Effects of Cardiac Overexpression of Type 6 Adenylyl Cyclase Affects on the Response to Chronic Pressure Overload

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ABSTRACT

Adenylyl cyclase (AC) type 5 (AC5) and type 6 (AC6) are the two major AC isoforms in the heart. Cardiac overexpression of AC6 (AC6 Tg) has been shown to be protective in response to several interventions. In this investigation, we examined the effects of chronic pressure overload in AC6 Tg. 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 (7850±526 vs. 6374±315 mmHg/sec). Forskolin (25 μg/kg/min) increased LVEF more, $p<0.05$, in AC6 Tg (16±1.1% or 15±1.0%, see results) than WT (7 or 8, see results±1.3%). Pressure overload, induced by 4 weeks of transverse aortic constriction (TAC), similarly increased the LV weight to body weight ratio (LV/BW) and myocyte cross-sectional area in both groups, but reduced LVEF more in AC6 Tg (22%) compared with WT (9 %), despite the higher starting level of LVEF in the AC6 Tg mice, and also increased LV systolic wall stress more in AC6 Tg. In addition, LV dP/dt was no longer elevated in AC6 Tg mice after TAC, compared to WT. LV end diastolic diameter was also greater ($p<0.05$) in AC6 Tg (3.8±0.07 mm) than WT (3.6±0.05 mm) after TAC. Thus, cardiac AC6 overexpression improves cardiac function at baseline, but adversely affects the response of LV function to chronic pressure overload. Thus, cardiac AC6 overexpression improves cardiac function at baseline, but in contrast to other interventions reported previously to be salutary, the response to chronic pressure overload was not; actually, the AC6 Tg fared worse than WT. The mechanism may be due to the increased LV systolic wall stress in the AC6 Tg with chronic pressure overload.

Keywords: adenylyl cyclase, cardiac function, transverse aortic constriction, hypertrophy, apoptosis

INTRODUCTION

Among nine mammalian isoforms of adenylyl cyclase (AC), types 5 and type 6 of AC (AC5 and AC6) are the two major isoforms in the heart. Similar to other AC isoforms, AC5 and AC6 primarily function as an effective enzyme that catalyzes the production of cAMP from ATP upon sympathetic stimulation mediated by coupling of the β-adrenergic receptors and the G-protein, G_s. Despite sharing a high amino acid sequence identity (65%) and several regulatory characteristics such as activation by G_s and forskolin and inhibition by G_i, PKA and low concentration of calcium (Hanoune and Defer 2001; Beazely and Watts 2006), AC5 and AC6 have shown differential expression of with age , and opposite expression of their protein, e.g. an upregulation of AC5 and a downregulation of AC6, in pressure overload LV hypertrophy (Hu, Chandra et al. 2009). More interestingly, AC5 and AC6 appear to act differently when overexpressed or disrupted. AC5 disruption has been shown to prolong longevity (21) and improve LV function following either chronic catecholamine stress (13) or chronic pressure overload (12), whereas AC6 deletion impaired cardiac cAMP generation and calcium handling which resulted in depressed LV function (Tang, Gao et al. 2008). Cardiac overexpression of AC6 has been shown to improve cardiac function in response to myocardial ischemia (14, 20) or rescuing dilated cardiomyopathy (14, 15). More recently, Tang, et al, 2010, found that AC 6 KO mice were protected from chronic pressure overload, which would be consistent with our findings in AC5 KO mice with chronic pressure overload(12); 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 weeks of transverse aortic constriction (TAC) in mice with cardiac overexpression of AC6 and in their WT littermates.

MATERIALS & METHODS

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

Transgenic Mice. AC6 Tg mice were generated as previously described (Hu, Chandra et al. 2009). Briefly, in order to get cardiac-specific overexpression of adenylyl cyclase type 6 (AC6 Tg), full length cDNA of the canine AC6 gene (4Kb, Dr. Yoshihiro Ishikawa) was used under the control of the α-myosin heavy chain (αMHC) promoter (Subramaniam, Jones et al. 1991). Briefly, the cDNA fragment was excised and subcloned into the SalI-and HindIII poly linker site on a pBlueScript vector followed by a 0.6 Kb hGH poly A signal. AC6 Tg mice were generated on an FVB background. Heterozygous mice and their wild type (WT) littermates were used for this study.

