Level of β-Adrenergic Receptor Kinase 1 Inhibition Determines Degree of Cardiac Dysfunction After Chronic Pressure Overload –Induced Heart Failure

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Background—Heart failure is characterized by abnormalities in β-adrenergic receptor (βAR) signaling, including increased level of myocardial βAR kinase 1 (βARK1). Our previous studies have shown that inhibition of βARK1 with the use of the Gβγ sequestering peptide of βARK1 (βARKct) can prevent cardiac dysfunction in models of heart failure. Because inhibition of βARK activity is pivotal for amelioration of cardiac dysfunction, we investigated whether the level of βARK1 inhibition correlates with the degree of heart failure.

Methods and Results—Transgenic (TG) mice with varying degrees of cardiac-specific expression of βARKct peptide underwent transverse aortic constriction (TAC) for 12 weeks. Cardiac function was assessed by serial echocardiography in conscious mice, and the level of myocardial βARKct protein was quantified at termination of the study. TG mice showed a positive linear relationship between the level of βARKct protein expression and fractional shortening at 12 weeks after TAC. TG mice with low βARKct expression developed severe heart failure, whereas mice with high βARKct expression showed significantly less cardiac deterioration than wild-type (WT) mice. Importantly, mice with a high level of βARKct expression had preserved isoproterenol-stimulated adenylyl cyclase activity and normal βAR densities in the cardiac membranes. In contrast, mice with low expression of the transgene had marked abnormalities in βAR function, similar to the WT mice.

Conclusions—These data show that the level of βARK1 inhibition determines the degree to which cardiac function can be preserved in response to pressure overload and has important therapeutic implications when βARK1 inhibition is considered as a molecular target. **(*Circulation*. 2005;111:591-597.)**

Key Words: receptors, adrenergic, beta ■ heart failure ■ signal transduction ■ mice, transgenic ■ gene therapy

Catecholamine-stimulated β-adrenergic receptor (βAR) signaling is one of the most powerful regulators of cardiac function. Abnormalities in βAR signaling are a prominent characteristic of failing hearts and may contribute to the progressive deterioration in cardiac function.[1,2] Chronic stimulation of βARs in conditions of heart failure due to high levels of circulating catecholamines leads to desensitization and impaired βAR responsiveness, in part as a result of increased levels of βAR kinase 1 (βARK1) (also known as GRK2).[3] Stimulation of βAR by catecholamines leads to dissociation of heterotrimeric G protein into Gα and Gβγ subunits. βARK1 is recruited to the plasma membrane through its interaction with dissociated membrane-bound Gβγ subunits and phosphorylates the agonist-occupied receptor. β-Arrestin binds to the phosphorylated βAR and sterically interdicts further coupling of the receptor with Gsα subunit, leading to decreased βAR signaling.[4,5] Thus, phosphorylation of receptor by βARK1 is a critical step in regulating βAR function and is consistent with in vivo studies showing that βARK1 is a critical modulator of cardiac function.[6]

A peptide inhibitor of βARK1, βARKct is composed of the last 194 amino acids of βARK1 and contains the binding site for Gβγ subunits. Overexpression of βARKct peptide sequesters the dissociated Gβγ subunits of heterotrimeric G proteins, leading to the inhibition of βARK1 recruitment to the membrane.[6] Our previous studies have shown that inhibition of βARK1 has an important role in the pathophysiology of heart failure with the use of genetically engineered mouse models of heart failure, such as the muscle *lim* protein–knockout[7] model and cardiac-specific overexpression of calsequestrin.[8] Overexpression of βARKct led to less deterioration in cardiac function and prolonged survival in these genetic models of heart failure.[8] Similarly, βARKct

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expression through adenoviral gene delivery in experimental models of myocardial infarction significantly delayed the development of heart failure.[9] Importantly, recent experimental data support the concept that normalizing cardiac βAR function leads to improved in vivo cardiac function in conditions of chronic pressure overload.[2] Because the levels of myocardial βARK1 have been shown to be elevated in several cardiovascular disorders, including myocardial hypertrophy,[10] ischemia,[11] hypertension,[12] and heart failure,[13] βARK1 inhibition is a potential novel therapeutic strategy for conditions accompanied by marked ventricular dysfunction.

