Effects of cardiac overexpression of type 6 adenylyl cyclase affects on the response to chronic pressure overload

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Guellich A, Gao S, Hong C, Yan L, Wagner TE, Dhar SK, Ghaleh B, Hittinger L, Iwatsubo K, Ishikawa Y, Vatner SF, Vatner DE. Effects of cardiac overexpression of type 6 adenylyl cyclase affects on the response to chronic pressure overload. Am J Physiol Heart Circ Physiol 299: H707–H712, 2010. First published June 18, 2010; doi:10.1152/ajpheart.00148.2010.—Adenylyl cyclase (AC) type 5 (AC5) and AC type 6 (AC6) are the two major AC isoforms in the heart. Cardiac overexpression of AC6 has been shown to be protective in response to several interventions. In this investigation, we examined the effects of chronic pressure overload in AC6 transgenic (TG) mice. In the absence of any stress, AC6 TG mice exhibited enhanced contractile function compared with their wild-type (WT) littermates, i.e., increased (P < 0.05) left ventricular (LV) ejection fraction (EF) (75 ± 0.9 vs. 71 ± 0.5%) and LV dp/dt (7,850 ± 526 vs. 6,374 ± 315 mmHg/s). Forskolin (25 μg·kg−1·min−1 for 5 min) increased LVEF more (P < 0.05) in AC6 TG mice (14.8 ± 1.0%) than in WT mice (7.7 ± 1.0%). Also, isoproterenol (0.04 μg·kg−1·min−1 for 5 min) increased LVEF more (P < 0.05) in AC6 TG mice (18.0 ± 1.2%) than in WT mice (11.6 ± 2.1%). Pressure overload, induced by 4 wk of transverse aortic constriction (TAC), increased the LV weight-to-body weight ratio and myocyte cross-sectional area similarly in both groups, but reduced LVEF more in AC6 TG mice (14.8% vs. 3.6%) after TAC. Thus, cardiac function; transverse aortic constriction; hypertrophy; apoptosis; transgenic

AMONG NINE MAMMALLIAN ISOFORMS of adenylyl cyclase (AC), AC type 5 (AC5) and AC type 6 (AC6) are the two major isoforms in the heart. Similar to other AC isoforms, AC5 and AC6 primarily function as effective enzymes that catalyze the production of cAMP from ATP upon sympathetic stimulation mediated by the coupling of β-adrenergic receptors and the G protein Goα. Despite sharing a high amino acid sequence identity (65%) and several regulatory characteristics, such as activation by Goα and forskolin and inhibition by Goα, PKA, and low concentrations of Ca2+ (1, 6), AC5 and AC6 have shown differential expression of their mRNA with age (21) and opposite expression of their protein, e.g., an upregulation of AC5 and a downregulation of AC6 in pressure overload left ventricular (LV) hypertrophy (7). More interestingly, AC5 and AC6 appear to act differently when overexpressed or disrupted. AC5 disruption has been shown to prolong longevity (27) and improve LV function after either chronic catecholamine stress (16) or chronic pressure overload (15), whereas AC6 deletion impaired cardiac cAMP generation and Ca2+ handling, which resulted in depressed LV function (24). Cardiac overexpression of AC6 has been shown to improve cardiac function in response to myocardial ischemia (12) or rescuing dilated cardiomyopathy (17, 18, 25). More recently, Tang et al. (26) found that female AC6 knockout (KO) mice were protected from chronic pressure overload, which would be consistent with our findings in AC5 KO mice with chronic pressure overload (15), but, in contrast, male AC6 KO mice were not protected. However, this intervention has not been examined with AC6 overexpression, which was the goal of the present investigation. Accordingly, we examined the effects of 4 wk of transverse aortic constriction (TAC) in mice with cardiac overexpression of AC6 [AC6 transgenic (TG) mice] and in their wild-type (WT) littermates.

