Disruption of type 5 adenylyl cyclase prevents β-adrenergic receptor cardiomyopathy: A novel approach to β-adrenergic receptor blockade

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Yan L, Vatner SF, Vatner DE. Disruption of type 5 adenylyl cyclase prevents β-adrenergic receptor cardiomyopathy: A novel approach to β-adrenergic receptor blockade. Am J Physiol Heart Circ Physiol 307: H1521–H1528, 2014. First published September 5, 2014; doi:10.1152/ajpheart.00491.2014.—β-Adrenergic receptor (β-AR) blockade is widely used to treat heart failure, since the adverse effects of chronic β-AR stimulation are central to the pathogenesis of this disease state. Transgenic (Tg) mice, where β-AR signaling is chronically enhanced by overexpression of cardiac β2-ARs, is a surrogate for this mechanism, since these mice develop cardiomyopathy as reflected by reduced left ventricular (LV) function, increased fibrosis, apoptosis, and myocyte hypertrophy. We hypothesized that disruption of type 5 adenylyl cyclase (AC5), which is in the β-AR signaling pathway in the heart, but exerts only a minor β-AR blocking effect, could prevent the cardiomyopathy in β2-AR Tg mice without the negative effects of full β-AR blockade. Accordingly, we mated β2-AR Tg mice with AC5 knockout (KO) mice. The β2-AR Tg × AC5 KO bigenic mice prevented the cardiomyopathy as reflected by improved LV ejection fraction, reduced apoptosis, fibrosis, and myocyte size and preserved exercise capacity. The rescue was not simply due to a β-blocking effect of AC5 KO, since neither baseline LV function nor the response to isoproterenol was diminished substantially compared with the negative inotropic effects of β-blockade. However, AC5 disruption in β2-AR Tg activates the antioxidant, manganese superoxide dismutase, an important mechanism protecting the heart from cardiomyopathy. These results indicate that disruption of AC5 prevents the cardiomyopathy induced by chronically enhanced β-AR signaling in mice with overexpressed β2-AR, potentially by enhancing resistance to oxidative stress and apoptosis, suggesting a novel, alternative approach to β-AR blockade.

β-adrenergic receptor signaling; oxidative stress

AFTER SOME INITIAL controversy, it is now well accepted that chronic β-adrenergic receptor (β-AR) stimulation is deleterious and is involved in the pathogenesis of heart failure (13, 14, 21, 24, 34, 52, 60), and that β-AR blockade is an important adjunct to heart failure therapy (7, 43a). However, these drugs also reduce cardiac function, which sometimes cannot be tolerated in patients with heart failure (7, 29, 35). Accordingly, it would be desirable to have a therapeutic agent that inhibits β-AR signaling distal to the β-AR, and that does not have a significant negative inotropic effect. One candidate is inhibition of adenylyl cyclase (AC), which transduces β-AR stimulation to increase cAMP. There are two major isoforms of AC in the heart, AC types 5 and 6. Type 5 regulates a lesser fraction of AC and cAMP in the heart (roughly 25–30%) (30, 47), suggesting it would be less likely to exert a strong negative inotropic effect when blocked.

Therefore, the goal of the present investigation was to determine if inhibiting chronic β-AR stimulation, specifically by inhibiting only type 5 adenylyl cyclase (AC5), using an AC5 knockout (KO) model, would be useful in countering the adverse effects of chronic β-AR stimulation, i.e., the decrease in function and cardiomyopathy that develops (13, 14, 52, 60). Transgenic mice with cardiac overexpressed β2-AR (β2-AR Tg) were used as the model to elicit chronic β-AR stimulation, since they exhibit increased cardiac function at a young age but develop decreased cardiac function and cardiomyopathy as they age (13, 14, 52, 60). To accomplish this goal, β2-AR Tg mice were mated with AC5 KO mice and were examined for cardiac dysfunction and other manifestations of cardiomyopathy, e.g., cardiac fibrosis, apoptosis, and increased myocyte cross-sectional area, and compared with wild-type (WT) controls and β2-AR Tg mice as they aged to determine if the cardiomyopathy was prevented in the bigenic mice. We also examined if the AC5 KO would also prevent other clinical signs of cardiomyopathy, almost always observed in patients with heart disease, e.g., exercise intolerance (5, 8, 11, 23, 37). Because our hypothesis was that prevention of the cardiomyopathy would not be mediated by simply decreasing AC activity and cAMP, we also measured effects on oxidative stress as a potential mechanism based on our prior studies demonstrating manganese superoxide dismutase (MnSOD) involvement in longevity (64) and catecholamine stress (36) in AC5 KO.