Although beneficial effects of βARKct have been demonstrated, it is not known whether the level of βARK1 inhibition correlates directly with the degree of heart failure or its amelioration. In the present study, we tested whether there is a gene-dosage effect of βARKct on preservation of cardiac function in heart failure by monitoring cardiac function in βARKct transgenic (TG) mice with varying levels of transgene expression that underwent pressure overload–induced heart failure.

Methods

Experimental Animals

TG mice overexpressing βARKct peptide were generated as previously described.[6] Briefly, the coding sequence for the last 194 aa of the bovine βARK1 (βARK1ct) was fused to the α-myosin heavy-chain promoter. βARKct TG and wild-type (WT) littermate mice of either sex and 3 months of age were used for this study. Animals were handled according to the approved protocols and animal welfare regulations of the institutional review board at Duke University Medical Center.

Echocardiography

Echocardiography was performed on conscious mice with an HDI 5000 echocardiograph as previously described.[7]

In Vivo Pressure Overload

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg), and TAC was performed as previously described.[2,14] Twelve weeks after surgery, the transstenotic pressure gradient (TSPG) was assessed by recording simultaneous measurements of right carotid and left axillary arterial pressures.

βAR Density and Adenylyl Cyclase Activity

Membrane fractions were prepared as previously described.[2,14] Twenty-five micrograms of the membrane fraction was used to perform receptor binding with the use of 250 pmol/L of [^{125}I]cyanopindolol.[2,14] Receptor density (fmol) was normalized to milligrams of membrane protein. Adenylyl cyclase assays were performed with the use of 20 μg of the membrane fraction. Generated cAMP was quantified with a liquid scintillation counter (MINAXIβ-4000).[2,14]

Immunoblotting

Immunodetection of myocardial levels of βARK1 and βARKct was performed on cytosolic extracts with a GRK2 antibody (Santa Cruz Biotechnology) after 12 weeks of TAC. Detection was performed with the use of enhanced chemiluminescence (ECL, Amersham), and the bands were quantified with the use of Bio-Rad Flouro-S Multimage software. βARK1 and βARKct values were normalized to actin (Santa Cruz Biotechnology) as a loading control. Reproducibility was confirmed by loading the same concentration of protein from each heart on multiple gels.

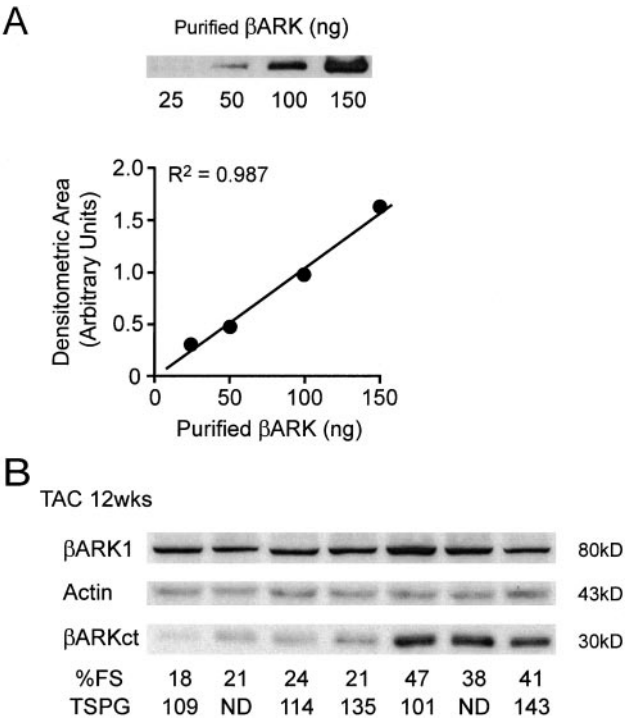

Figure 1. Quantification of βARK and βARKct protein levels in TG βARKct mice. A, Different amounts of purified βARK (25, 50, 100, and 150 ng) were loaded onto SDS-PAGE gel, and Western blots were quantified by densitometry. Positive linear correlation was observed between purified βARK and densitometry. B, Representative Western blot in TG βARKct mice after 12 weeks of TAC, showing variability of βARK expression and correlation with percent fractional shortening (%FS). ND indicates not determined.