Western Blotting. Mouse hearts were washed in saline (NaCl 0.9%) and the LV was quickly frozen. Membrane protein preparation was obtained as previously described (Ihnatovych, Novotny et al. 2002). Cardiac membrane proteins were separated on 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and then incubated with anti-AC5 antibody (1/1000, developed in our laboratory, (Hu, Chandra et al. 2009)) or anti-AC6 antibody (1/800, C-20, Santa Cruz, CA). The obtained bands were quantified by densitometry and the data are presented as arbitrary units of density (AU).

Experimental protocol. AC6 Tg mice and their WT littermates were subjected to 4 weeks TAC or sham surgery. At the end of the 4 weeks, 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. Since the mice with TAC were not studied prior to transverse aortic constriction, the baseline data reported in this manuscript 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 (Meguro, Hong et al. 1999). Mice were anesthetized (ketamine: 0.065 mg/g, xylazine: 0.013 mg/g, and acepromazine: 0.002 mg/g i.p.) and then ventilated via endotracheal intubation with a tidal volume of 0.2-0.3 ml and a respiratory rate of 110 breaths per minute. The left side of the chest was opened at the second intercostal space, and the transverse thoracic aorta between the innominate and the carotid arteries was constricted against a 28G needle. 3-7 month old mice underwent TAC or sham operation for 4 weeks.

Echocardiography. Transthoracic echocardiography was performed using an Acuson Sequoia 256 ultrasound system with a 13-MHz linear transducer. Echocardiography studies were performed under light anesthesia (Avertin, 2.5%-0.012 ml/g, i.p), the chest was shaved, and the animal was then placed on a warmed pad. Electrode needles were connected to each limb (Grass Technologies) and electrocardiogram was simultaneously recorded. Mice were imaged in a shallow left lateral decubitis position. Two-dimensional parasternal short-axis imaging plane was used to obtain M-mode tracings at the level of the papillary muscles. LV internal dimensions

were determined at systole and diastole (LVESD and LVEDD respectively) using the leading-edge methods and guidelines of the American Society of Echocardiography (Sahn, DeMaria et al. 1978). 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 percentage of LV ejection fraction (LVEF): LVEF (%) = $[(LVEDD^3-LVESD^3) /LVEDD^3] \times 100$.

Hemodynamics. A high-fidelity catheter (1.4F Millar catheter SPR-839, Millar Instruments) was inserted into the right carotid then advanced into the LV to measure the LV systolic pressure (LVSP), its first derivative (LV dP/dt) and end diastolic pressure (LVEDP). To measure the pressure gradient across the aortic constriction, the catheter was retracted to the ascending aorta and a second one was inserted to the abdominal aorta through the right femoral artery. Pressures were measured simultaneously.

CALCULATION OF LV SYSTOLIC WALL STRESS IS MISSING (see last para of results)

Histology. After *in vivo* studies, 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 6-μm thick, deparaffinized and used for staining for cell size and TUNEL. Images were obtained using an Olympus CCD video camera (DP 71, Olympus,) attached to an Olympus microscope (Olympus BX 51) with a 40X objective lens.

Myocyte size. Rhodamine-labeled wheat germ agglutinin (WGA, 1/250, Vector) was used on transverse paraffin sections of the LV to detect plasma membranes. Myocyte cross-sectional areas were quantified using ImagePro plus 5.0 Software System (Media cybernetics, Inc.). 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 the 3'-hydroxyl ends (Terminal Transferase, recombinant kit, Roche Diagnostics). After the TUNEL procedure, the slides were washed in PBS, mounted in 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 rate was measured in conscious AC6 Tg (n=3) and WT (n=3) mice. Following anesthesia (ketamine mixture), a miniaturized telemetry device (DSI model TA11-PAC20, Datascience Corp) was subcutaneously implanted. After at least 5 days of post surgical 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, the experimental data were recorded continuously for 6 hours. The heart rate for each hour interval was estimated by analyzing the recording for 2 minutes with a stable signal and without fluctuations.

