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PRE-CLINICAL RESEARCH

Adenylyl Cyclase 6 Improves Calcium Uptake and Left Ventricular Function in Aged Hearts

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Objectives This study tested the hypothesis that activation of adenylyl cyclase 6 (AC6) expression in cardiac myocytes improves calcium uptake and left ventricular (LV) function in aging mice.

Background Aging hearts exhibit impaired β-adrenergic receptor signaling and LV dysfunction.

Methods Twenty-month-old mice with cardiac-directed and regulated AC6 expression were randomized into 2 groups, and AC6 expression was activated in 1 group (AC6-On) but not the other (AC6-Off). One month later, LV function and sarcoplasmic reticulum calcium uptake were assessed.

Results AC6 expression was associated with increased LV contractility, as reflected by ejection fraction ($p = 0.02$), rate of pressure development ($p = 0.002$), and slope of the LV end-systolic pressure-volume relationship ($p = 0.04$). No changes in LV weight to tibial length ratio, LV fibrosis, and expression of fetal genes (atrial natriuretic factor, α-skeletal muscle actin, and β-myosin heavy chain) and collagens were observed between AC6-On and AC6-Off groups. However, LV samples from AC6-On mice showed increases in: isoproterenol-stimulated cAMP production ($p = 0.04$), cAMP-dependent protein kinase activity ($p < 0.0004$), phosphorylation of phospholamban (at Ser16 site; $p = 0.04$) and cardiac troponin I (at Ser23/24 sites; $p = 0.01$), velocity of sarcoplasmic reticulum calcium uptake ($p < 0.0001$), and sarcoplasmic reticulum calcium-ATPase2a (SERCA2a) affinity for calcium ($p < 0.0001$). Finally, we found that AC6 expression increased sarcoplasmic reticulum calcium storage in cardiac myocytes isolated from 23-month-old rats. In contrast, AC6 expression in 7-month-old mice did not change LV function and calcium uptake.

Conclusions These results indicate that activation of cardiac AC6 expression improves impaired function of aged hearts through improved calcium uptake. (J Am Coll Cardiol 2011;57:1846–55) © 2011 by the American College of Cardiology Foundation

Cardiac senescence is associated with reduced left ventricular (LV) function (1–4) and impaired cardiac β-adrenergic receptor (βAR) responsiveness (5). In humans, LV diastolic and systolic functions in response to βAR stimulation progressively decrease after the age of 20 years. At age 80 years, LV contractile reserve is less than one-half of what it was at age 20 years (6). In addition, congestive heart failure (CHF) is a common disease of the elderly (7–9), and older patients with CHF have a particularly poor prognosis (8).

Adenylyl cyclase (AC) is the effector molecule for βAR signaling (10), playing a pivotal role in contractile responsiveness, cardiac relaxation, and LV diastolic function (11). AC catalyzes ATP to generate cAMP, a second messenger that is required for many intracellular events (12). Reduced βAR responsiveness in aging hearts occurs in the presence of increased plasma catecholamine levels (13), underscoring the abnormality in AC signaling (5,14). Indeed, impaired LV cAMP production is associated with decreased cardiac AC content in hearts from animals of advanced age (15,16). However, the precise mechanism by which AC regulates cardiac function in aging hearts is not known and is the focus of the current investigation.

Cardiac-directed expression of AC type 6 (AC6) increases LV function in CHF (17,18), in which impaired βAR-AC signaling and impaired calcium uptake are prominent (19). Increased cardiac cAMP production and improved sarcoplasmic reticulum (SR) calcium uptake are of mechanistic importance for the beneficial effect of AC6 on failing hearts (18,20). In this study, we used a transgenic line with cardiac-directed tetracycline-regulated

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AC6 expression (21,22) to test the hypothesis that activation of cardiac AC6 expression improves LV function by increasing cardiac cAMP production and correcting calcium uptake impairment in hearts from older mice.