Statistical Analysis

Data are expressed as mean±SEM. Two-way repeated-measures ANOVA was used to evaluate the echo measurements for analysis of cardiac function after TAC. Post hoc analysis was performed with Newman-Keuls test. Multigroup comparisons were made with 1-way ANOVA and Tukey test. For all analyses, a value of $P<0.05$ was considered significant.

Results

Quantification of Myocardial βARK1 and βARKct Protein

We generated standard curves for the level of βARK1 protein by loading different amounts of purified βARK1 (25 to 150 ng) onto a 10% SDS-PAGE gel and then quantified the amount of βARK protein on the Western immunoblot by densitometry using Bio-Rad Flouro-S Multimage software for analysis (Figure 1A). There was a highly linear and reproducible relationship ($R^2=0.987$) between the quantified band and the amount of purified βARK1 (Figure 1A). We then quantified the level of myocardial βARK1 and βARKct protein expression from the standard curves. Interestingly, we found that the level of βARKct protein in the cytosol of pressure-overloaded βARKct TG mice was quite variable (Figure 1B). Importantly, the highest levels of βARKct expression were associated with the greatest percent fractional shortening despite having similar TSPG and βARK1 levels (Figure 1B).

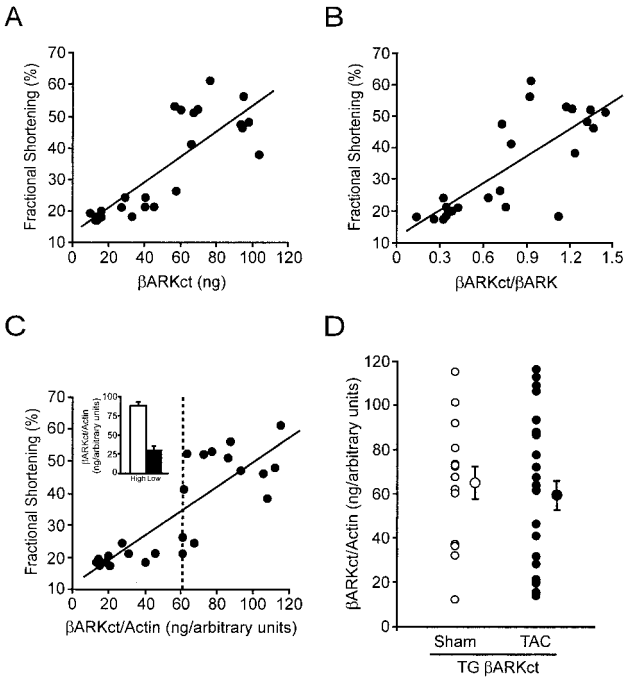

Figure 2. Level of βARKct protein expression correlates directly with degree of cardiac dysfunction after 12 weeks of TAC. A, Positive linear correlation is seen between βARKct and percent fractional shortening (R^2=0.683) (n=24). B, Positive linear correlation exists between βARKct/βARK and percent fractional shortening (R^2=0.598) (n=24). C, Normalized βARKct values with actin (βARKct/Actin) correlate with percent fractional shortening (R^2=0.695) (n=24). TG βARKct mice were divided into high- and low-expression groups by a median value (61.7 ng/arbitrary units) of βARKct/actin ratio (βARKct/actin [ng/arbitrary units]: inset, 95.7±5.3 for high expression, 35.2±5.9 for low expression). Moreover, there was a significant intergroup difference when the TG βARKct mice were divided into high, medium, and low βARKct expression (P<0.0005, 1-factor ANOVA). D, No difference of variability on βARKct/actin expression was observed in sham-operated (n=14) and TAC TG βARKct (n=24) mice (βARKct/actin [ng/arbitrary units]: 65.2±7.6 for sham, 59.4±7.0 for TAC).

We next determined whether the level of βARK1 inhibition correlated directly with the degree of cardiac dysfunction by quantifying the level of myocardial βARKct protein expression and the associated percent fractional shortening in 24 TG mice after 12 weeks of TAC. TG βARKct mice showed a positive linear relationship between the level of βARKct protein expression and percent fractional shortening (Figure 2A), as well as when βARKct expression was normalized to the level of βARK1 (Figure 2B) or actin (Figure 2C). On the basis of βARKct transgene expression, mice were divided into high- and low-expression groups by a median of βARKct/actin ratio of 61.7 ng/arbitrary units (Figure 2C).