Inotropic response to forskolin and ISO. AC6 Tg (n=7) and WT (n=7) mice were anesthetized with Avertin, isoproterenol (ISO) was infused via a catheter implanted into the external jugular vein at a dose of 0.02 µg/kg/min for 5 minutes and then forskolin was infused at a dose of 25 µg/kg/min for 5 minutes. Echocardiography was performed at baseline and following AC stimulation with forskolin and ISO.

Statistics. Data reported are mean ± SEM. Statistical significance was assessed using Student's *t*-test or ANOVA with Fisher's PLSD post-hoc using StatView software (StatView 5.0, SAS Institute Inc.). Differences in slopes were assessed by the comparison of two independent regression data sets. A *p* value of less than 0.05 was considered significant.

RESULTS

Characterization of AC6 Tg model. In AC6 Tg mice, AC6 protein levels were approximately 7 times greater than in WT (Fig. 1, *B*), but there was no change in the protein level of AC5, the other major cardiac isoform.

Baseline cardiac function and response to forskolin and ISO. There was no difference in heart rate between WT and AC6 Tg mice either measured in the conscious state with telemetry (687±24 vs. 695±11 beats/min, respectively) or following anesthesia in the echocardiography studies (475±14 vs 456±13 beats/min). However, as shown in Table 1, LVEF was higher, $p<0.05$, in AC6 Tg (75±0.9%) compared to WT (71±0.5%), as was LV dP/dt (7850±526 vs. 6374±315 mmHg/sec). Other than LVSP, no other hemodynamic variables were different between the two groups. In response to forskolin, LVEF increased in AC6 Tg (15±1.0%) more, $p<0.05$, than in WT (8±1.0 %). The increases in LVEF with ISO were not different in the two groups. The higher baseline of LVEF in AC6 Tg could have masked an enhanced response. There was no change in LV systolic pressure with either drug on both WT and AC6 Tg.

Effects of AC6 overexpression on response to chronic pressure overload. Only one of 16 AC6 Tg mice died from TAC and there was no mortality in the WT TAC operated group. Pressure overload for 4 weeks increased the aortic pressure gradient and LVW/BW (Table 2) and myocyte cross-sectional area (Table 3) similarly in both WT and AC6 Tg, but significantly ($p<0.05$) reduced LVEF to lower levels in AC6 Tg (58±1.3%) than WT (65±0.9%), despite the higher starting level of LVEF in AC6 Tg, LV dP/dt was no longer elevated in AC6 Tg compared

to WT. LV end diastolic diameter was also greater ($p<0.05$) in AC6 Tg (3.8±0.07 mm) than WT (3.6±0.05 mm) after TAC. There were no significant differences in LV end diastolic pressure and the lung weight/BW ratio between TAC operated WT and AC6 Tg. However, LV systolic wall stress increased more, $p<0.05$, in AC6 Tg (92.2±5.9 Kdyn/cm^2) vs. WT (73.9±4.6 Kdyn/cm^2), which could be the mechanism underlying the adverse effects on LVEF in AC6 Tg 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 (Yan, Vatner et al. 2007) and improve LV function following either chronic catecholamine stress (Okumura, Vatner et al. 2007) or chronic pressure overload (Okumura, Takagi et al. 2003), whereas cardiac overexpression of AC6 has been shown to improve cardiac function in response to myocardial ischemia (Roth, Bayat et al. 2002; Tang, Gao et al. 2004) or rescuing dilated cardiomyopathy (Roth, Gao et al. 1999; Roth, Bayat et al. 2002).

