Bitransgenesis with β_2-Adrenergic Receptors or Adenylyl Cyclase Fails to Improve β_1-Adrenergic Receptor Cardiomyopathy

Natalia Petrashevskaya, Ph.D.[1], Brigitte R. Gaume, Ph.D.[1], Kathryn A. Mihlbachler, B.S.[1], Gerald W. Dorn II, M.D.[2], and Stephen B. Liggett, M.D.[1]

Abstract

Cardiomyopathic effects of β-adrenergic receptor (βAR) signaling are primarily due to the β_1AR subtype. β_1/β_2AR and β_1/adenylyl cyclase type 5 (AC5) bitransgenic mice were created to test the hypothesis that β_2AR or AC5 co-overexpression has beneficial effects in β_1AR-mediated cardiomyopathy. In young mice, β_1/β_2 hearts had a greater increase in basal and isoproterenol-stimulated contractility compared to β_1/AC5 and β_1AR hearts. By 6 months, β_1AR and β_1/β_2 hearts retained elevated basal contractility but were unresponsive to agonist. In contrast, β_1/AC5 hearts maintained a small degree of agonist responsiveness, which may be due to a lack of β_1AR downregulation that was noted in β_1- and β_1/β_2 hearts. However, by 9 -months, β_1, β_1/β_2, and β_1/AC5 mice had all developed severely depressed fractional shortening *in vivo* and little response to agonist. p38 mitogen activated protein kinase (MAPK) was minimally activated by β_1AR, but was markedly enhanced in the bitransgenics. Akt activation was only found with the bitransgenics. The small increase in cystosolic second mitochondria-derived activator of caspase (Smac), indicative of apoptosis in 9-month β_1AR hearts, was suppressed in β_1/AC5, but not in β_1/β_2, hearts. Taken together, the unique signaling effects of enhanced β_2AR and AC5, which have the potential to afford benefit in heart failure, failed to salvage ventricular function in β_1AR-mediated cardiomyopathy.

Keywords: heart failure, β-adrenergic receptors, adenylyl cyclase, apoptosis, transgenes

Introduction

Heart failure from virtually every etiology is accompanied by enhanced sympathetic activity, an adaptation in response to decreased cardiac output. While this response is effective in increasing contractility during acute decompensation, prolonged activation is deleterious, leading to worsening failure.[1,2] Both β_1-adrenergic receptors (β_1AR) and β_2AR are expressed on cardiomyocytes and participate in catecholamine-mediated enhancement of cardiac inotropy or chronotropy. The deleterious effects of catecholamine signaling at the cardiomyocytes have generally been attributed to their activation of the β_1AR. Indeed, we and others have shown that moderate overexpression of β_1AR in cardiomyocytes of transgenic mice results in a time-dependent heart failure,[3–5] while β_2AR expression at similar levels is well tolerated.[6] This difference in the propensity to evoke failure is not readily reconciled with the enhanced contractility observed in young transgenic overexpressing mice, as the degree of increased contractility is similar in β_1- and β_2AR-overexpressing mice.[3,6] Nor is it altogether apparent that the pathogenic effects of β_1AR activation are entirely due to cAMP/protein kinase A (PKA) activation; cardiac adenylyl cyclase type 5 (AC5)-overexpressing mice do not develop failure, yet have levels of (elevated) AC activities similar to those of young β_1AR-overexpressing transgenic mice.[3,7] It has been postulated that intrinsic differences between β_1AR and β_2AR signaling accounts for the more pathogenic nature of β_1AR.[1] And furthermore, certain properties of the β_2AR subtype may be "protective" in heart failure.[1] These properties include coupling to the inhibitory G-protein $G_{\alpha i}$, signaling to antiapoptotic pathways, and receptor/cAMP microdomain localization. In addition to such potential distinct signaling events evoked by the two subtypes in cardiomyocytes, the heart failure milieu also includes stimuli (elevated catecholamines) for desensitization and downregulation of βAR. And indeed, the β_1- and β_2AR vary in a number of ways in regard to agonist-promoted desensitization and trafficking.[1] Taken together, these differences have suggested that β_2AR activation might mitigate against β_1AR-mediated heart failure, and that stabilizing, activating, or mimicking the signaling of this subtype might have therapeutic potential.[8] Similarly, AC5/6 levels are reduced in β_1AR-mediated cardiomyopathy,[9] and methods to replace, or overexpress, AC5 or AC6 have been considered as therapeutic interventions.[10] While AC6 overexpression has "rescued" certain forms of left ventricular dysfunction from genetic manipulation,[11] such an approach has not been taken with a model of transgenic overexpression of β_1AR, which leads to a time-dependent heart failure. To investigate these two potential avenues for altering β_1AR-mediated cardiomyopathy, we utilized overexpressing transgenic mice that we have previously developed to create β_1/β_2AR and β_1/AC5 bitransgenic mice, which were compared to β_1AR-overexpressing mice over a 9-month time period for physiologic or biochemical modification of the β_1AR phenotype.

Materials and Methods

Transgenic mice

Transgenic mice overexpressing the human β_1AR (the most common variant, Arg389), β_2AR, and AC5 were generated using the α-myosin heavy chain (α-MHC) promoter to target expression to cardiomyocytes, and have each been previously described.[7,9,12] All mice were of the FVB/N background. Heterozygous β_1AR transgenics were mated with heterozygous β_2AR or AC5 transgenics to create the bitransgenic mice, which are denoted as β_1/β_2AR and β_1/AC5 mice. Genomic DNA from tail-cuts was screened for the presence of transgenes by targeted PCRs, which included one primer in the α-MHC promoter and one in the cDNA of transgene. Mice were fed a normal diet and maintained under identical conditions, and either sex was studied.

Physiologic studies

The studies were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. A

[1]Cardiopulmonary Genomics Program, University of Maryland School of Medicine, Baltimore, Maryland, USA; [2]Center for Pharmacogenomics, Washington University School of Medicine, St. Louis, Missouri, USA.
Correspondence: SB Liggett (sligg001@umaryland.edu)
DOI: 10.1111/j.1752-8062.2008.00061.x

hemodynamic evaluation was performed, as described previously, using the work-performing mouse heart preparation.[3] Mice were anesthetized via intraperitoneal injection with 100 mg/kg sodium nembutal and 1.5 units of heparin to prevent microthrombi. The hearts were removed and attached by the aorta to a 20-gauge cannula and temporarily retrogradely perfused with oxygenated Krebs–Henseleit solution (in mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 0.5 Na-EDTA, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 glucose) saturated with 95% O_2, 5% CO_2. A polyethelene-50 catheter was inserted into the apex of the left ventricle for the measurement of intraventricular pressure. The pulmonary vein was connected to a second cannula, and antegrade perfusion with oxygenated Krebs–Henseleit solution was initiated with a basal workload of 300 mmHg × mL/min (6 mL venous return and 50 mmHg mean aortic pressure). Hearts were allowed to equilibrate for 20 minutes. Atrial pressure was monitored through a sidearm in the left atrial cannula. The left ventricular pressure signals were digitized at 1 kHz and analyzed offline by the computer software Biobench (National Instruments, Austin, TX, USA). The first positive and negative derivatives of the left intraventricular pressure curve (+dP/dt and –dP/dt) and duration of contraction and relaxation (time to peak pressure: TPP) and time to half relaxation ($TR_{1/2}$) were calculated. After establishment of baselines, infusions of the nonselective βAR agonist isoproterenol were undertaken using doses from 0.1 nM to 0.1 μM. The maximal response during the 5-minute infusion was utilized to construct the dose–response curves.