To exclude the possibility that cardiac remodeling by pressure overload might alter the level of βARKct protein expression, we measured βARKct levels in sham-operated mice and compared them with levels in the 12-week TAC mice. Importantly, we found a variability in the level of βARKct transgene expression in sham mice similar to that found in the TAC-operated animals (Figure 2D). In addition, Southern blotting confirmed that high and low βARKct expression was due to high and low copy numbers of the transgene (data not shown). These data show that the variability in βARKct expression occurred in both sham and TAC-operated mice and that the induction of heart failure by pressure overload did not alter the expression of βARKct transgene.

Level of βARKct Transgene Expression Directly Regulates In Vivo Cardiac Function in Response to Chronic Pressure Overload

We plotted fractional shortening against the range of TSPG in the low- and high-expressing βARKct mice after 12 weeks of banding. Fractional shortening between high- and low-expressing βARKct TG mice was clearly different even across a wide range of TSPG (Figure 3A), showing that mice with higher expression of βARKct had better cardiac function even after 12 weeks of chronic TAC. As expected, mean TSPG between the high- and low-expressing βARKct groups was not significantly different and spread over a broad range of pressures (Table and Figure 3A).

Because equivalence of pressure gradients does not necessarily mean that the average area of stenosis was the same across the groups, we calculated stenotic area across the transverse aorta by approximating stroke volume on the basis of the echocardiographic parameters of chamber size[15] and by applying the Gorlin equation.[16] TAC stenotic area for the low βARKct expressors was 0.4±0.2 mm^2, and that for the high βARKct expressors was 0.2±0.3 mm^2. In addition, pressure proximal to the transverse aortic stenosis was measured and found to be similar for the 3 groups (proximal pressure=170±10 mm Hg for WT, 190±9 mm Hg for high βARKct expressors, 173±12 mm Hg for low βARKct expressors; P=NS for any of the groups). These data support our assessment that the stenotic area and the load on the left ventricles (LV) were similar across the 3 groups.

To determine the time course for the development of heart failure, we monitored cardiac function in TG βARKct and WT littermate control mice by serial echocardiography at 4, 8, and 12 weeks after TAC. Echocardiography showed progressive LV enlargement and deterioration in cardiac function in the WT and TG mice with low level of βARKct expression (Figure 3B). These mice showed a 70% reduction in percent fractional shortening, a 270% increase in LV end-systolic dimension, and a 70% increase in LV end-diastolic dimension at 12 weeks after TAC compared with their basal measurements (Figures 3B and 4, Table). In contrast, TG mice with a high level of βARKct expression showed a significant preservation of cardiac function over the same period after chronic pressure overload, with only mild increases in LV end-systolic dimension or LV end-diastolic dimension (Figures 3B and 4, Table). Interestingly, chronic pressure overload led to a thinning of the septal and posterior myocardial walls in the WT and low-expressing βARKct mice, whereas wall thickness was increased in the high-expressing βARKct mice (Figure 3B and Table). At 12 weeks after TAC, the increase in the ratio of LV weight to body weight was significantly blunted in high-expression βARKct mice compared with WT and low-expression βARKct mice