MATERIALS AND METHODS

All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School. All investigations conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

TG Mice

Briefly, to obtain cardiac-specific overexpression of AC6, full-length cDNA of the canine AC6 gene (4 kb, Dr. Yoshihiro Ishikawa) was used under the control of the α-myosin heavy chain promoter (22). The cDNA fragment was excised and subcloned into the SalI and HindIII polylinker site on a pBlueScript vector followed by a 0.6-kb human growth hormone poly A signal. AC6 TG mice were generated on an FVB background. Heterozygous mice and their WT littermates were used for this study.

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H707
Western Blot Analysis

Mouse hearts were washed in saline (0.9% NaCl), and the LV was quickly frozen. Membrane protein preparation was obtained as previously described (8). Cardiac membrane proteins were separated on a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and then incubated with anti-AC5 antibody [1:1,000, developed in our laboratory (7)] or anti-AC6 antibody (1:800, C-20, Santa Cruz Biotechnology, Santa Cruz, CA). The obtained bands were quantified by densitometry, and the data are presented as arbitrary units of density.

Experimental Protocol

AC6 TG mice and their WT littermates were subjected to 4 wk of TAC or sham surgery. At the end of 4 wk, animals were anesthetized with Avertin, echocardiographic and hemodynamic measurements were obtained, and tissue was harvested for myocyte size and apoptosis measurements. Separate groups of AC6 TG and WT mice were used for conscious measurements of heart rate (via telemetry) and for responses to forskolin and isoproterenol (Iso). Since the mice with TAC were not studied before the TAC, the baseline data reported in this article are actually those values in sham-operated AC6 TG and WT mice.

Surgery. TAC or sham operation was performed on AC6 TG mice and their WT littermates as previously described (14). Mice were anesthetized [ketamine (0.065 mg/g), xylazine (0.013 mg/g), and acepromazine (0.002 mg/g ip)] and then ventilated via endotracheal intubation with a tidal volume of 0.2–0.3 ml and a respiratory rate of 110 breaths/min. The left side of the chest was opened at the second intercostal space, and the transverse thoracic aorta between the innominate and carotid arteries was constricted against a 28-gauge needle. Three- to seven-month-old mice underwent TAC or sham operation and were studied 4 wk later.

Echocardiography. Transthoracic echocardiography was performed using an Acuson Sequoia 256 ultrasound system with a 13-MHz linear transducer. Echocardiographic experiments were performed under light anesthesia [Avertin (2.5%, 0.012 ml/g ip)]. The chest was shaved, and the animal was then placed on a warmed pad. Electrode needles were connected to each limb (Grass Technologies), and the electrocardiogram was simultaneously recorded. Mice were imaged in a shallow left lateral decubitus position. The two-dimensional parasternal short-axis imaging plane was used to...
obtain M-mode tracings at the level of the papillary muscles. LV internal dimensions and LV wall thickness were determined at systole and diastole using leading-edge methods and guidelines of the American Society of Echocardiography (20). End-diastolic measurements were taken at the maximal LV diastolic dimension, and end systole was defined as the time of the most anterior systolic excursion of the posterior wall. Measurements were taken from three consecutive beats for each mouse. Systolic function was estimated from LV dimensions by the cubed method as a percentage of the LV ejection fraction (LVEF) as follows: LVEF (in %) = \( \frac{[(LVEDD^3 - LVEDD^3)/LVEDD^3] \times 100}{LVEDD} \), where LVEDD is LV end-diastolic diameter and LVEDD is LV end-diastolic diameter.

**Hemodynamics**

A high-fidelity catheter (1.4-Fr Millar catheter SPR-839, Millar Instruments) was inserted into the right carotid and then advanced into the LV to measure LV systolic pressure (LVSP), its first derivative (LV dP/dt), and LV end-diastolic pressure. To measure the pressure gradient across the aortic constriction, the catheter was retracted to the ascending aorta, and a second catheter was inserted to the abdominal aorta through the right femoral artery. Pressures were measured simultaneously. LV systolic wall stress (LVSWS) was calculated as follows: LVSWS (in kdyn/cm²) = \( \frac{1}{2} \times LVSP \times LVID^2 \times \frac{1}{4} \times \frac{LVPW \times (1 + LVPWs/LVIDs)}{LVIDs} \), where LVIDs and LVPWs are the LV internal diameter and LV posterior wall thickness at systole, respectively.