Echocardiography

Mice were sedated with isoflurane delivered by a nasal cone and secured in the supine position to a warming pad maintained at 37°C. Transthoracic echocardiography was performed with a Vev0770 echocardiograph with the 707B probe (Visualsonics, Toronto, CA, USA), as previously described.[3] The heart was imaged in the two-dimensional mode (M-mode) in the parasternal long-axis views. The measurements of intraventricular septal (IVS) thickness, left ventricular posterior wall (LVPW) thickness, and left ventricular internal diameter were made from the left ventricle in systole and diastole. The diastolic measurements were made at the time of maximal left ventricular end-diastolic dimension (LVEDD). Left ventricular end-systolic dimensions (LVESD) were performed at the time of the most anterior systolic excursion of the LVPW. Left ventricular percent fractional shortening, chamber volume, and mass were calculated using methods as previously described.[13] In some mice, after these baseline measurements were obtained, echocardiography was repeated 3 minutes after a 2-μg/g body weight intraperitoneal injection of isoproterenol was administered.

Radioligand binding and Western blots

For radioligand binding, the hearts were homogenized in 5 mM Tris, 2 mM EDTA, pH 7.4 buffer at 4°C with a Polytron for 15 seconds, diluted, and centrifuged at 400 × *g* for 10 minutes. The supernatant was recovered and centrifuged at 30,000 × *g* for 15 minutes, and the pellet was resuspended in 75 mM Tris/12 mM $MgCl_2$/2 mM EDTA pH 7.4 at 25°C. Quantitative radioligand binding was performed, as previously described,[14] using the βAR radioligand ^{125}I-cyanopindolol (^{125}I-CYP) with 1.0-μM propranolol used to define nonspecific binding. In the β_1/β_2AR mouse hearts, differentiation of the densities of the two subtypes was determined using competition with the β_1AR-specific antagonist CGP20712 and β_2AR-specific antagonist ICI118551, as previously described.[14] The results are provided as fmol/mg protein and are from six hearts from each group. Protein was determined by the copper bicinchoninic method.[15] Western blots were carried out as previously described.[3] Briefly, homogenized hearts were solubilized in 10 mM Tris and 1 mM EDTA pH 7.6 with 1% SDS. Protease inhibitor cocktail (Roche, Nutley, NJ, USA), and phosphatase inhibitor cocktails 1 and 11 (Calbiochem, San Diego, CA, USA) were included in all steps. The samples were clarified by centrifugation at 10,000 × *g*, the proteins fractionated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was carried out with antibodies (from Cell Signaling, Danvers, MA, USA) using the following titers: ERK1/2 MAPK (1:2,000), phospho-ERK1/2 MAPK(1:1,000), p38 MAPK(1:2,000), phospho-p38 MAPK (1:1,000), Akt (1:1,500), and phospho-Akt (1:1,000). Detection was by enhanced chemiluminescence (PerkinElmer, Waltham, MA, USA), and the signals were acquired directly from the membranes using a Fuji LAS-3000 charged coupled device camera and quantitated with the included software. For each blot, the ratios of phosphorylated signal to the total signal was calculated and then normalized to the mean nontransgenics (NTG) ratio.

Statistical analysis

Unpaired *t*-tests were used to compare data from the indicated groups, typically comparing results from β_1AR transgenic hearts with β_1/β_2AR and β_1/AC5 transgenics, or between time periods (2, 6, or 9 months). When dose–response studies were performed, the minimal response (R_{min}) and maximal response (R_{max}) were determined by fitting the data to a sigmoid curve using Prism (GraphPad, San Diego, CA, USA). Paired *t*-tests were utilized for radioligand binding and Western blot data as indicated. *p* values <0.05 were considered significant.

Results

Physiologic function at 2 months of age

βAR expression in 2-month-old mice as determined by ^{125}I-CYP radioligand binding was: NTG mice 31 ± 3.5 fmol/mg (~70% β_1AR), β_1AR mice 3,446 ± 352 fmol/mg (essentially all β_1AR), and β_1/AC5 mice 1,666 ± 285 fmol/mg (essentially all β_1AR; $p < 0.01$ vs. β_1AR mice). For the β_1/β_2AR mice, β_1AR expression was 2,624 ± 256 fmol/mg ($p < 0.05$ vs. β_1AR mice) and β_2AR expression was 1,350 ± 60 fmol/mg. None of the transgenic mice had heart/body weight ratios that differed from NTG mice (*Table 1*). The hearts from age- and sex-matched mice were studied using the work-performing model at baseline (the absence of agonist) and in response to the nonselective βAR agonist isoproterenol. In the hearts from

	2-month $n = 5$	6-month $n = 5$	9-month $n = 5$
NTG	3.5 ± 0.04	3.6 ± 0.1	3.8 ± 0.2*
β_1	3.5 ± 0.1	3.9 ± 0.2	5.3 ± 0.2
β_1/β_2	3.6 ± 0.1	5.1 ± 0.2*	6.7 ± 0.2*
β_1/AC5	3.6 ± 0.2	4.6 ± 0.1*	5.2 ± 0.2
β_2	3.5 ± 0.2	3.9 ± 0.1	4.2 ± 0.1*
AC5	3.6 ± 0.1	3.7 ± 0.2	3.9 ± 0.1*

*Ratio different from β_1AR transgenic hearts of the same age, $p < 0.01$.

Table 1. Heart-to-body weight ratios stratified by age and transgene.