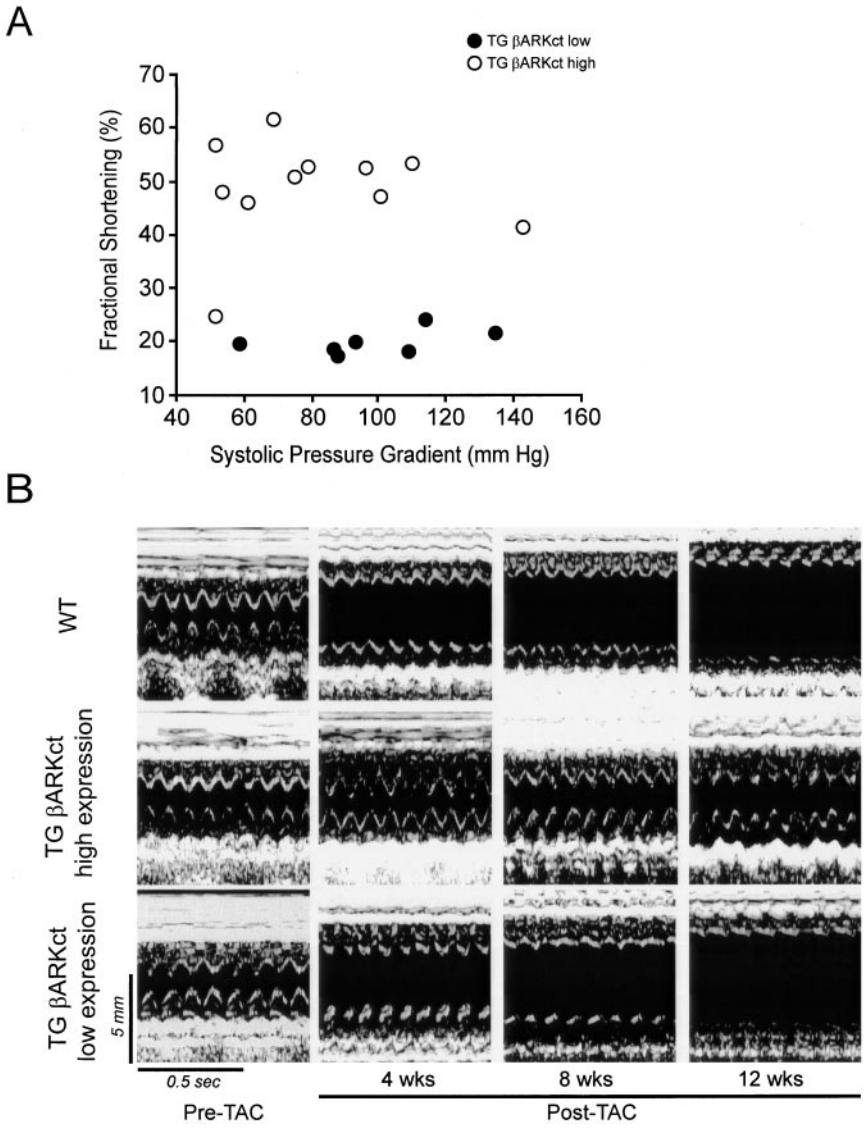

Figure 3. A, Fractional shortening was plotted against the systolic pressure gradient between high- (n=11) and low-expressing (n=7) βARKct mice. B, Representative serial M-mode echocardiography in conscious WT and TG βARKct mice measured before TAC and 4, 8, and 12 weeks after TAC.

(Table). These morphometric and echocardiographic data show that a high level of βARKct protein expression in hearts of TG mice results in a significant blunting in heart size and mass and is associated with preservation of cardiac function in response to chronic pressure overload in vivo.

βAR Signaling in WT and βARKct Mice Under Conditions of Chronic Banding

Because previous studies have shown that βARK1 inhibition leads to preservation of βAR function,[6] we determined whether inhibition of βARK1 through cardiac-specific overexpression of βARKct could normalize βAR function in the mice with a high level of βARKct expression under conditions of chronic pressure overload–induced heart failure. βAR levels and receptor-effector coupling were evaluated in membrane fractions from the WT and TG βARKct mice after 12 weeks of banding. βAR density was significantly reduced by ≈35% in the hearts of the WT and low-expressing βARKct mice on 12 weeks of banding (Figure 5A). In contrast, we found no significant decrease in receptor density after 12 weeks of TAC in the TG mice with high levels of βARKct expression (Figure 5A).

Receptor-effector coupling was assessed by adenylyl cyclase activity from the membrane fractions of sham and chronic banded WT and TG βARKct mice. Hearts from WT and low-expression βARKct banded mice showed significant desensitization, as measured by markedly diminished isoproterenol-stimulated membrane adenylyl cyclase activity (Figure 5B). Importantly, however, we found that isoproterenol-stimulated membrane adenylyl cyclase activity was preserved in the TG mice with high βARKct expression (Figure 5B). Taken together, these studies show that high levels of βARKct protein expression in the heart lead to normalization of βAR density and isoproterenol-stimulated adenylyl cyclase activity, indicating preservation of βAR–G protein coupling after chronic banding.

