAR-Gi coupling in a genetic mouse model in which myocardial βARK1 activity was reduced by 50% also showed an increase in high-affinity agonist binding similar to that observed in dbh−/− hearts (Figure 6B). The significantly greater number of high-affinity receptors is consistent with an increased ability of βARs to form the coupled hormone-receptor–G-protein high-affinity state. These changes occur when receptors are less desensitized.23

Discussion

The present study demonstrates that adult dbh−/− mice that lack endogenous NE and epinephrine have the following characteristics: (1) myocardial growth is proportionate to...
body weight; (2) cellular contractility in isolated myocytes is increased; (3) βAR responsiveness in vivo is significantly enhanced; and (4) this enhanced contractile state is associated with a significant reduction in βARK1 and increased high-affinity agonist binding without changes in βAR density or G-protein levels. Thus, the enhanced inotropy and βAR responsiveness likely results from decreased βARK desensitization.

Previous studies have shown that a tissue subjected to either surgical or chemical sympathectomy elicits an exaggerated response to catecholamine stimulation, a process termed catecholamine supersensitivity. In the mouse model used in the present study, which is devoid of endogenous NE and epinephrine, we demonstrate that enhanced responsiveness to βAR stimulation is likely due to an increase in the level of βARK1 protein and reduction in βARK1 activity. Thus, we have further demonstrated that the level of βARK1 in the normal heart may be modulated by activity of the sympathetic nervous system, an observation supported by previous experimental studies.

Immunoblotting of sarcolemmal membranes with an antibody to either Goa or Gsa showed no appreciable difference in the level of these G proteins in dbh−/− compared with control hearts. In contrast, the changes in βARK1 are significant and likely account for the enhancement in βAR responsiveness. We have recently shown that mice heterozygous for the βARK1 gene deletion, which possess 50% less βARK1 enzyme than wild-type animals, have enhanced LV contractility and sensitivity to βAR agonists, a cardiac phenotype that is similar to the dbh−/− mice described herein. In the present study, we also show that both dbh−/− and βARK1+/− hearts have a significantly greater percentage of receptors in the high-affinity state (Figure 6). This enhanced βAR-effector coupling in dbh−/− hearts, which is identical to the findings when myocardial βARK1 is reduced by 50%, is most consistent with a mechanism of diminished desensitization resulting from the reduction in βARK1 that allows for a greater number of βARs to form the coupled hormone-receptor–G-protein high-affinity state.

Despite multiple lines of evidence showing enhanced βAR responsiveness in dbh−/− mice, we were unable to show an increase in membrane adenyl cyclase activity (basal conditions: control versus dbh−/−, 20.6±5.9 versus 25.4±7.9 pmol·mg protein−1·min−1, P=NS; isoproterenol [10−4 mol/L]: control versus dbh−/−, 35.7±5.9 versus 42.3±10.2 pmol·mg protein−1·min−1, P=NS; n=6 for both groups). Although we can only speculate about the apparent lack of enhanced activity, we postulate that the βARK1 activity present in purified membranes isolated from dbh−/− hearts is sufficient to desensitize βARs to the same extent as controls in this in vitro assay. Importantly, however, we document that dbh−/− mice have enhanced βAR responsiveness and reduced βARK1 activity, which does lead to more βARs in the high-affinity state.

The dbh−/− mice showed a marked enhancement of LV dP/dtmax in response to β-agonist stimulation consistent with a state of diminished βAR desensitization. Desensitization of βARs requires not only GRK-mediated phosphorylation but also the binding of β-arrestins, which bind to phosphorylated receptors and interdict further activation of G proteins. The GRKs expressed in the heart are GRK2 (commonly known as βARK1), GRK3 (βARK2), GRK5, and GRK6, with βARK1 being the most abundant. A pivotal role for βARK1 in the regulation of βAR signaling is suggested by recent experimental and clinical studies. In a series of studies in genetically targeted mice, we have shown that cardiac-targeted overexpression of βARK1 results in marked βAR desensitization, whereas overexpression of a peptide inhibitor of βARK123 or 50% reduction in βARK15 results in enhanced βAR sensitivity to β-agonist stimulation. Furthermore, impaired βAR responsiveness, which occurs in response to pressure overload hypertrophy, is caused by the increase in myocardial βARK1 and can be completely reversed on βARK inhibition.20 Finally, βARK1 levels are increased in heart extracts from human end-stage heart failure.5,9 In this regard, using several gene-targeted mouse models, we have recently shown that βARK1 can play a primary role in the pathogenesis of the failing heart.21

A characteristic of chronic heart failure is βAR desensitization. The trigger for βAR desensitization in the failing heart is thought to be an increase in cardiac neural activity that causes elevation in circulating plasma NE levels. It has been generally assumed that activation of the sympathetic nervous system, which precedes the onset of clinically recognized heart failure, is detrimental to the failing heart and promotes progressive deterioration in cardiac function.10,12,13 Although the level of plasma NE is a prognostic indicator of survival, it is not known whether the increase in plasma catecholamines is responsible for the long-term deterioration in LV function. A recent study29 showed that selective ventricular denervation in an experimental model of progressive heart
failure induced by rapid ventricular pacing could attenuate the decline in cardiac function, which suggests that cardiac nerves play a role in the progression of heart failure. In this regard, the dhh<sup>−/−</sup> mouse provides a unique opportunity to test the roles the sympathetic nervous system and plasma NE play in the pathogenesis of the failing heart.

In summary, dhh<sup>−/−</sup> mice that lack the ability to generate NE or epinephrine show enhanced cellular contractility and βAR responsiveness that are associated with a decrease in the level of βARK1 protein and kinase activity. The loss of βARK1-mediated βAR phosphorylation appears to be the most likely mechanism accounting for this cardiac phenotype. The dhh<sup>−/−</sup> animal model will prove valuable to test whether NE and epinephrine are required for the pathogenesis of heart failure through mating strategies that cross the dhh<sup>−/−</sup> mouse into genetically engineered heart failure backgrounds.

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