MATERIALS AND METHODS

Animal models. The development and characterization of mice with cardiac-specific overexpression of the β2-AR used in this study have been described previously (13, 44). Parent β2-AR mice were obtained from Jackson Laboratories. Parent AC5 KO mice used in this study have been described previously (48, 64). The mice with cardiac-specific overexpression of the β2-AR were mated with AC5 KO mice to generate littermate wild-type (WT), β2-AR, AC5 KO, and β2-AR × AC5 KO bigenic mice. Four groups of animals were studied (WT, AC5 KO, β2-AR Tg, and β2-AR Tg × AC5 KO bigenic mice) at 13–16 mo of age, at a time when cardiomyopathy develops in the β2-AR Tg mice. All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee at the New Jersey Medical School. All of the investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Echocardiography. Transthoracic echocardiography was performed in 13- to 16-mo age-matched mice using an Acuson Sequoia with a 13-MHz transducer. Mice were anesthetized with 2.5% tribromoethanol (Avertin) injected intraperitoneally and placed on a warmed saline bag. Electrocardiographic leads were attached to each limb using needle electrodes (Grass Technologies). After a short-axis

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two-dimensional (2-D) image of the left ventricle (LV) was obtained at the level of the papillary muscles, a 2-D guided M-mode tracing crossing the anterior and posterior wall was recorded at a sweep speed of 200 mm/s. The following parameters were measured on the M-mode tracings using the leading-edge technique: LV internal dimensions of diastole and systole (LVIDd, LVIDs), LV external dimensions of diastole and systole, and wall thickness at diastole and systole. LV ejection fraction (LVEF) was calculated by the cubed method: LVEF = [(LVIDd)³ - (LVIDs)³]/(LVIDd)³.

Exercise capacity. Mice were exercised on the treadmill. All mice were subjected to a practice trial 3 days before the experiment to adapt to the treadmill testing environment. Food was withdrawn at least 3 h before the exercise. At the time of the experiment, each mouse was placed on a treadmill at a constant 10° angle. The treadmill was started at 4 m/min, and the speed incrementally increased 2 m/min every 2 min until the mice reached exhaustion. Exhaustion was defined as spending time (10 s) on the electric stimulus platform without attempting to reengage the treadmill belt. The maximal running distance was calculated based on the time and speed of running.

Isoproterenol challenge. Echocardiography was performed in WT and AC5 KO mice anesthetized with 2.5% tribromoethanol (290 mg/kg). For acute injection of isoproterenol (ISO), a PE-10 catheter was inserted in the right jugular vein, ISO was injected at the rate of 0.04 μg·kg⁻¹·min⁻¹ for 5 min, and LVEF fraction was measured. In WT mice, after recovery, 0.5 mg/kg propranolol was administered intravenously followed by LVEF measurement. ISO was given again 3 min after propranolol was infused followed by LVEF measurement.

Western blot analysis. Total proteins were extracted from the LV of hearts as previously described (28, 64). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was performed using polyclonal antibodies to MnSOD from Millipore and cleaved caspase-3 from Cell Signaling. Immunodetection was accomplished using a donkey anti-rabbit secondary antibody (1:2,000 dilution) and the enhanced chemiluminescence kit (Amer sham Biosciences). Expression of these proteins was quantified by densitometry, and the data are presented as arbitrary units of density. GAPDH antibody Western blotting was used to verify equal protein loading of the blots.

Superoxide dismutase activity. The superoxide dismutase (SOD) activity was measured by an SOD Assay kit from Cayman Chemicals (Ann Arbor, MI) following the manufacturer’s instructions.

Histology. The heart was excised and washed in cold PBS. A ring of LV tissue, cut at the level of the papillary muscles, was fixed in 10% buffered formalin, processed, and embedded in paraffin. Sections (4 μm thickness) were stained with 10% buffered formalin, processed, and embedded in paraffin. Sections were cut 6-μm thick and deparaffinized. Images were obtained using an Olympus microscope (Olympus BX 51) with a ×40 objective lens. Collagen volume percent was assessed using samples stained with picric sirius red. Myocyte cross-sectional area was determined on sections stained with rhodamine-labeled wheat germ agglutinin (1: 250; Vector). Apoptosis was determined by using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling, which detects apoptosis-induced DNA fragmentation by nick-end labeling of the fragmented DNA at the 3'-hydroxyl ends (Terminal Transferase, recombinant kit; Roche Diagnostics). The nuclei were costained with 4',6-diamino-2-phenylindole. These techniques have all been used extensively (36, 50–52, 64). To further discriminate apoptosis in myocytes from smaller nonmyocytes, tissue sections (5 μm thickness) were costained with α-actinin for myocytes as previously described (18, 50, 51).