Parameters	NTG 2m $n = 7$	β_1 2m $n = 5$	β_1/β_2 2m $n = 5$	β_1/AC5 2m $n = 5$	NTG 6m $n = 5$	β_1 6m $n = 6$	β_1/β_2 6m $n = 5$	β_1/AC5 6m $n = 5$
+dP/dt, mmHg/s	4104 ± 118*	5588 ± 217	5928 ± 408	5988 ± 356	3863 ± 85*	5718 ± 594	3021 ± 311*	4420 ± 205
–dP/dt, mmHg/s	3128 ± 169*	4639 ± 360	5608 ± 344*	4567 ± 321	2852 ± 272*	5085 ± 603	3393 ± *280	4264 ± 225*
TPP, ms/mmHg	0.37 ± 0.04*	0.29 ± 0.03	0.22 ± 0.01*	0.24 ± 0.01	0.41 ± 0.04*	0.26 ± 0.02	0.34* ± 0.03	0.28 ± 0.02*
TR1/2, ms/mmHg	0.60 ± 0.03*	0.47 ± 0.02	0.38 ± 0.02*	0.37 ± 0.03*	0.57 ± 0.03*	0.46 ± 0.03	0.49 ± 0.04	0.44 ± 0.03

*Different from β_1AR transgenic hearts at the same age, $p < 0.01$

Table 2. Cardiac contractile parameters at baseline stratified by age and transgene.

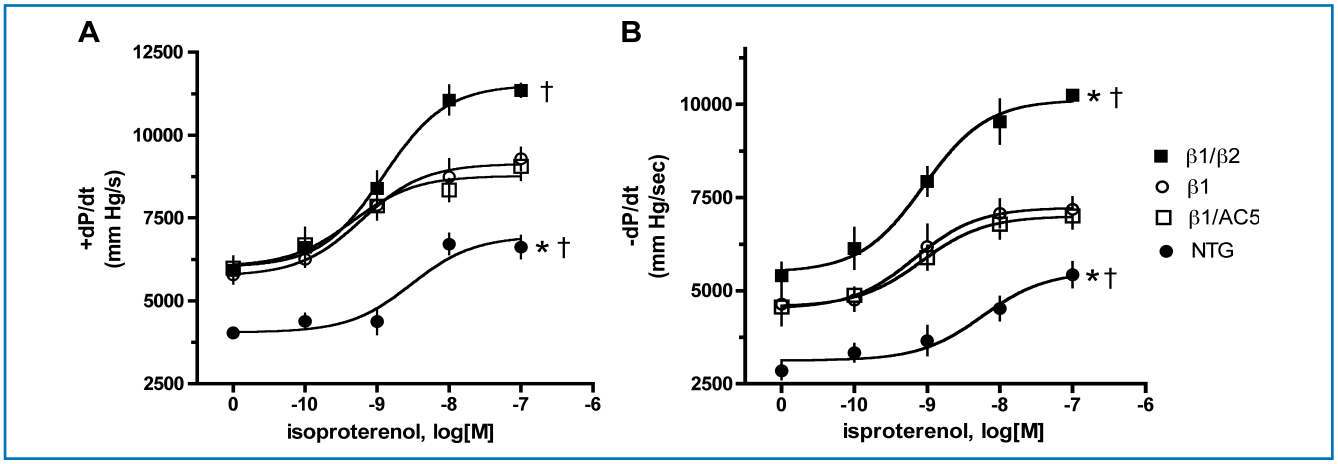

Figure 1. Agonist-promoted contractile responses in hearts from β_1AR, β_1/β_2AR, and β_1/AC5 transgenic mice at 2 months of age. Results are from 5–6 experiments performed with each of the indicated lines using the *ex vivo* work-performing model. (**A**) +dP/dt, (**B**) –dP/dt. *R_{min}, †R_{max}, different from β_1AR transgenic hearts at $p < 0.01$.

these young mice, overexpression of β_1AR resulted in an increased baseline +dP/dt and –dP/dt (*Table 2*). Co-overexpression in the β_1/β_2AR bitransgenic mice did not significantly enhance +dP/dt over β_1AR overexpression alone, nor did co-overexpression of AC5, as observed in the β_1/AC5 bitransgenic mice, enhance +dP/dt over β_1AR overexpression. However, a modest increase in baseline –dP/dt was observed in the β_1/β_2AR bitransgenic hearts compared to β_1AR hearts. This increase in –dP/dt was only found when β_1AR was co-expressed with β_2AR, and not with the β_1/AC5 bitransgenics (*Table 1*).

The contractile response to isoproterenol in these young mice is shown in *Figure 1*. While baseline +dP/dt was equivalent in β_1AR, β_1/β_2AR, and β_1/AC5 hearts at 2 months of age (as discussed above), the response to agonist was significantly greater for β_1/β_2AR hearts compared to the other two transgenics, which were not different between each other (*Figure 1A*). Thus, the co-expression of β_2AR further enhanced agonist-promoted contractility in β_1AR-overexpressing hearts, but co-overexpression of AC5 with β_1AR had no effect over β_1AR alone. For relaxation in the 2-month-old hearts, essentially the same pattern was found, except that, as reported above, the baseline –dP/dt was somewhat increased for β_1AR and β_1/AC5 hearts compared to β_1/β_2AR hearts (*Figure 1B*).

Physiologic function at 6 months of age

In these older mice, the β_1AR transgenic mice had heart/body weight ratios (*Table 1*) that trended toward being greater than those of NTG mice (3.9 ± 0.2 mg/g vs. 3.6 ± 0.1 mg/g, $p = 0.20$). However, both β_1/β_2 and β_1/AC5 hearts had increased heart-to-body weight ratios (5.1 ± 0.2 and 4.6 ± 0.1, respectively) compared to NTG hearts ($p < 0.005$) as well as β_1AR transgenics ($p < 0.005$). In these 6-month-old mice, the baseline +dP/dt remained increased over NTG in the β_1AR hearts; however, in β_1/β_2AR hearts, this effect was not observed, and indeed they did not differ from NTG (*Table 2*). β_1/AC5 mice at 6 months of age also displayed a decrease in +dP/dt compared to 2-month-old hearts (4,420 ± 205 mmHg/s vs. 5,986 ± 356 mmHg/s, $p < 0.01$), but this parameter was still slightly greater than NTG (3803 ± 85 mmHg/s, $p < 0.05$). This pattern was mimicked in regard to baseline –dP/dt.