Methods

Animals. The Animal Use and Care Committee of the VA San Diego Healthcare System, in accordance with National Institutes of Health and Association for Assessment and Accreditation of Laboratory Animal Care guidelines, approved this study. Twenty-month-old transgenic mice with cardiac myocyte–specific tetracycline-regulated (tet-off) AC6 expression (22) were used for echocardiography, in vivo physiology, and biochemistry studies. Adenylyl cyclase 6 transgene expression is completely suppressed until tetracycline is removed from the water supply, and chronic tetracycline treatment did not affect cardiac function. Mice were randomized into 2 groups. AC6 expression was activated (by removing tetracycline suppression) in one group (AC6-On) and was continuously suppressed by tetracycline in the other group (AC6-Off). These mice were studied 1 month after activation (or continued inactivation) of AC6 transgene expression. For comparison, echocardiography, in vivo physiology, and biochemistry studies were also performed on 7-month-old tetracycline-regulated AC6 mice. For calcium transients study, cardiac myocytes were isolated from 23-month-old Sprague-Dawley rats (Harlan Laboratories, Indianapolis, Indiana).

Echocardiography. Echocardiography was performed under light anesthesia as previously reported (23). Data were acquired and analyzed without knowledge of group identity.

LV in vivo physiologic studies. A 1.4-F conductance-micromanometer catheter was used to measure LV pressure and volume to assess the LV end-systolic pressure-volume relationship (ESPVR) as previously reported (18). Data were acquired and analyzed without knowledge of group identity.

Necropsy and LV fibrosis assessment. Body and LV weights (including septum) and tibial lengths were recorded. A short-axis midwall LV ring was formalin fixed and paraffin embedded. The LV sections were stained with picrosirius red, and collagen fractional area was quantified using NIH ImageJ software (24).

Biochemistry studies. Total RNA extraction and quantitative reverse transcriptase-polymerase chain reaction were performed as previously reported (24). LV samples were homogenized and used for Western blotting as described previously (23). AC activity, cAMP-dependent protein kinase (PKA) activity, and caspase 3/7 activity in LV samples were measured as reported (18).

Calcium uptake. LV tissues were homogenized, and the ATP-dependent initial rate of SR calcium uptake was measured by a modified Millipore filtration technique as reported (20).

Calcium transients. Cardiac myocytes were isolated from adult rats as previously described (25), plated on laminin-coated 25-mm glass coverslips, and infected with adenovirus encoding green fluorescent protein or murine AC6 (400 viral particles/cell). Forty hours after infection, cells were loaded with the calcium-sensitive fluorescent indicator Fura-2 AM (3 μM), and the intracellular calcium concentration was monitored using a digital fluorescence imaging system (Intracellular Imaging, Cincinnati, Ohio), as described previously (23). To assess SR calcium load, caffeine-induced calcium release was initiated by addition of 10 mM caffeine to Tyrode's solution. The peak amplitude of calcium transients was calculated from the baseline and the transient rise after caffeine treatment. Data were acquired and analyzed without knowledge of group identity.

Abbreviations and Acronyms

- **AC** = adenylyl cyclase
- **βAR** = β-adrenergic receptor
- **CHF** = congestive heart failure
- **cTnI** = cardiac troponin I
- **ESPVR** = end-systolic pressure-volume relationship
- **LV** = left ventricular
- **LV/TL** = LV weight to tibial length ratio
- **MMP** = matrix metalloproteinase
- **PKA** = cAMP-dependent protein kinase
- **PLN** = phospholamban
- **SERCA2a** = sarcoplasmic reticulum calcium-ATPase2a
- **SR** = sarcoplasmic reticulum

Statistical analysis. Results are shown as mean ± SE. Group differences were compared using unpaired, 2-tailed Student *t* test. The null hypothesis was rejected when $p < 0.05$.