Discussion

In this study we demonstrate that inhibition of βARK1 through cardiac-specific overexpression of the βARKct peptide amelio-

Physiological Parameters in WT and TG βARKct Mice

	WT (n=10)		TGβARKct High-Expression (n=12)		TGβARKct Low-Expression (n=12)	
	Before TAC	12 wk After TAC	Before TAC	12 wk After TAC	Before TAC	12 wk After TAC
Heart rate, bpm	677±14	602±14*	648±16	645±12	637±10	543±16*
LVEDD, mm	3.24±0.06	5.47±0.41*	3.14±0.07	3.46±0.14†	3.21±0.07	5.56±0.17*
LVESD, mm	1.18±0.08	4.43±0.46*	1.13±0.03	1.86±0.18†	1.26±0.07	4.45±0.15*
%FS	64±2	21±3*	64±1	48±3*†	61±2	20±1*
Septal wall thickness, mm	0.77±0.02	0.79±0.08	0.76±0.03	1.17±0.07*†	0.80±0.02	0.72±0.03
Posterior wall thickness, mm	0.79±0.03	0.79±0.08	0.78±0.03	1.09±0.07	0.78±0.02	0.72±0.04
Mean Vcfc, cir/s	3.95±0.16	1.31±0.02*	4.17±0.14	3.06±0.15*†	4.02±0.16	1.33±0.08*
Body weight, g	26.7±1.4	27.7±1.3	29.4±1.2	31.5±1.5	29.4±1.2	24.7±1.1*
LV weight/body weight, mg/g	...	9.03±0.49	...	5.87±0.35‡	...	7.31±0.30
LV weight/tibial length, mg/mm	...	12.86±0.56	...	9.10±0.39‡	...	11.42±0.40
TSPG, mm Hg	...	83.3±5.2	...	81.1±8.3	...	97.9±7.0

LVEDD indicates LV end-diastolic dimension; LVESD, LV end-systolic dimension; %FS, percent fractional shortening, calculated as (LVEDD−LVESD)×100/LVEDD; Mean Vcfc, heart rate−corrected mean velocity of circumferential fiber shortening, calculated as FS divided by ejection time multiplied by the square root of the R-R interval; cir, circumference; and TSPG, transstenotic systolic pressure gradient, measured as the difference between right carotid and left axillary artery systolic pressures.

*$P<0.001$ vs before TAC in same group; †$P<0.001$, βARKct high-expression vs WT or βARKct low-expression group at same point; ‡$P<0.05$, βARKct high-expression vs either WT or βARKct low-expression group.

rates the development of cardiac dysfunction under conditions of chronic in vivo pressure overload. This amelioration of cardiac dysfunction seems to depend primarily on the level of βARKct protein expression in the TG mice. TG mice with high βARKct expression showed preserved isoproterenol-stimulated adenylyl cyclase activity and normal βAR density after 12 weeks of chronic banding. In contrast, WT and TG mice with low βARKct expression showed marked abnormalities in βAR