While at 6 months of age β_1AR-overexpressing hearts maintained a somewhat increased baseline +dP/dt, as we have previously noted,[3] they were unresponsive to isoproterenol (*Figure 2A*). Similarly, β_1/β_2AR mice were unresponsive to agonist. In contrast, β_1/AC5 mice had a positive inotropic response that was similar to that of NTG, but the maximal response was depressed compared to that of the hearts from 2-month-old β_1/AC5 bitransgenic mice (6,240 ± 526 mmHg/s vs. 9,065 ± 240 mmHg/s, $p < 0.005$). For relaxation, neither β_1AR nor β_1/β_2AR hearts responded to agonists. The maximal β_1/AC5 relaxation response was greater than NTG, but essentially parallel in nature, with the difference in maximal increase attributable to the increased baseline –dP/dt. Nevertheless, the maximal isoproterenol-promoted –dP/dt in 6-month-old β_1/AC5 bitransgenics was not different from that in 2-month-old mice of the same genotype,

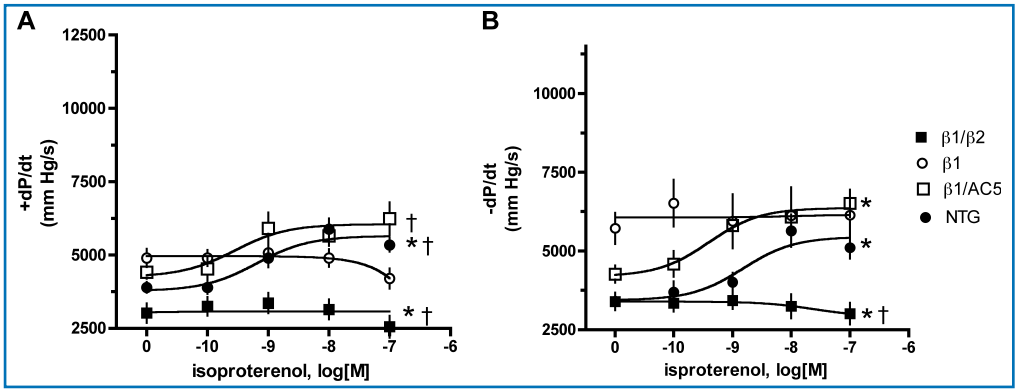

Figure 2. Agonist-promoted contractile responses in hearts from β_1AR, β_1/β_2AR, and β_1/AC5 transgenic mice at 6 months of age. Results are from 5–6 experiments performed with each of the indicated lines using the *ex vivo* work-performing model. (**A**) +dP/dt, (**B**) –dP/dt. *R_{min}, †R_{max}, different from β_1AR transgenic hearts at $p < 0.01$

nor was the change in –dP/dt from the baseline affected by age in these mice (*Figure 1B* and *2B*).

Physiologic function at 9 months of age

At 9 months of age, an increase in the heart-to-body weight ratio was apparent for the β_1AR-overexpressing hearts compared to NTG. And, the β_1/β_2 and β_1/AC5 hearts continued to have increased ratios, as was observed at 6 months. At this age, a number of the transgenic mouse hearts were unstable once they were removed and thus could not be studied by the *ex vivo* method. So, noninvasive M-mode echocardiography at rest and in response to a single subcutaneous dose of isoproterenol was carried out in the 9-month-old mice (*Table 3* and *Figure 3*). Left ventricular chamber dilatation was observed for β_1AR, β_1/β_2AR, and β_1/AC5 mice compared to NTG, readily observed in the LVEDD and LVESD measurements. Substantial increases in calculated left ventricular systolic (2-fold) and diastolic (2–5-fold) volumes were noted. As previously reported,[3] β_1AR-mediated cardiomyopathy results in markedly reduced fractional shortening compared to NTG at 9 months of age (*Table 2*). Neither co-expression of β_2AR or AC5 had any notable effect on this phenotype, and indeed β_1/β_2AR mice had the lowest baseline fractional shortening of all transgenics (11.2 ± 1.85%). Consistent with the 6-month *ex vivo* contractile studies, β_1AR and β_1/β_2AR mice at 9 months had minimal increases in fractional shortening in response to isoproterenol (*Figure 3*). However, while some contractile responsiveness was observed at 6 months with the β_1/AC5 mice, by 9 months of age, isoproterenol stimulation of fractional shortening in these mice was virtually absent (from 19 ± 1.4% at baseline to 26 ± 1.6% after isoproterenol).

Selected protein expression or activity by genotype and age

Although the primary goals of this study relate to physiologic function, we also examined expression or activity of several proteins previously identified as playing important roles in adrenergic signaling and heart failure progression. β_1AR expression decreased over time in some mice, as summarized in *Figure 4*. β_1AR expression in 6-month-old β_1AR transgenic hearts decreased compared to 2-month-old hearts (1,983 ± 203 fmol/mg vs. 3,446 ± 352 fmol/mg, $p < 0.01$). In contrast, there was no change in β_1AR expression in the β_1/AC5 bitransgenic mice over this time period (1,666 ± 248 fM/mg vs. 2,005 ± 720 fM/mg). While overall βAR expression did not change over time in the β_1/β_2 bitransgenic hearts (3,694 ± 290 fM/mg vs. 3,727 ± 235 fM/mg), the absolute levels of the two subtypes, and their ratios, clearly changed. By 6 months of age, β_1AR expression in these mice decreased to 989 ± 204 fM/mg (from 2,624 ± 256 fM/mg, $p < 0.01$), while β_2AR expression actually increased (2,746 ± 207 fM/mg at 6 months, from 1,350 ± 60 fM/mg at 2 months, $p < 0.01$). Thus, β_1AR expression decreased in β_1AR and $\beta_1/(\beta_2$AR hearts, but not β_1AR/AC5 hearts, from

Parameter	NTG* $n = 5$	β_1 $n = 9$	β_1/β_2 $n = 7$	β_1/AC5 $n = 5$
IVSd, mm	1.1 ± 0.07	0.78 ± 0.06	0.65 ± 0.04	0.83 ± 0.03
IVSs, mm	1.56 ± 0.11	1.05 ± 0.09	0.76 ± 0.15	1.11 ± 0.10
LVPDd, mm	1.3 ± 0.05	1.0 ± 0.03	0.79 ± 0.1†	0.97 ± 0.04
LVPDs, mm	1.7 ± 0.09	1.37 ± 0.15	1.17 ± 0.11	1.42 ± 0.06
LVESD, mm	2.37 ± 0.2	3.68 ± 0.09	4.91 ± 0.07	3.80 ± 0.15
LVEDD, mm	3.76 ± 0.2	4.71 ± 0.15	5.43 ± 0.02	4.63 ± 0.11
LV% fractional shortening	35 ± 3.9	19 ± 1.6	11 ± 1.85	19 ± 1.4
LVVD, µL	67 ± 11.0	128 ± 11.5	156 ± 14.8	101 ± 10
LVVS, µL	20.1 ± 5.9	84 ± 9.7	125.1 ± 14.9	78.8 ± 6.9
LVM, mg	135 ± 9.3	215 ± 11	238 ± 31	177 ± 18

*Parameters different from those of β_1AR mice at $p < 0.01$.
†Different from β_1AR mice at $p < 0.01$. LVVS, left ventricular volume (systole); LVM, left ventricular mass; LVPD, left ventricular – dimension.