Statistical analysis. All data were expressed as means ± SE. To compare two independent groups, we used Student’s unpaired t-test. For a comparison of three or more groups, one-way ANOVA with Newman-Keuls post hoc test was used. P < 0.05 was taken as a minimal level of significance.
tolerance, which has not been noted previously, prior studies also demonstrated the development of cardiomyopathy and increased mortality in β2-AR Tg mice (13, 52). Mortality was not examined in the current study because many mice had to be killed for the histological and biochemical studies, which reduced the power and statistical validity of a mortality analysis. A prior study indicated that the cause of the increased mortality in β2-AR Tg mice was due primarily to heart failure and potentially to arrhythmias, since ectopic beats were observed on the ECG (13). It is likely that the AC5 KO also protects against arrhythmias, since AC5 Tg mice are more susceptible to arrhythmias than AC5 KO or WT (65).

The potentially most clinically attractive feature of this prevention is that the older AC5 KO do not have reduced LV ejection fraction (LVEF) compared with wild-type mice (WT, n = 17). In contrast to the depressed left ventricle (LV) function observed in old β2 Tg mice, the old β2 Tg × AC5 KO bigenic mice (n = 30) exhibited normal cardiac function. Exercise capacity as determined by maximal running distance was significantly reduced in β2 Tg mice (n = 8) compared with WT mice (n = 8). However, reduced exercise capacity was not observed in the β2 Tg × AC5 KO bigenic mice (n = 9). The representative images (left) and quantification (right) of fibrosis (C) and myocyte size (D) showed significantly higher levels in β2 Tg hearts compared with WT. In contrast to the β2 Tg mice, fibrosis and myocyte size in the bigenic mice were no longer different from WT; n = 4/group. The original magnification of hematoxylin and eosin (H&E) staining images for fibrosis was ×20. The original magnification of wheat germ agglutinin (WGA) staining images for myocyte size was ×40. Results are expressed as means ± SE. *P <0.05 by one-way ANOVA. WT and AC5 KO values were not significantly different, except for exercise capacity (n = 6 for AC5 KO), which was increased significantly, P <0.05, in AC5 KO.
DISRUPTION OF AC5 PREVENTS β-ADRENERGIC RECEPTOR CARDIOMYOPATHY

Table 1. LV function and pathology

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>AC5 KO</th>
<th>β2-AR Tg</th>
<th>β2-AR Tg × AC5 KO</th>
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<tbody>
<tr>
<td>Echocardiographic results</td>
<td></td>
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<td></td>
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<tr>
<td>Age, mo</td>
<td>17</td>
<td>11</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>LV EF, %</td>
<td>16.2</td>
<td>15.1</td>
<td>15.6</td>
<td>15.1</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>63 ± 1</td>
<td>68 ± 2*</td>
<td>53 ± 3*</td>
<td>70 ± 1†</td>
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<tr>
<td>Fraction shortening, %</td>
<td>28 ± 1</td>
<td>32 ± 1*</td>
<td>23 ± 1*</td>
<td>33 ± 1†</td>
</tr>
<tr>
<td>End-diastolic diameter, mm</td>
<td>4.0 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>4.5 ± 0.2*</td>
<td>3.8 ± 0.1†</td>
</tr>
<tr>
<td>End-systolic diameter, mm</td>
<td>2.9 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>3.5 ± 0.2*</td>
<td>2.5 ± 0.1†</td>
</tr>
<tr>
<td>Pathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body wt, g</td>
<td>36 ± 1</td>
<td>29 ± 1*</td>
<td>36 ± 2</td>
<td>30 ± 2†</td>
</tr>
<tr>
<td>LV wt, mg</td>
<td>117 ± 7</td>
<td>96 ± 6*</td>
<td>136 ± 6*</td>
<td>95 ± 5†</td>
</tr>
<tr>
<td>Lung wt, mg</td>
<td>171 ± 5</td>
<td>160 ± 5</td>
<td>196 ± 9*</td>
<td>166 ± 6†</td>
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<tr>
<td>LV wt/body wt</td>
<td>3.3 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>3.9 ± 0.3*</td>
<td>3.2 ± 0.1†</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>18.3 ± 0.2</td>
<td>18.1 ± 0.1</td>
<td>18.4 ± 0.1</td>
<td>18.4 ± 0.2</td>
</tr>
<tr>
<td>LV wt/TL</td>
<td>6.1 ± 0.3</td>
<td>5.3 ± 0.3</td>
<td>7.4 ± 0.3*</td>
<td>5.1 ± 0.2†</td>
</tr>
<tr>
<td>Lung wt/TL</td>
<td>9.4 ± 0.3</td>
<td>8.9 ± 0.2</td>
<td>10.6 ± 0.5*</td>
<td>9.0 ± 0.3†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of mice. LV, left ventricle; WT, wild type; AC5, type 5 adenyl cyclase; KO, knockout; β2-AR, β2-adrenergic receptor; LVEF, left ventricular ejection fraction. *P < 0.005 vs. WT; †P < 0.05 vs. β2 Tg.