**Histology**

After the in vivo experiments, 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 were cut to 6 μm thick, deparaffinized, and used for staining for cell size and TUNEL. Images were obtained using an Olympus charge-coupled device video camera (DP 71, Olympus) attached to an Olympus microscope (Olympus BX 51) with a x40 objective lens.

**Myocyte size.** Rhodamine-labeled wheat germ agglutinin (1:250, Vector) was used on transverse paraffin sections of the LV to detect plasma membranes. Myocyte cross-sectional areas were quantified using the ImagePro plus 5.0 Software System (Media Cybernetics). The mean area was calculated for the LV in each animal, and the group mean was calculated for each group.

**Apoptosis.** TUNEL was used to quantify apoptosis. This technique detects apoptosis-induced DNA fragmentation by nick-end labeling of the fragmented DNA at 3’-hydroxyl ends (Terminal Transferase, recombinant kit, Roche Diagnostics). After the TUNEL procedure, slides were washed in PBS, mounted in 4’,6-diamidino-2-phenylindole (DAPI) medium, and observed under a fluorescence microscope. The number of positive nuclei per cross section was determined by manual counting of double-positive DAPI-TUNEL nuclei and normalized to the cross-sectional area.

**Telemetry.** Heart rates were measured in conscious AC6 TG (n = 3) and WT (n = 3) mice. After anesthesia (ketamine mixture), a miniaturized telemetry device (DSI model TA11-PAC20, DataScience) was subcutaneously implanted. After at least 5 days of postsurgical recovery, the probes were magnetically activated, and the ECG signal was obtained and digitized at a sampling rate of 1 kHz before being processed by an algorithm able to detect ECG cycles (Notocord-hem, Notocord Systems, SAS). Using the acquisition software, experimental data were recorded continuously for 6 h. The heart rate for each hour interval was estimated by analyzing the recording for 2 min with a stable signal and without fluctuations and then averaged for the 6 h.

**Inotropic response to forskolin and Iso.** AC6 TG (n = 7) and WT (n = 7) mice were anesthetized with Avertin. Iso was infused via a catheter implanted into the external jugular vein at a dose of 0.04

### Table 1. Echocardiographic and hemodynamic parameters

<table>
<thead>
<tr>
<th></th>
<th>Sham Operation</th>
<th>TAC Operation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WT Mice</td>
<td>AC6 TG Mice</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.7 ± 0.04</td>
<td>22</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.4 ± 0.03</td>
<td>22</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>71 ± 0.5</td>
<td>22</td>
</tr>
<tr>
<td>FS, %</td>
<td>34 ± 0.4</td>
<td>22</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>475 ± 14</td>
<td>19</td>
</tr>
<tr>
<td>LV dP/dt, mmHg/min</td>
<td>6,374 ± 315</td>
<td>19</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>84 ± 2</td>
<td>19</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>3 ± 1</td>
<td>19</td>
</tr>
<tr>
<td>Pressure gradient, mmHg</td>
<td>101 ± 5</td>
<td>14</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of animals used in each group (*3 animals in this group were studied for echocardiography but were not euthanized). TAC, transverse aortic constriction; WT, wild-type; AC6 TG, adenylyl cyclase type 6 transgenic; LVEDD, left ventricular (LV) end-diastolic diameter; LVESD, LV end-systolic diameter; LVEF, LV ejection fraction; FS, fractional shortening; HR, heart rate; LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure. *P < 0.05, sham-operated AC6 TG mice vs. sham-operated WT mice; †P < 0.05, TAC-operated AC6 TG mice vs. TAC-operated WT mice; §P < 0.05, sham-operated mice vs. TAC-operated mice.