Table 3. Echocardiography results in 9-month-old mice.

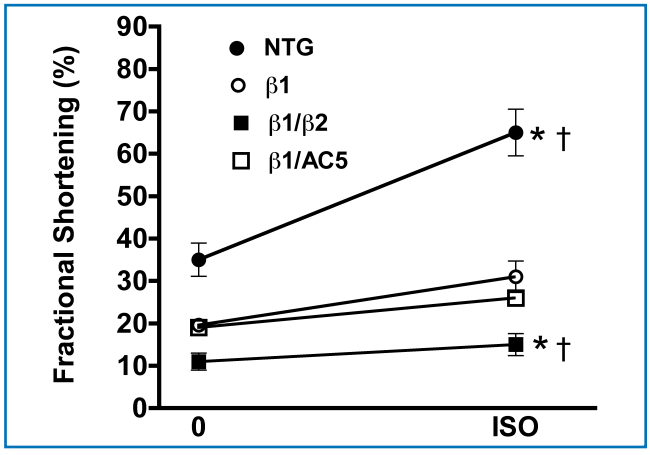

Figure 3. Baseline and agonist-stimulated fractional shortening in hearts from β_1AR, β_1/β_2AR and β_1/AC5 transgenic mice at 9 months of age. Results are from 5–9 experiments performed with each line using echocardiography in the anesthetized mouse. *Basal, †isoproterenol stimulated, LVEFs different from β_1AR transgenics at $p < 0.01$.

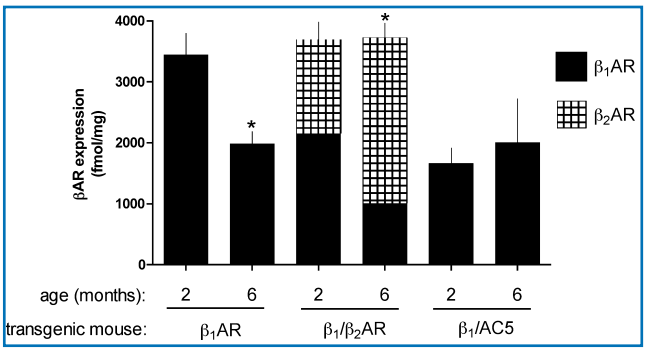

Figure 4. Age-dependent changes in β_1- and β_2AR expression in hearts from β_1AR, β_1/β_2AR, and β_1/AC5 transgenic mice. Results are from five experiments. *β_1AR expression decreased compared to 2 months of age, $p < 0.01$.

2 to 6 months. At 2 months, ERK1/2 MAPK activity was not elevated in any heart over NTG except for the β_2AR transgenic, which was utilized as a positive control (*Figure 5A*). At 6 months of age, these β_2AR-overexpressing hearts showed no enhancement of ERK1/2 MAPK activity compared to NTG (*Figure 5D*), but the β_1/β_2 hearts revealed a small increase. In young mice, β_1AR overexpression resulted in a 2-fold increase in p38 MAPK activation; co-expression of β_2AR, and AC5, with β_1AR resulted in an even more marked increase in the activity of this kinase at 2 months, and this pattern was maintained in the 6-month-old mice (*Figure 5B* and *5E*). Akt was not activated by β_1AR overexpression; however, both of the bitransgenics at 2 and 6 months of age revealed activation of Akt by 3–4-fold (*Figure 5C* and *5F*).

Finally, we measured an index of apoptosis to assess the potential for modification by co-expression. β_1AR transgenic hearts overexpressing the receptor at the levels utilized here do not exhibit overt apoptosis (such as would be detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining) at any age, including at 9 months.[3] We thus utilized a highly sensitive immunoblot assay for cytosolic second mitochondria-derived activator of caspase (Smac) (also termed direct IAp binding protein, DIABLO). In the intrinsic cell death pathway, activation of caspases is by release from the mitochondria of proapoptotic proteins such as Smac.[16] As shown in *Figure 6*, β_1AR-overexpressing hearts display a small increase in cytosolic Smac only at 9 months of age. This was also observed with β_2AR hearts, and the extent of increase in cytosolic Smac from 2 months to 9 months was the same in the bitransgenic β_1/β_2AR hearts as the single βAR transgenics. However, β_1/AC5 bitransgenics showed no evidence of enhanced Smac release, indicating a potential protective role for overexpressed AC5.

Discussion

Enhancement of cardiac contractility can be accomplished by increasing βAR signaling with transgenic overexpression of the β_1AR or β_2AR subtype, AC5, or AC6.[3,6,7,17] Early studies with these mice revealed several intriguing findings that have provided new insights into how the heart responds over time to these different interventions and the potential for new therapeutics. Two early studies, one in the C57BL/6 background[18] and one in the FVB/N background,[6] showed that overexpression of the β_2AR increased resting contractility and the response to the agonist isoproterenol, with no deleterious effects throughout the life of the animal. In addition, we showed that transgenic mice overexpressing mutated β_2AR, known to have depressed coupling in transfected cells, also displayed depressed contractility and agonist responsiveness compared to wild-type β_2AR.[6] This indicated that despite background levels of β_1- and β_2AR expression together (<100 fmol/mg), transgenic overexpression provides a model that, within limits, is useful for linking receptor signaling to physiologic function. Subsequent studies in the FVB/N background revealed that as β_2AR expression levels were increased (from ~60- to ~350-fold over background) a progressive, time-dependent cardiomyopathy developed in those with overexpression of

