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

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β-Adrenergic Receptor Kinase-1 Levels in Catecholamine-Induced Myocardial Hypertrophy

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Abstract—Pressure overload ventricular hypertrophy is accompanied by dysfunctional β-adrenergic receptor signaling due to increased levels of the β-adrenergic receptor kinase-1, which phosphorylates and desensitizes β-adrenergic receptors. In this study, we examined whether increased β-adrenergic receptor kinase 1 expression is associated with myocardial hypertrophy induced by adrenergic stimulation. With use of implanted mini-osmotic pumps, we treated mice with isoproterenol, phenylephrine, or vehicle to distinguish between α_1- and β-adrenergic stimulation. Both treatments resulted in cardiac hypertrophy, but only isoproterenol induced significant increases in β-adrenergic receptor kinase-1 protein levels and activity. Similarly, in isolated adult rat cardiac myocytes, 24 hours of isoproterenol stimulation resulted in a significant 2.8-fold increase in β-adrenergic receptor kinase-1 protein levels, whereas 24 hours of phenylephrine treatment did not alter β-adrenergic receptor kinase-1 expression. Our results indicate that increased β-adrenergic receptor kinase-1 is not invariably associated with myocardial hypertrophy but apparently is controlled by the state of β-adrenergic receptor activation. **(*Hypertension*. 1999;33[part II]:396-401.)**

Key Words: myocardial hypertrophy ■ β-adrenergic receptor ■ G protein–coupled receptor kinases ■ desensitization ■ catecholamines

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 activity[5] or expression[6] 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 α_1- 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 α_1ARs 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 α_1- and βARs. Cardiac hypertrophy was induced in mice by chronic administration of the β-agonist isoproterenol (ISO) or the α_1-agonist phenylephrine (PE). We also studied a transgenic model of direct myocardial α_1AR-induced cardiac hypertrophy[15] and agonist-treated cultured adult rat ventricular myocytes.

Methods

Animals, Study Design, and Pump Implantation

C57/Bl6 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

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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 $MgCl_2$, 2 mmol/L EDTA] and binding was performed with the βAR ligand [^{125}I]cyanopindolol as described.[5,16] All assays were performed in triplicate, and receptor density (femtomoles) was normalized to milligrams of membrane protein.

Adenylyl Cyclase Activity

Crude myocardial membranes were prepared as described above. Membranes (20 to 30 μg of protein) were incubated for 15 minutes at 37°C with [α-^{32}P]ATP under basal conditions or in the presence of either 0.1 mmol/L ISO or 10 mmol/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.[4,16] Excised hearts were homogenized and βARK1 was immunoprecipitated from 1 mL of clarified extract (equal protein amounts) with 1:2000 (0.5 μL) monoclonal βARK1 antibody[4,16] 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 Gαi 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 Gαi was visualized with a commercially available polyclonal antibody to $G\alpha i_{1-3}$ (Santa-Cruz). Quantitation of immunoreactive βARK1, GRK5, and Gαi 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.[4,5,16] Cytosolic protein (100 to 150 μg) was incubated with rhodopsin-enriched rod outer segment (ROS) membranes in lysis buffer with 10 mmol/L $MgCl_2$ and 0.1 mmol/L ATP (containing [γ-^{32}P]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 13 000*g*. 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, bacterial protease, and 0.0125 mmol/L $CaCl_2$ 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^{-6} mol/L) or PE (10^{-6} 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 Gαi Expression and Activity

To assess the possible involvement of βARK1 in uncoupled βAR signaling, we examined βARK1 levels in control, ISO-,