The major finding of the current investigation is that cardiac function is not preserved as well in AC6 Tg, compared with WT, in response to chronic pressure overload. This is based on measurements of LVEF, which despite being higher at baseline than in WT, fell to a significantly lower level with chronic pressure overload, accompanied by greater LV dilatation. These findings are at odds with the 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 from 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 prior studies examined AC6 Tg with 10- (Gao, Bayat et al. 2002), 17- (Takahashi, Tang et al. 2006) and 20-fold (Gao, Lai et al. 1999) increases in AC6 protein. In addition, the current baseline values of similar levels of heart rate and elevated LV inotropic function are similar to what has been reported previously (Gao, Lai et al. 1999).

Although it is still controversial as to whether enhancing the beta-adrenergic pathway is beneficial or deleterious in the therapy of cardiac stress and heart failure, it is clear that

overexpression of either beta 1- or beta 2-adrenergic receptors in the heart will lead to cardiomyopathy and heart failure (Engelhardt, Hein et al. 1999), (Liggett, Tepe et al. 2000). This was also observed with overexpression of cardiac Gsα (Iwase, Uechi et al. 1997). Conversely, inhibition of the other major cardiac isoform, AC5, appears to be salutary in the response to pressure overload and chronic catecholamine stress (Okumura, Vatner et al. 2007). Most clinically relevant are the studies in patients, demonstrating the adverse effects of chronic sympathomimetic amine therapy in heart failure (Roubin, Choong et al. 1984) and the striking beneficial effects of beta blockers (Bristow 2000; Javed and Deedwania 2009), which has now become a staple in the armamentarium for the clinical treatment of heart failure (Jessup, Abraham et al. 2009).

Thus, cardiac AC6 overexpression improves cardiac function at baseline, but in contrast to other interventions reported previously (Roth, Gao et al. 1999; Takahashi, Tang et al. 2006) to be salutary, the response to chronic pressure overload was not; actually, the AC6 Tg fared worse than WT. Increased LV systolic wall stress could be the mechanism mediating the adverse effects in AC6 Tg with chronic pressure overload.

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DISCLOSURES:

None.

FIGURE LEGENDS:

Figure 1. Generation of transgenic mice overexpressing AC6 in the cardiomyocyte. (*A*) Schematic representation of the construct used for microinjection, α-MHC promoter construct was used to generate AC6 Tg mice. (*B*) Western blot analysis of the expression of AC6 transgene. Membrane protein preparation from WT, and AC6 Tg were separated by 12% polyacrylamide gel electrophoresis, visualized by autoradiography and quantified by densitometry. Pancadherin was used to normalize the intensity of AC6 or AC5 specific bands. * indicates significant difference compared to WT.

Figure 2. Effects of chronic pressure overload on AC6 Tg LV function. (*A*) TAC results in a significantly greater decrease, $p<0.05$, in LVEF in AC6 Tg compared to WT. (*B*) TAC increased, $p<0.05$, LV end-diastolic dimension (LVEDD) in AC6 Tg but not in WT. *(C)* LVEF is plotted as a function of LV systolic wall stress (LVSWS) in WT and AC6 Tg. Comparing 2 independent regression data sets (large sample theory) showed that the slope for AC6 was significantly lower, $p<0.05$, than for WT. *(D)* The mean ± SE values for LV systolic wall stress are compared in AC6 Tg and WT. LV systolic wall stress increased more, $p<0.05$, in AC6 Tg than WT with chronic pressure overload. * $P < 0.05$ AC6 Tg sham vs. WT sham; $^{\#}$ $P < 0.05$ AC6 Tg TAC vs. WT TAC; † $P < 0.05$ sham vs. TAC.