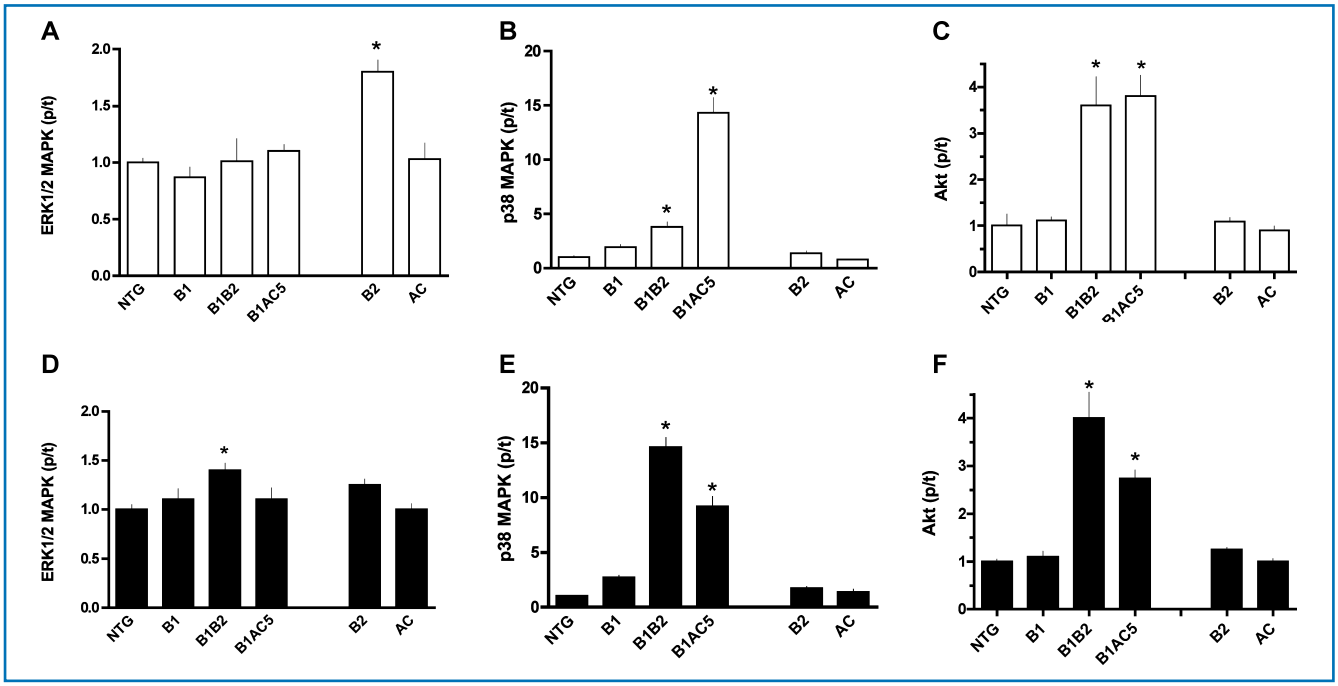

Figure 5. Alterations in cardiac ERK1/2 MAPK, p38 MAPK, and Akt presented by age and transgene. Results are plotted as the ratio of phosphorylated to total kinase expression, normalized to the mean NTG values. Results are from five experiments. *, p values of < 0.05 to < 0.01 versus β_1AR.

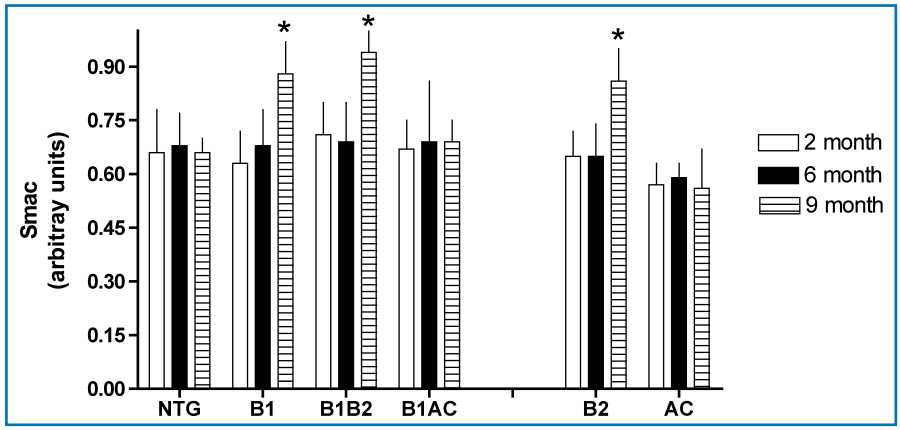

Figure 6. Alterations in the apoptosis-related expression of cardiac Smac by age and transgene. Results are from four experiments. *$p < 0.05$ versus 2-month values within each line.

approximately 100-fold or more.[19] In contrast, several reports have indicated that relatively low levels of β_1AR overexpression (5–20-fold), while initially increasing contractility and the response to agonist, ultimately result in cardiomyopathy and heart failure.[3,4] While the age of onset of ventricular failure and the upper limits of nonpathogenic expression levels differ between investigators and strains, the paradigm that modest cardiac β_2AR overexpression is well tolerated in mice, while β_1AR overexpression is not, has been generally accepted. With the mice generated in our laboratory,[3] β_1AR (the most common human allele, Arg389) overexpression at levels of 1,000–3,000 fmol/mg results in three time-dependent physiologic phases: an early (≤ 4-month-old period) enhancement of contractility and agonist response, an approximately 5–7-month-old period where agonist responsiveness is absent but fractional shortening is maintained, and a ≥ 9-month-old period where chamber dilatation, depressed fractional shortening, and heart failure are observed. Cardiac overexpression of AC5 and AC6 has also been reported.[7,17] Enhanced baseline and agonist-stimulated contractility were observed with AC5 overexpression,[7] while the AC6 overexpressors had enhanced agonist-stimulated contractility without an increase in the baseline contractility.[17] None of these AC transgenic mouse lines displayed loss of agonist responsiveness or overt cardiomyopathy with age.

These studies of single-gene transgenics prompted the development of potential therapeutic strategies for heart failure. For example, AC5 and AC6 overexpression has been reported to "rescue" ventricular function evoked by $G_{\alpha q}$ overexpression,[11,20] as has overexpression of the β_2AR.[21] It has been hypothesized that unique properties of AC5/6 or the β_2ARs, aside from their cAMP/PKA-dependent effects, may be responsible for these salutary effects. With AC6 overexpression, phospholamban expression is reduced due to enhancement of the transcriptional repressor ATF3, which binds to the phospholamban promoter.[22] This effect is not observed with isoproterenol or forskolin treatment, indicating cAMP-independent effect. The cardiac effects of altering AC5/6 expression is nevertheless still somewhat unclear, as AC5 (–/–) mice show protective effects in heart failure models. AC5 (–/–) mice have been reported to be resistant to apoptosis during chronic isoproterenol infusion, with an accompanying increase in phosphorylated Akt and Bcl2.[23] Furthermore, AC5 (–/–) mice have been shown to be largely protected from pressure overload-induced ventricular dysfunction and apoptosis.[24] The β_2AR is now recognized to have distinct properties compared to β_1AR. The β_2AR subtype has been shown to couple to G_i (after PKA-mediated receptor phosphorylation), which leads to activation of ERK1/2 MAPK, Akt and c-Src family members. β_2AR also activate proapoptotic pathways including p38 MAPK, but collectively, β_2AR signaling has been considered to be antiapoptotic. A recent study has shown that inhibition of G_i via transgenesis with a G_i-inhibitory peptide resulted in mice with greater infarct size and apoptosis during ischemia/reperfusion,[25] supporting the notion that β_2AR-G_i signaling is protective under such conditions. In addition, β_2AR when co-overexpressed with β_1AR using adenovirus vectors with isolated cardiomyocytes enhances isoproterenol-stimulated myocyte contractile responses.[26] This has been suggested to be due to heterodimerization of the two subtypes that form a distinct signaling unit. However, the physiologic effects of such co-expression in the intact heart have not been demonstrated.