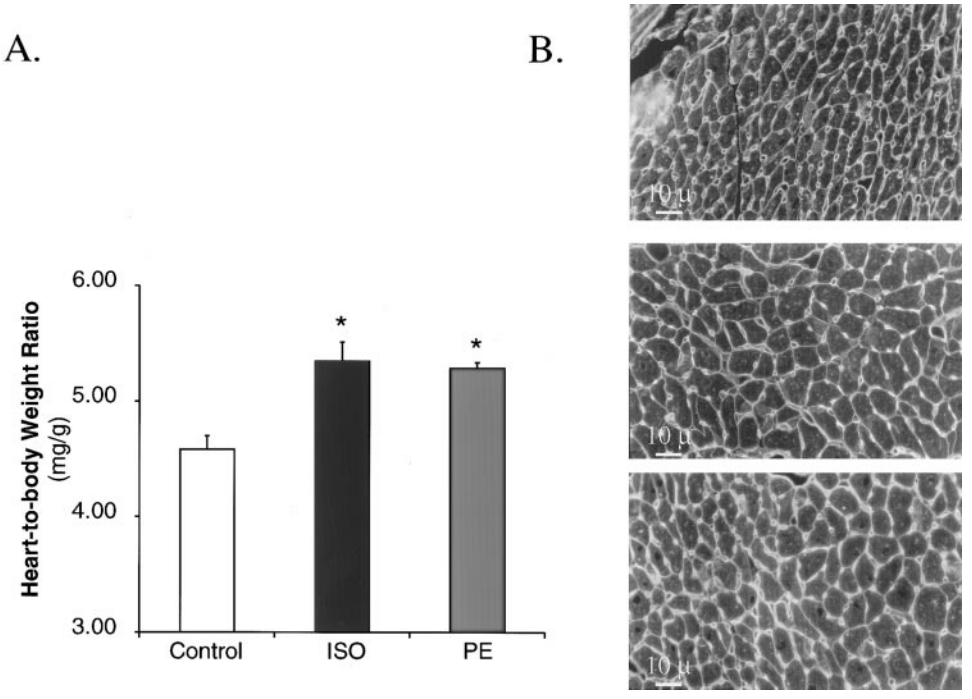

Figure 1. Effects of chronic ISO or PE on cardiac size. A, Bars show the heart weight-to-body weight ratio (milligrams/gram), calculated in control (vehicle), ISO-treated, and PE-treated mice. Both of the catecholamine treatments were able to induce similar myocardial hypertrophy, without affecting body 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 μm^2; ISO: 249±21 μm^2; $P<0.05$ versus Control; PE: 216±23 μm^2; $P<0.05$ versus Control; NS versus ISO; ANOVA). The white bar shown is equal in length to 10 μm.

and PE-treated hearts. As shown in Figure 2A, βARK1 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 α_1AR-mediated myocardial hypertrophy. This is a transgenic mouse with myocardial-targeted overexpression of a constitutively activated mutant (CAM) α_{1B}AR. This mouse develops myocardial hypertrophy because of enhanced signaling through the CAM-α_{1B}ARs.[15] βARK1 content in these CAM-α_{1B}AR hearts was similar to βARK1 content in their nontransgenic littermates (Figure 3) confirming the inability of α_1-adrenergic signaling to regulate myocardial βARK1 levels. This also demonstrated that increases in βARK1 expression are not associated with this model of myocardial hypertrophy.

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

	Control	ISO	PE
βAR density (fmol/mg)	36±2	23±1*	39±4
Adenylyl cyclase activity (pmol · min^{-1} · mg^{-1})			
Basal	42±3	26±3*	21±4*
ISO (10^{-4} mol/L)	61±5	31±5*	32±7*
NaF (10^{-2} mol/L)	347±30	309±42	391±11
βAR responsiveness (% ISO over basal)	45±2	22±3*	52±3

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

*$P<.05$ vs control (vehicle), ANOVA.

Because the different patterns of alteration in βARK1 content in ISO- and PE-treated mice could be caused by differences induced by these two agents in peripheral and central hemodynamics, we examined the regulation of βARK1 in cultured adult rat ventricular myocytes. Cells were exposed for 24 hours to either ISO or PE, and βARK1 levels were assessed. In this model, the effects on βARK1 expression were apparently due to the direct activation of myocardial βARs as ISO significantly enhanced βARK1 content, whereas PE did not alter βARK1 expression (Figure 4).

Our results fit with the hypothesis of a specific impairment of βAR signaling induced by chronic activation of cardiac βARs but not α_1ARs. 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.