function at baseline, so adding AC5 inhibition to heart failure should not have the potential to reduce cardiac function further, yet the AC5 KO prevention of the adverse effects of chronic β-adrenergic receptor stimulation is still observed. One might predict that reducing AC activity will, by itself, be responsible for rescuing β-AR cardiomyopathy. However, the main source of AC activity in the heart is AC6, not AC5. We have demonstrated that AC5 KO reduces total AC activity in the heart by only 25–30% (30, 47). Our data in this study showing the source of AC activity in the heart is AC6, not AC5. We have previously demonstrated that upregulation of MnSOD is a powerful mechanism leading to longevity (64) as well as involved in resistance to cardiomyopathy induced by catecholamine stress in the AC5 KO mice (36). It is conceivable that resistance to oxidative stress could be an important mechanism in the protection against cell death and prevention of cardiomyopathy in β2-AR Tg × AC5 KO bigenic mice. Indeed, the β2-AR Tg mice showed increased necrosis, as reflected by myocardial fibrosis, and increased apoptosis when cardiomyopathy develops, and these Tg mice are less tolerant to oxidative stress, whereas fibrosis and apoptosis were normalized in β2-AR Tg × AC5 KO bigenic mice, and MnSOD was augmented in β2-AR Tg × AC5 KO mice, supporting the conclusion that prevention of oxidative stress in the AC5 KO mice plays a role in preventing the cardiomyopathy in β2-AR Tg. Moreover, we previously demonstrated that upregulation of MnSOD and resistance to oxidative stress in the AC5 KO heart are mediated by activation of MEK/ERK and SIRT1/FoxO3a pathways (36, 64). It is conceivable that MEK/ERK and SIRT1/FoxO3a-mediated activation of MnSOD is also involved in prevention of cardiomyopathy developed in β2 Tg mice.

Interestingly, the predominant fraction of the increased apoptosis in the cardiomyopathy and the decrease, when the cardiomyopathy was prevented by the AC5 KO mechanism, occurred in nonmyocytes. Whereas this might be understood, recognizing that over 70% of cells in the heart are nonmyocytes, it seems inconsistent with the currently held concept that apoptosis leads to cardiac dysfunction through reducing myocyte numbers (4, 19). However, the current results are consistent with our previous findings that the apoptosis occurs predominantly in nonmyocytes in several models of cardiomyopathy, including ischemic cardiomyopathy (50) and hypertrophic cardiomyopathy (18, 51). Based on our previous studies, the cell types of apoptotic nonmyocytes mainly include macrophages, neutrophils, fibroblasts, and endothelial cells (50, 51). The role of nonmyocyte apoptosis in the heart in mediating or preventing the cardiomyopathy is not clear, since the nonmyocytes are a double-edged sword. On the one hand, many of these cell types have been linked to myocyte cell damage (1, 17, 18, 33, 41, 42, 50), whereas on the other hand they have been linked to rescue (25, 38, 58, 61). For example, activation of both macrophages or myofibroblasts plays a beneficial role in tissue repair and the improvement of cardiac remodeling and function after myocardial infarction (38, 62).

Exercise intolerance is not only uniformly observed in patients with heart failure but is generally an early sign of cardiac dysfunction that precedes evidence of reduced baseline function at rest (2, 5, 26, 37, 53). However, exercise capacity is rarely examined in mouse models of cardiomyopathy, and has not been examined in transgenic mice, where β-AR signaling is chronically enhanced. However, it is known that, when β-AR signaling is enhanced in young mice, either genetically by increasing components of the β-AR signaling pathway (16) or pharmacologically (45), exercise performance is improved. AC5 KO mice exhibit enhanced exercise performance, whereas the older β2-AR Tg mice that developed cardiomyopathy had depressed exercise capacity, which was rescued in the bigenic mice. This finding is important because...
simply rescuing baseline cardiac function in patients with cardiomyopathy does not necessarily indicate that exercise tolerance will be normalized, leading to an improved quality of life.