In the current report, we examined the physiologic and signaling consequences of β_2AR and AC5 overexpression in the setting of β_1AR-mediated cardiomyopathy by developing β_1/β_2 and β_1/AC5 bitransgenic mice. In young mice, co-overexpression of β_2- with β_1AR resulted in enhanced agonist-promoted contraction and relaxation compared to β_1AR overexpression alone. This is consistent with the reports in isolated myocytes,[26] but as we show in the intact heart, over time, β_2AR co-overexpression does not attenuate the cardiomyopathic effects of β_1AR. In contrast, no enhancement was observed when AC5 was overexpressed with β_1AR. These findings suggest that (a) enhancement of βAR responsiveness can occur over that of β_1AR by co-expression of β_2AR, despite the fact that the latter can inhibit AC via G_i coupling, and (b) the level of AC is not the "limiting" component in βAR-mediated cardiac contraction coupling, as has been claimed by some.[17,27] In regard to the latter, additional signaling mechanisms other than via AC could also explain the enhanced agonist-stimulated inotropy of the β_1/β_2 hearts compared to that of the β_1/AC5 hearts[28]. By 6 months of age, β_1AR overexpressors maintained elevated contractility at baseline but failed to respond to agonist. β_1/β_2 hearts had a lower baseline contractility, potentially due to the increased G_i, which is known to occur with β_1AR overexpression[3] working in concert with β_2AR-G_i coupling, and also failed to respond to agonist. β_1/AC5 mice displayed an approximately 2-fold increase in agonist-promoted contraction and relaxation. The former was similar to that seen with NTG mice, and was clearly depressed compared to the 2-month response. Relaxation at 6 months in the β_1/AC5 mice was not significantly altered compared to young hearts. We have previously shown that AC5/6 expression is depressed at 6 months in β_1AR overexpressors, so concomitant transgenic expression of AC5 may have compensated to some extent. However, at least for contractility, while responsiveness is preserved, the maximal amplitude of the response is not maintained from 2 to 6 months, and thus frank "rescue" is not afforded.

The downstream signals measured relative to apoptosis failed to reveal a robust pattern, indicative of a beneficial effect of β_2AR or AC5 on β_1AR-mediated cardiomyopathy. Of note, our results with the single β_1AR and β_2AR transgenics differ from those of a

recent paper by Peter et al.,[29] but this may be attributable to the differences in strain or receptor expression levels. In that paper, β_2AR overexpression promoted a cardiomyopathy accompanied by significant apoptosis, p38 MAPK and Akt activation, and reduced left ventricular ejection fraction (LVEF). Although not specifically stated, the cited source[18] of these mice indicate that β_2AR expression was 40 pmol/mg (~30-fold greater than in this report) and the strain was C57BL/6, as compared to FVB/N used here. Interestingly, we did find activation of p38 MAPK in our study with the β_1AR, but the activation was substantially amplified in the β_1/β_2 and β_1/AC5 bitransgenics. This suggests that a threshold effect (potentially cAMP –dependent, since it occurs with AC5 co-overexpression) may be at play for activation of p38 MAPK. Peter et al., also found activation of p38 MAPK in a β_1AR overexpressor, as well as Akt in older mice. Based on the reference[4] for the source, these mice were in the FVB/N background and presumably expressed 600 fmol/mg (not unlike our β_1AR overexpressor). However, we do not find increases in these kinases in our β_1AR overexpressors. Indeed, the only hearts with elevated Akt were the bitransgenics.

Taken together, our findings fail to reveal a significant long-term physiologic improvement in ventricular function, or changes in the development of heart failure, by β_2AR or AC5 overexpression in β_1AR-mediated cardiomyopathy. There were differences in some phenotypes between β_1/β_2 and β_1/AC5 hearts. The latter had some preservation of ventricular function and agonist responsiveness at 6 months of age, did not show a progressive downregulation of β_1AR expression, had little or no effect on cardiac mass, and had no increase in cystosolic Smac. However, by 9 months of age, the relevance of these differences appeared to be minimal, as β_1AR, β_1/β_2, and β_1/AC5 hearts all had markedly depressed fractional shortening, with little response to agonist. We cannot exclude the possibility that different levels of overexpression of β_2AR or AC5 than used here may have given different results. Or, that AC6 overexpression may have given different results compared to our AC5 bitransgenic. Nor does this study address other approaches to maintain βAR responsiveness in heart failure, such as reversal of desensitization by inhibition of G-protein coupled receptor kinase (CRKs).[30] Nevertheless, the current study places into question whether maintenance or enhancement of β_2AR signaling, or AC activity, by overexpression of this receptor or its effector has a long-term beneficial effect in heart failure.

Acknowledgments

The authors thank Esther Moses for manuscript preparation. The work was supported by an NIH grant HL077101.

References

1. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of beta-adrenergic signaling in heart failure? *Circ Res*. 2003; 93: 896–906.

2. Dorn GW II, Molkentin JD. Manipulating cardiac contractility in heart failure: data from mice and men. *Circ*. 2004; 109: 150–158.

3. Mialet-Perez J, Rathz DA, Petrashevskaya NN, Hahn HS, Wagoner LE, Schwartz A, Dorn GW, II, Liggett SB. Beta 1-adrenergic receptor polymorphisms confer differential function and predisposition to heart failure. *Nat Med*. 2003; 9: 1300–1305.

4. Engelhardt S, Hein L, Wiesmann F, Lohse MJ. Progressive hypertrophy and heart failure in beta1-adrenergic receptor transgenic mice. *Proc Natl Acad Sci U S A*. 1999; 96: 7059–7064.

5. Bisognano JD, Weinberger HD, Bohlmeyer TJ, Pende A, Raynolds MV, Sastravaha A, Roden R, Asano K, Blaxall BC, Wu SC, Communal C, Singh K, Colucci W, Bristow MR, Port DJ. Myocardial-directed overexpression of the human beta(1)-adrenergic receptor in transgenic mice. *J Mol Cell Cardiol*. 2000; 32: 817–830.

6. Turki J, Lorenz JN, Green SA, Donnelly ET, Jacinto M, Liggett SB. Myocardial signalling defects and impaired cardiac function of a human beta2-adrenergic receptor polymorphism expressed in transgenic mice. *Proc Natl Acad Sci U S A*. 1996; 93: 10483–10488.