### Table 2. Morphometry

<table>
<thead>
<tr>
<th></th>
<th>Sham Operation</th>
<th>TAC Operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT Mice</td>
<td>AC6 TG Mice</td>
</tr>
<tr>
<td>BW, g</td>
<td>30 ± 0.8</td>
<td>22</td>
</tr>
<tr>
<td>LV weight/BW, mg/g</td>
<td>3.1 ± 0.1</td>
<td>22</td>
</tr>
<tr>
<td>LV weight/TI, mg/mm</td>
<td>5.1 ± 0.2</td>
<td>22</td>
</tr>
<tr>
<td>Lung weight/BW, mg/g</td>
<td>5.2 ± 0.2</td>
<td>22</td>
</tr>
<tr>
<td>Lung weight/TI, mg/mm</td>
<td>8.5 ± 0.2</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>27 ± 0.9</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>4.8 ± 0.2*</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>7.4 ± 0.4*</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>7.1 ± 0.8*</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>10.6 ± 1.1*</td>
<td>17</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of animals used in each group. BW, body weight; TL, tibia length. No differences were found between WT and AC6 TG animals. *P < 0.05, sham-operated mice vs. TAC-operated mice.

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μg·kg⁻¹·min⁻¹ for 5 min. Forskolin was infused at a dose of 25 μg·kg⁻¹·min⁻¹ for 5 min. Echocardiography was performed at baseline and after AC stimulation with forskolin and Iso.

**Statistics**

Data are reported as means ± SE. Statistical significance was assessed using Student’s *t*-test or ANOVA with Fisher’s protected least-significant-difference post hoc test using StatView software (StatView 5.0, SAS Institute). Differences in slopes were assessed by the comparison of two independent regression data sets. *P* values of <0.05 were considered significant.

**RESULTS**

**Characterization of the AC6 TG Mouse Model**

In AC6 TG mice, AC6 protein levels were approximately seven times greater than in WT mice (Fig. 1B), but there were no changes in the protein levels of AC5, the other major cardiac isoform.

**Table 3. Histology**

<table>
<thead>
<tr>
<th></th>
<th>Sham Operation</th>
<th>TAC Operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT Mice</td>
<td>AC6 TG Mice</td>
</tr>
<tr>
<td>Cross-sectional area, μm²</td>
<td>288 ± 10.9</td>
<td>291 ± 13.9</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>TUNEL-positive nuclei/mm²</td>
<td>0.50 ± 0.04</td>
<td>0.59 ± 0.02</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are means ± SE. No differences were noted between WT and AC6 TG mice. *P < 0.05, sham-operated mice vs. TAC-operated mice.

**Fig. 2.** Effects of chronic pressure overload on AC6 TG left ventricular (LV) function. A: transverse aortic constriction (TAC) resulted in a significantly greater decrease (*P < 0.05) in LV ejection fraction (EF) in AC6 TG mice compared with WT mice. B: TAC increased (*P < 0.05) LV end-diastolic dimension (LVEDD) in AC6 TG mice but not in WT mice. C: LVEF was plotted as a function of LV systolic wall stress (LVWS) in WT and AC6 TG mice. The comparison of two independent regression data sets (large sample theory) showed that the slope for AC6 TG mice was significantly lower (*P < 0.05) than for WT mice. D: mean ± SE values for LVWS in AC6 TG mice compared with WT mice. LVWS increased more (*P < 0.05) in AC6 TG mice than in WT mice with chronic pressure overload. *P < 0.05, AC6 TG sham-operated mice vs. WT sham-operated mice; #P < 0.05, AC6 TG TAC-operated mice vs. WT TAC-operated mice; †P < 0.05, sham-operated mice vs. TAC-operated mice.
in WT mice (11.6 ± 2.1%). There were no changes in LVSP with either drug in either WT and AC6 TG mice.