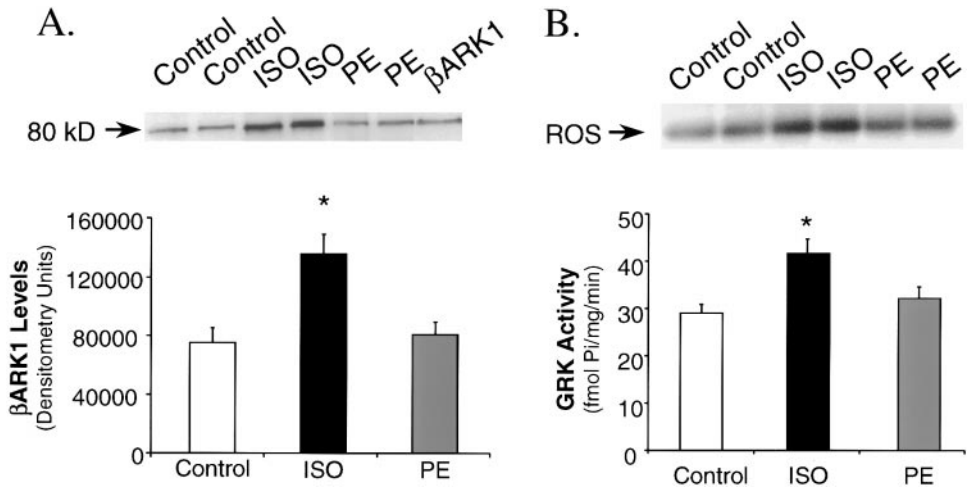

Figure 2. Myocardial βARK1 levels and activity after control, ISO, or PE treatment. A, Myocardial βARK1 protein levels. The histograms represent the mean±SEM in densitometry units (DU) of scanned chemiluminescent immunoblots from 8 to 15 hearts per treatment. The inset shows representative immunoblot with purified βARK1 used as a control for protein migration. *$P<0.05$ versus vehicle (ANOVA). B, Myocardial GRK activity. Results shown are the mean±SEM of 4 to 6 hearts per treatment. The inset is a representative autoradiograph from a dried gel showing phosphorylated rhodopsin (ROS). *$P<0.05$ versus control (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 βARK1. Previously, we showed that chronic treatment with ISO can lead to increased expression of βARK1 in the heart, which in turn impairs βAR signaling[16]; however, PE-induced hypertrophy is not associated with a similar enhancement of βARK1 expression, which demonstrates a differential regulation of βARK1 in response to SNS activity. Thus, βAR but not α_1AR stimulation selectively regulates myocardial βARK1 expression.

Increased expression of βARK1 can have detrimental effects on myocardial βAR signaling and function, and enhanced βARK1 actually appears to be an early defect in ventricular hypertrophy induced by pressure overload.[4] The increased βARK1 found in pressure overload hypertrophy is likely responsible for impaired βAR contractile responses, because pressure overload in transgenic mice expressing a βARK1 inhibitor does not lead to impaired in vivo cardiac function.[4] The mechanisms that underlie increases in myocardial βARK1 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 βARK1 through the stimulation of the myocardial ARs. The results of the present study, in fact, indicate a major role of βARs in βARK1 upregulation, because the chronic stimulation of α_1ARs does not induce any change in cardiac βARK1 content. The selective increase of βARK1 only in the presence of ISO-induced hypertrophy, but not in PE-induced hypertrophy, also rules out the possibility that hypertrophy per se increases βARK1.

The mechanisms of PE- and ISO-induced cardiac hypertrophy are not completely understood. We know that in

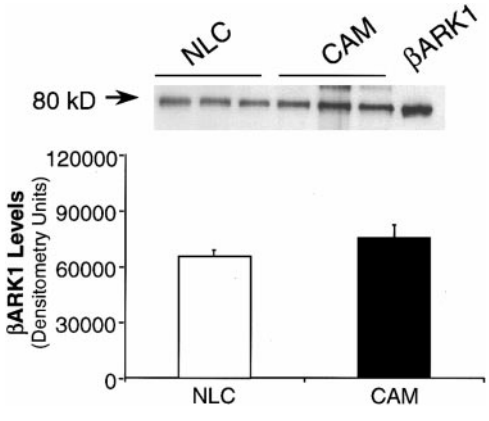

Figure 3. Myocardial βARK1 levels in transgenic mouse hearts overexpressing a CAMα_{1B}AR. Histograms represent the mean±SEM densitometry units of scanned βARK1 immunoblots from 5 hypertrophic CAMα_{1B}AR transgenic mouse hearts and 5 normotrophic nontransgenic littermate controls. The inset shows a representative immunoblot with purified βARK1 included in the last lane as a control for protein migration (P=NS).

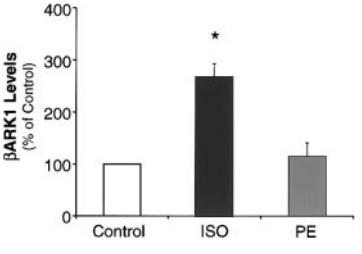

Figure 4. Myocardial βARK1 levels in treated adult rat ventricular myocytes. βARK1 levels in isolated adult rat cardiac myocytes treated for 24 hours with either vehicle (Control, ascorbic acid 0.002%), ISO (10^{-6} mol/L) or PE (10^{-6} mol/L). Data are expressed as percent of basal values found in control hearts. Histograms show mean±SEM from 4 experiments. *$P<0.05$ versus control (ANOVA).