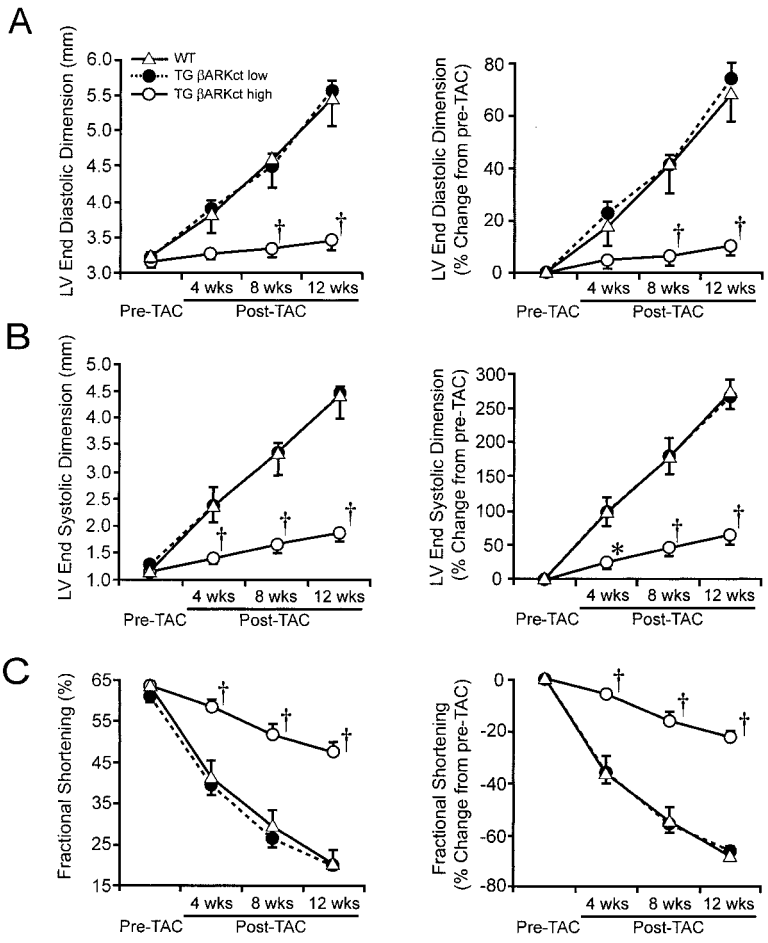

Figure 4. High expression of the βARKct transgene significantly ameliorated the development of cardiac dysfunction after chronic pressure overload–induced heart failure. Shown are LV end-diastolic dimension (A), LV end-systolic dimension (B), and percent fractional shortening (C) in WT (n=10), high-expression (n=12), and low-expression (n=12) TG βARKct mice measured by serial echocardiography at indicated time intervals after TAC. *$P<0.01$, †$P<0.001$ for high-expression βARKct mice vs WT or low-expression mice.

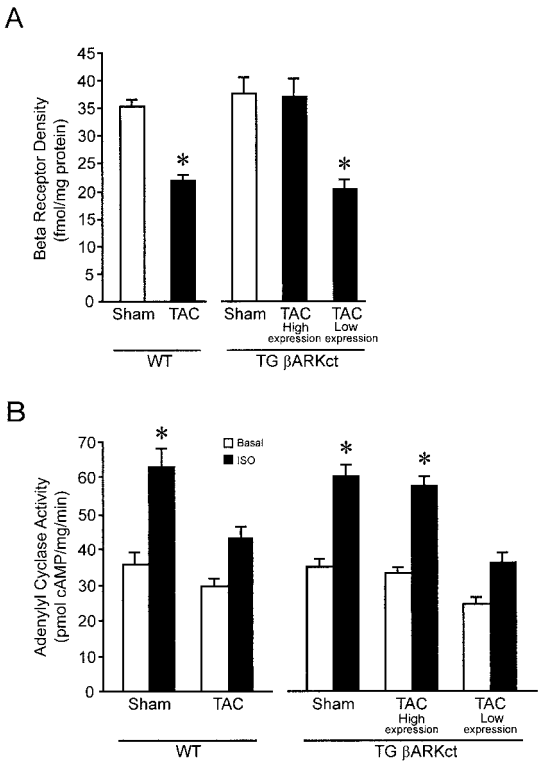

Figure 5. Inhibition of βARK by overexpression of the βARKct peptide prevents βAR dysfunction on 12 weeks of banding. A, βAR density of WT and TG βARKct mice. Shown are WT sham (n=5), WT TAC (n=6), TG βARKct sham (n=4), and TG βARKct TAC mice with high expression (n=6) and low expression (n=7) of transgene; *$P<0.01$. B, Adenylyl cyclase activity in membrane fractions from WT and TG βARKct mice. Shown are WT sham (n=6), WT TAC (n=6), TG βARKct sham (n=6), and TG βARKct-TAC mice with high expression (n=6) and low expression (n=4) of transgene; *$P<0.001$. ISO indicates isoproterenol.

function, similar to that described in human heart failure.[1,17] Strikingly, the extent of βARK1 inhibition, assessed by the level of βARKct protein expression, correlated positively with the attenuation of cardiac dysfunction after 12 weeks of chronic pressure overload. Thus, βARK1 as a therapeutic target would have immense clinical implications because βARK1 levels are increased in several cardiovascular disorders, including myocardial hypertrophy,[10] myocardial ischemia,[11] hypertension,[12] and heart failure.[13]