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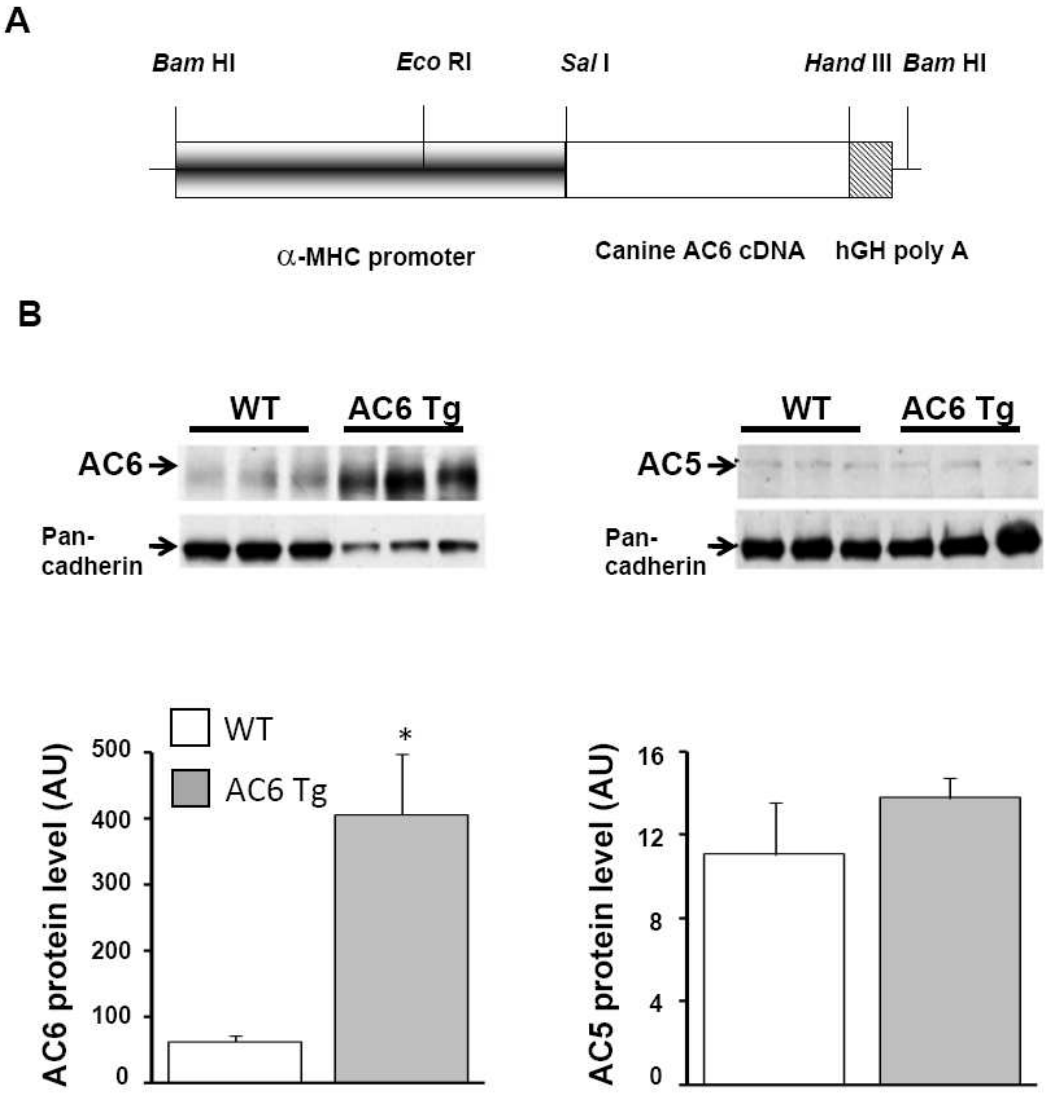
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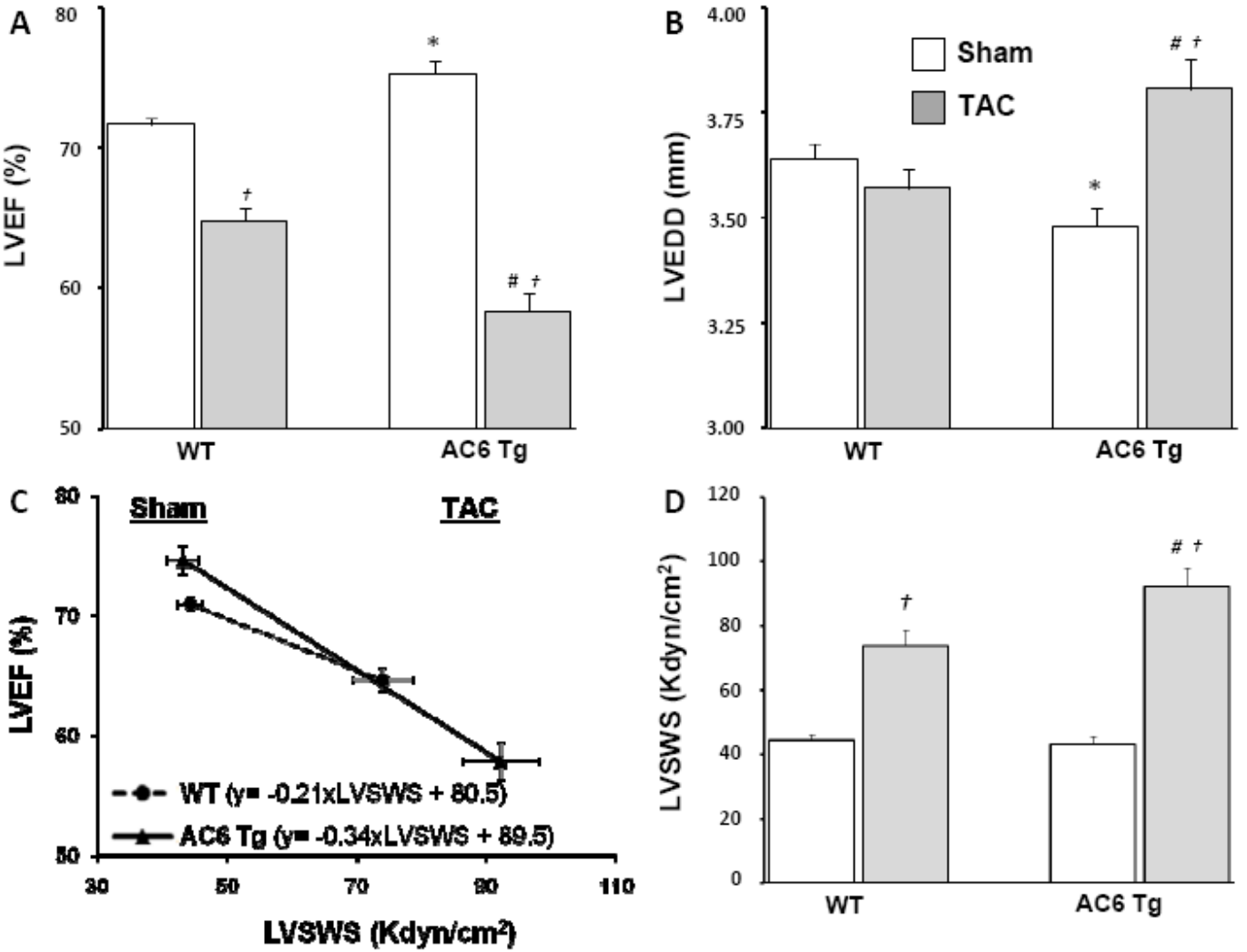
Fig. 1


A
LVEF (%)
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70
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50
*
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WT
AC6 Tg
B
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3.00
Sham
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TAC
LVEF (%)
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60
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WT (y= -0.21xLVSWS + 80.5)
AC6 Tg (y= -0.34xLVSWS + 89.5)
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110
LVSWS (Kdyn/cm²)
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LVSWS (Kdyn/cm²)
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WT
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