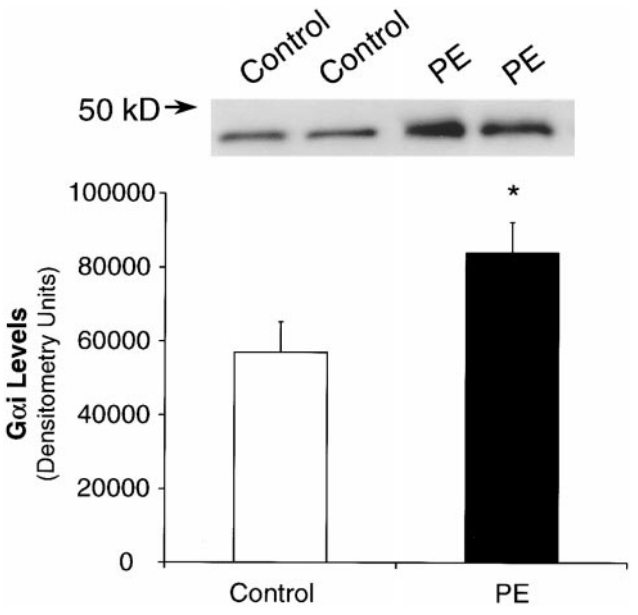

Figure 5. Myocardial Gαi content in control versus PE-treated mice. Histograms represent the mean±SEM densitometry units of scanned Gαi immunoblots with use of cardiac membranes purified from vehicle-treated control hearts and PE-treated mice. In the inset, the ~40-kd Gαi protein was visualized with 1:2500 dilution of polyclonal antibody for $G\alpha i_{1\text{-}3}$ (Santa Cruz Biotechnology, I-20). The 50-kd marker is shown. *$P<0.05$ versus control (Student's *t* test).

isolated neonatal cardiac myocytes treatment with PE or ISO leads to the activation of different nuclear factors, which results in increased myocardial protein synthesis.[14] 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 α_1ARs. To rule out this possibility, we added two controls to our experimental design. First, we used a transgenic mouse model of α_1AR-induced myocardial hypertrophy (CAM-α_{1B}AR).[15] In this model, cardiac hypertrophy develops because of the direct activation of the cardiac α_1AR signaling pathway in the absence of changes in blood pressure.[15] We observed no changes in the cardiac content of βARK1 of these transgenic animals, further strengthening our conclusion that chronic α_1AR stimulation does not induce changes in the expression of cardiac βARK1. Second, we examined βARK1 levels in isolated adult rat myocytes treated for 24 hours with ISO or PE. In these studies, we demonstrated conclusively that only the direct action of βAR stimulation causes enhanced βARK1 expression, because α_1AR signaling did not alter myocyte βARK1 levels.

βARK1 upregulation was observed in several pathophysiological conditions, such as heart failure,[9] ischemia,[7] hypertension,[8] and myocardial hypertrophy.[4] All those conditions are characterized by adrenergic activation,[10,11] by early impairment of the βAR signaling pathway,[3,19–21] and by the eventual development of heart failure.[22,23] Thus, the results of the present study add new information to this scenario, showing that the chronic and selective activation of βARs results in the worsening of βAR signaling through the increase in myocardial βARK1 levels and activity. We have demonstrated that the increase in βARK1 expression arises independently from the general development of cardiac hypertrophy. Thus, βARK1 appears to play a pivotal role in the regulation of contractility in the heart, and the selective and chronic activation of βARs is the mechanism that triggers βARK1 upregulation in apparently all conditions where the SNS is activated.

In ISO-induced hypertrophy, increased βARK1 expression and activity resulted in the impairment of βAR signaling. This result confirms the central role of βARK1 in the regulation of βAR signaling observed in transgenic mice overexpressing βARK1 or a βARK1 peptide inhibitor and also in βARK1 heterozygous knockout animals.[5,6] The absence of the upregulation of βARK1 in PE-treated mice can explain why the βAR responsiveness in these mice was not blunted as in the ISO-treated group. Baseline membrane adenylyl cyclase activity, however, was reduced in PE-treated animals, which suggests that changes are occurring in myocardial adenylyl cyclase signaling. In rat models of hypertension and hypertrophy an increase in the myocardial Gαi content was observed.[3,24] 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[4] 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 α_1ARs. 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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