The most likely mechanism for βARKct in preventing βAR abnormalities and cardiac dysfunction is that the transgene sequesters Gβγ subunits and inhibits receptor phosphorylation by blocking the recruitment of βARK1 to the agonist-stimulated receptor. Our present study shows that overexpression of the βARKct transgene effectively ameliorates heart failure in conditions of a molar ratio of 3:1 for βARKct to βARK. Previous studies have shown that βARK1 plays a critical role in the pathophysiology of heart failure, and inhibition of βARK1 with the use of βARKct in mouse models of heart failure has been shown to be beneficial.[7,8,18] The mouse models of heart failure that have been rescued through βARKct include muscle *lim* protein knockout,[7] cardiac overexpression of calsequestrin,[8] and mice that developed hypertrophic cardiomyopathy.[18] In all 3 models of heart failure, mice containing cardiac-specific overexpression of the βARKct showed less deterioration of cardiac function, and the cardiac overexpression of calsequestrin/βARKct mice even exhibited an increase in mean survival age.[8] Interestingly, overexpression of βARKct did not prevent the development of heart failure in all genetically modified mice, particularly in those in which βARK1 was not elevated, such as the TG Gαq mice.[19,20] Although these studies suggest that an increase in βARK1 level is important for βARKct to have an effect on cardiac function, we show in this study that the level of βARKct expression is far more important than the upregulation of βARK1 in determining response to pressure overload. This point is particularly salient because in our study the level of βARK1 did not change significantly among the banded TG hearts.

We show that βARKct transgene expression among littermates is variable and is probably due to the number of copies of the transgene in the genome. At the time of generation of TG mice, the integration of the transgene occurs at different loci across the genome as well as in multiple copies at any given loci.[21] Therefore, segregation of the number of integrated copies of the transgene among the littermates likely accounts for the variable amounts of the expressed protein in the progeny. One of the standard procedures used to overcome variable transgene expression would be to backcross selected progeny for numerous generations to get uniform expression. Although the littermates have varying levels of transgene expression, this procedure has allowed us to directly correlate the effect of transgene expression with amelioration of heart failure.

Our data in the present study support our hypothesis that a critical determinant for the preservation of cardiac function by βARKct is normalization of βAR function in conditions of chronic overload. This is consistent with our recent data showing that preventing downregulation and desensitization of βARs through a mechanism of receptor-localized phosphatidylinositol 3–kinase inhibition also ameliorates the development of heart failure.[2] Because multiple pathways are involved in development of heart failure, other possible complementary mechanisms for the beneficial effects of βARKct transgene must be considered as well. It is possible that inhibition of other Gβγ-mediated signaling events such as activation of phosphatidylinositol 3–kinase[2,22,23] and $I_{K,Ach}$ channels[24] may contribute to the mechanism of action of βARKct in heart failure. In addition, it is possible that overexpression of βARKct inhibits the phosphorylation of other G protein–coupled receptors, such as endothelin and angiotensin receptors, although this would lead to enhanced signaling of the receptor systems.

As discussed earlier, βARK-mediated phosphorylation of βARs leads to their internalization through a variety of mechanisms, including classic clathrin-coated, pit-mediated processes, caveolae, and noncoated pit mechanisms as well.[25–28] It has been observed recently that the agonist-promoted internalization of βARs can lead to the assembly of complex signaling cascades that activate cellular growth pathways, such as the various mitogen-activated protein kinase pathways.[29–31] We postulate that one of the mechanisms for the beneficial effects of βARKct overexpression is by inhibiting the activation of these maladaptive growth pathways of the cardiac myocyte.[32]

Our previous studies have shown that TG mice develop cardiac hypertrophy to an extent similar to that of the WT littermate controls after 7 days of banding, indicating that the βARKct transgene does not alter the development of cardiac hypertrophy.[10] These data support our hypothesis that the devel-

opment of cardiac hypertrophy is not sufficient to preserve cardiac function under conditions of pressure overload; rather, normalization of detrimental signaling pathways such as the βAR pathway may be the critical determinant.[14,33]

In summary, we demonstrate that inhibition of βARK1 leads to preservation of βAR function and attenuates deterioration of cardiac function under conditions of chronic pressure overload in vivo. Importantly, the level of βARK1 inhibition determines the degree of cardiac preservation, consistent with a gene-dosage effect by βARKct on the pathological phenotype. Although multiple mechanisms are likely to be involved in the development of heart failure, we show here that inhibition of βARK1 is a novel molecular therapeutic target sufficient to prevent cardiac dysfunction. These findings have important clinical implications in developing future therapeutic strategies for heart failure.

Acknowledgments

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Disclosure

Dr Lefkowitz is a founding scientist of and Dr Rockman is a consultant for Norak Biosciences, Inc, a company that is developing drugs that inhibit GRK2.

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