7. Tepe NM, Lorenz JN, Yatani A, Dash R, Kranias EG, Dorn GW2, Liggett SB. Altering the receptor-effector ratio by transgenic overexpression of type V adenylyl cyclase: enhanced basal catalytic activity and function without increased cardiomyocyte beta-adrenergic signalling. *Biochem*. 1999; 38: 16706–16713.

8. Zhu W, Zeng X, Zheng M, Xiao RP. The enigma of beta2-adrenergic receptor Gi signaling in the heart: the good, the bad, and the ugly. *Circ Res*. 2005; 97: 507–509.

9. Ghanouni P, Gryczynski Z, Steenhuis JJ, Lee TW, Farrens DL, Lakowicz JR, Kobilka BK. Functionally different agonists induce distinct conformations in the G protein coupling domain of the beta2 adrenergic receptor. *J Biol Chem*. 2001; 276: 24433–24436.

10. Phan HM, Gao MH, Lai NC, Tang T, Hammond HK. New signaling pathways associated with increased cardiac adenylyl cyclase 6 expression: implications for possible congestive heart failure therapy. *Trends Cardiovasc Med*. 2007; 17: 215–221.

11. Roth DM, Gao MH, Lai NC, Drumm J, Dalton N, Zhou L, Zhu J, Entrikin D, Hammond HK. Cardiac-directed adenylyl cyclase expression improves heart function in murine cardiomyopathy. *Circ*. 1999; 99: 3099–3102.

12. Odley A, Hahn HS, Lynch RA, Marreez Y, Osinska H, Robbins J, Dorn GW. Regulation of cardiac contractility by Rab4-modulated beta2-adrenergic receptor recycling. *Proc Natl Acad Sci U S A*. 2004; 101: 7082–7087.

13. Syed F, Diwan A, Hahn HS. Murine echocardiography: a practical approach for phenotyping genetically manipulated and surgically modeled mice. *J Am Soc Echocardiogr*. 2005; 18: 982–990.

14. Schwinn DA, Leone B, Spann DR, Chesnut LC, Page SO, McRay RL, Liggett SB. Desensitization of myocardial beta-adrenergic receptors during cardiopulmonary bypass: evidence for early uncoupling and late downregulation. *Circ*. 1991; 84: 2559–2567.

15. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem*. 1985; 150: 76–85.

16. Shiozaki EN, Shi Y. Caspases, IAPs and Smac/DIABLO: mechanisms from structural biology. *Trends Biochem Sci*. 2004; 29: 486–494.

17. Gao MH, Lai NC, Roth DM, Zhou J, Zhu J, Anzai T, Dalton N, Hammond HK. Adenylylcyclase increases responsiveness to catecholamine stimulation in transgenic mice. *Circulation*. 1999; 99: 1618–1622.

18. Milano CA, Allen LF, Rockman HA, Dolber PC, McMinn TR, Chien KR, Johnson TD, Bond RA, Lefkowitz RJ. Enhanced myocardial function in transgenic mice overexpressing the beta2-adrenergic receptor. *Science*. 1994; 264: 582–586.

19. Liggett SB, Tepe NM, Lorenz JN, Canning AM, Jantz TD, Mitarai S, Yatani A, Dorn GWII. Early and delayed consequences of beta2-adrenergic receptor overexpression in mouse hearts. *Circ*. 2000; 101: 1707–1714.

20. Tepe NM, Liggett SB. Transgenic replacement of type V adenylyl cyclase identifies a critical mechanism of beta-adrenergic receptor dysfunction in the G-alpha q-overexpressing mouse. *FEBS Lett*. 1999; 458(2): 236–240.

21. Dorn GWII, Tepe NM, Lorenz JN, Koch WJ, Liggett SB. Low- and high-level transgenic expression of beta2-adrenergic receptors differentially affect cardiac hypertrophy and function in Galpha q-overexpressing mice. *Proc Natl Acad Sci U S A*. 1999; 96: 6400–6405.

22. Gao MH, Tang T, Guo T, Sun SQ, Feramisco JR, Hammond HK. Adenylyl cyclase type VI gene transfer reduces phospholamban expression in cardiac myocytes via activating transcription factor 3. *J Biol Chem*. 2004; 279: 38797–38802.

23. Okumura S, Vatner DE, Kurotani R, Bai Y, Gao S, Yuan Z, Iwatsubo K, Ulucan C, Kawabe J, Ghosh K, Vatner SF, Ishikawa Y. Disruption of type 5 adenylyl cyclase enhances desensitization of cyclic adenosine monophosphate signal and increases Akt signal with chronic catecholamine stress. *Circ*. 2007; 116: 1776–1783.

24. Okumura S, Takagi G, Kawabe J, Yang G, Lee MC, Hong C, Liu J, Vatner DE, Sadoshima J, Vatner SF, Ishikawa Y. Disruption of type 5 adenylyl cyclase gene preserves cardiac function against pressure overload. *Proc Natl Acad Sci U S A*. 2003; 100: 9986–9990.

25. DeGeorge BR, Jr, Gao E, Boucher M, Vinge LE, Martini JS, Raake PW, Chuprun JK, Harris DM, Kim GW, Soltys S, Eckhart AD, Koch WJ. Targeted inhibition of cardiomyocyte Gi signaling enhances susceptibility to apoptotic cell death in response to ischemic stress. *Circ*. 2008; 117: 1378–1387.

26. Zhu WZ, Chakir K, Zhang S, Yang D, Lavoie C, Bouvier M, Hebert TE, Lakatta EG, Cheng H, Xiao RP. Heterodimerization of beta1- and beta2-adrenergic receptor subtypes optimizes beta-adrenergic modulation of cardiac contractility. *Circ Res*. 2005; 97: 244–251.

27. Gao M, Ping P, Post S, Insel PA, Tang R, Hammond HK. Increased expression of adenylylcyclase type VI proportionately increases beta-adrenergic receptor-stimulated production of cAMP in neonatal rat cardiac myocytes. *Proc Natl Acad Sci U S A*. 1998; 95: 1038–1043.

28. Liggett SB. Cardiac 7-transmembrane-spanning domain receptor portfolios: diversify, diversify, diversify. *J Clin Invest*. 2006; 116: 875–877.

29. Peter PS, Brady JE, Yan L, Chen W, Engelhardt S, Wang Y, Sadoshima J, Vatner SF, Vatner DE. Inhibition of p38 alpha MAPK rescues cardiomyopathy induced by overexpressed beta 2-adrenergic receptor, but not beta 1-adrenergic receptor. *J Clin Invest*. 2007; 117: 1335–1343.

30. Pleger ST, Boucher M, Most P, Koch WJ. Targeting myocardial beta-adrenergic receptor signaling and calcium cycling for heart failure gene therapy. *J Card Fail*. 2007; 13: 401–414.