**Effects of AC6 Overexpression on the Response to Chronic Pressure Overload**

Only 1 of 16 AC6 TG mice died from TAC, and there was no mortality in the WT TAC-operated group. Pressure overload for 4 wk increased the aortic pressure gradient (Table 1) and LV weight-to-body weight ratio (Table 2) as well as myocyte cross-sectional area (Table 3) similarly in both WT and AC6 TG mice, but significantly (P < 0.05) reduced LVEF to lower levels in AC6 TG mice (58 ± 1.3%) than in WT mice (65 ± 0.9%), despite the higher starting level of LVEF in AC6 TG mice. LV dP/dt was no longer elevated in AC6 TG mice compared with WT mice. LVEDD was also greater (P < 0.05) in AC6 TG mice (3.8 ± 0.07 mm) than in WT mice (3.6 ± 0.05 mm) after TAC (Fig. 2). There were no significant differences in LV end-diastolic pressure and the lung weight-to-body weight ratio between TAC-operated WT and AC6 TG mice, but significantly (P = 0.05) reduced LVEF to lower levels in AC6 TG mice (58 ± 1.3%) than in WT mice (65 ± 0.9%), despite the higher starting level of LVEF in AC6 TG mice. LV dP/dt was no longer elevated in AC6 TG mice compared with WT mice. LVEDD was also greater (P < 0.05) in AC6 TG mice (3.8 ± 0.07 mm) than in WT mice (3.6 ± 0.05 mm) after TAC (Fig. 2). There were no significant differences in LV end-diastolic pressure and the lung weight-to-body weight ratio between TAC-operated WT and AC6 TG mice. However, LVFSW increased more (P < 0.05) in AC6 TG mice (92.2 ± 5.9 kdyn/cm²) versus WT mice (73.9 ± 4.6 kdyn/cm²), which could be the mechanism underlying the adverse effects on LVEF in AC6 TG mice with chronic pressure overload. Cell death through apoptosis occurred equally in both groups in response to TAC.

**DISCUSSION**

AC5 and AC6 are the two major isoforms of AC in the heart. These two isoforms appear to act differently when overexpressed or disrupted, e.g., AC5 disruption has been shown to prolong longevity (27) and improve LV function after either chronic catecholamine stress (16) or chronic pressure overload (15), whereas cardiac overexpression of AC6 has been shown to improve cardiac function in response to myocardial ischemia (17, 25) or rescue dilated cardiomyopathy (17, 18).

The major finding of the present investigation is that cardiac function is not preserved as well in AC6 TG mice, compared with WT mice, in response to chronic pressure overload. This is based on measurements of LVEF, which, despite being higher at baseline in AC6 TG mice than in WT mice, fell to a significantly lower level with chronic pressure overload, accompanied by greater LV dilatation. These findings are at odds with other studies examining the interventions of myocardial ischemia or rescuing genetically induced cardiomyopathies. Other than the fact that chronic pressure overload is a different stress than the others studied previously, there is no obvious additional explanation for the discrepancy in these studies. One possibility is that the level of overexpression of AC6 differed, but this is unlikely because the previous studies examined AC6 TG animals with 10-fold (4), 17-fold (23), and 20-fold (5) increases in AC6 protein. In addition, the present baseline values of similar levels of heart rate and elevated LV inotropic function are similar to what have been previously reported (5).

Although it is still controversial as to whether enhancing the β-adrenergic pathway is beneficial or deleterious in the therapy of cardiac stress and heart failure, it is clear that overexpression of either β₁- or β₂-adrenergic receptors in the heart will lead to cardiomyopathy and heart failure (3, 13). This was first observed with overexpression of cardiac Go₅ (9). Conversely, inhibition of the other major cardiac isoform, AC5, appears to be salutary in the response to pressure overload (15) and chronic catecholamine stress (16). Most clinically relevant are the studies in patients demonstrating the adverse effects of chronic sympathomimetic amine therapy in heart failure (19) and the striking beneficial effects of β-blockers (2, 10), which have now become a staple in the armamentarium for the clinical treatment of heart failure (11).

Thus, cardiac AC6 overexpression improves cardiac function at baseline, but in contrast to other interventions reported previously (18, 23) to be salutary, the response to chronic pressure overload was not; actually, AC6 TG mice fared worse than WT mice. Increased LVFSW could be the mechanism mediating the adverse effects on LVEF in AC6 TG mice with chronic pressure overload.

**REFERENCES**