Our current study supports the concept that inhibition of AC5 may be a novel approach to the treatment of cardiomyopathy/heart failure. β-Blockers are well-established drugs for treating heart failure (43a). However, because β-blockers can cause contractile dysfunction (15, 27, 54), some patients are intolerant to the administration of β-blockers (7, 29, 35), and the number of such patients who cannot benefit from β-blockers is, roughly estimated, to be over 1.3 million (43, 56). Inhibition of AC5 may be superior to inhibition of β-AR stimulation, since it does not decrease LV contractility (47–49, 64), and may lead to more effective β-AR desensitization (49). To demonstrate this point, we compared the extent to which the response to β-AR stimulation was blocked either by the AC5 KO or by a β-AR blocker propranolol. AC5 KO reduced the response to isoproterenol minimally, whereas propranolol completely abolished it. Thus, the development of a pharmacological AC5 inhibitor could be a useful alternative to β-blockers.

Fig. 2. AC5 disruption prevents apoptosis in β2-AR cardiomyopathy predominantly in nonmyocytes, and to a much lesser extent, in myocytes. A: quantification of terminal deoxyribonucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay in LV sections of old mice showed that nonmyocyte apoptosis was significantly rescued in β2 Tg × AC5 KO bigenic mice, n = 4/group. B: representative images of the staining used to discriminate apoptosis in myocytes (bottom) from nonmyocytes (top). Myocytes were stained with sarcomeric α-actinin (red) and counterstained with TUNEL (green) and 4’,6-diamino-2-phenylindole (DAPI, blue). The original magnification of the images was ×40. C: the levels of cleaved caspase 3 were significantly higher in β2 Tg hearts compared with AC5 KO. In contrast to the β2 Tg mice, the bigenic mice were no longer different from WT; n = 4/group. Results are expressed as means ± SE. *P <0.05 by one-way ANOVA. WT and AC5 KO values were not significantly different.
In summary, AC5 inhibition prevented the cardiomyopathy and depressed exercise tolerance induced by chronic \(\beta\)-adrenergic receptor stimulation. The prevention of cardiomyopathy by AC5 inhibition is not simply due, as might be thought, to reduced AC activity, thereby inhibiting sympathetic stimulation, as occurs with \(\beta\)-AR blockade, since AC activity is regulated to a minor extent by AC5 in the heart and consequently sympathetic stimulation was not blocked in the AC5 KO as it was with propranolol. Therefore, it is likely that there are distal mechanisms mediating the prevention, e.g., activation of SOD, which enhances resistance to oxidative stress through AC5 inhibition. Thus, AC5 inhibition may be a useful adjunct to \(\beta\)-AR blockade as a treatment for heart failure with the advantage of less inhibition of contractility.

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DISCLOSURES

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Author contributions: L.Y. performed experiments; L.Y. analyzed data; L.Y., S.F.V., and D.E.V. interpreted results of experiments; L.Y. prepared figures; L.Y., S.F.V., and D.E.V. edited and revised manuscript; S.F.V. and D.E.V. conception and design of research; S.F.V. and D.E.V. approved final version of manuscript.

**Fig. 3.** The effect of AC5 KO is not simply due to \(\beta\)–blockade. There is no decrease in baseline LVEF in AC5 KO mice, whereas \(\beta\)–blocker, propranolol, treatment reduced baseline LVEF. The response to isoproterenol (ISO) challenge is similar in WT (\(n = 6\)) and AC5KO (\(n = 4\)) mice. In contrast, the increase in LVEF with ISO is fully abolished by propranolol (\(n = 5\)). *P < 0.05 vs. WT by Student’s t-test analysis.

**Fig. 4.** Manganese superoxide dismutase (MnSOD) is activated in \(\beta_2\)-Tg × AC5 KO bigenic mice. MnSOD expression levels determined by Western blotting (A) and superoxide dismutase (SOD) activity (B) were significantly downregulated in \(\beta_2\)-Tg mice but preserved in \(\beta_2\)-Tg × AC5 KO bigenic mice compared with WT mice; \(n = 4–7\) group. Results are expressed as means ± SE. *P < 0.05 by one-way ANOVA. MnSOD activity was increased significantly, \(P < 0.05\), in AC5 KO mice.
REFERENCES

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