Results

AC activity in aging hearts. To confirm that aging is associated with decreased AC activity in the heart (5,14,16,26), we measured LV cAMP production in 7- and 20-month-old mice. There were a 43% reduction in basal ($p = 0.0001$), a 56% reduction in isoproterenol-stimulated ($p = 0.04$), and a 58% reduction in NKH477-stimulated LV cAMP production ($p = 0.0001$) in 20- versus 7-month-old mice (Fig. 1A). We also found a 59% reduction of mRNA expression of AC6, a major cardiac AC isoform, in LV samples from 20- versus 7-month-old mouse hearts (Fig. 1B).

Echocardiography. Aging was associated with a decline in LV ejection fraction (7-month-old [n = 13]: 80 ± 3%; 20-month-old [n = 17]: 57 ± 10%; $p < 0.0001$). Both 7- and 20-month-old mice with cardiac-directed and regulated AC6 expression were randomized, and AC6 expression was activated in the AC6-On but not the AC6-Off group. There were no group differences (AC6-On vs. AC6-Off group) in any of the echocardiographic measures before activation of AC6 expression. However, in 20-month-old mice, activation of AC6 expression increased LV ejection fraction (Table 1). These mice also showed reduced LV end-systolic dimension after AC6 expression was activated

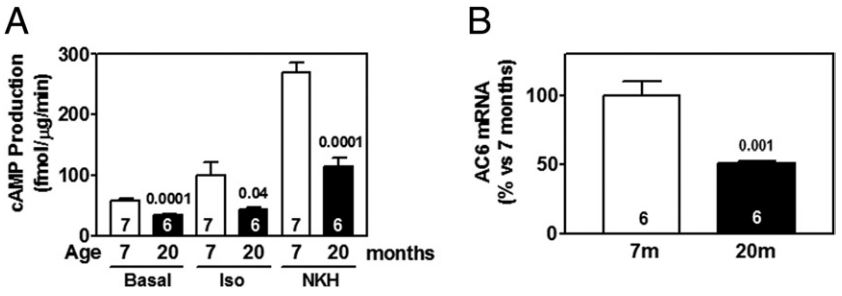

Figure 1 **Aging Associated With Reduced LV AC Activity and AC6 Expression**

(A) Basal and isoproterenol- (+GTPγs) and NKH477 (NKH)-stimulated cAMP production were reduced in left ventricular (LV) samples from 20-month-old versus 7-month-old mice. **(B)** Quantitative real-time reverse transcriptase polymerase chain reaction showed reduced LV expression of adenylyl cyclase 6 (AC6) mRNA in 20-month-old versus 7-month-old mice. Probability values **(values above bars)** are from the Student *t* test (unpaired, 2-tailed). **Error bars** denote 1 SE; **numbers in bars** indicate group size.

($p < 0.05$), consistent with improved LV ejection fraction. Other measures showed no group differences. In 7-month-old mice, activation of cardiac AC6 expression did not change LV ejection fraction or any of the other echocardiographic measures (Table 1).

LV contractile function. Activation of cardiac-directed AC6 expression increased the slope of ESPVR by 2.6-fold in 20-month-old mice (AC6-Off [n = 10]: 1.8 ± 0.6 mm Hg/μl; AC6-On [n = 9]: 4.6 ± 1.0 mm Hg/μl; p = 0.04) (Figs. 2A and 2B). AC6 expression in 20-month-old mice was also associated with an increased rate of LV pressure development (LV + dP/dt) (p = 0.002) (Fig. 2C) and increased stroke work (p = 0.04) (Fig. 2D). There were no group differences in stroke volume (AC6-Off [n = 10]: 19 ± 2 μl; AC6-On [n = 10]: 20 ± 3 μl; p = 0.78) and cardiac output (AC6-Off [n = 10]: 7 ± 1 ml/min; AC6-On [n = 10]: 10 ± 2 ml/min; p = 0.20) in 20-month-old mice. Heart rate was similar in both groups (AC6-Off [n = 10]: 444 ± 31 beats/min; AC6-On [n = 10]: 469 ± 27 beats/min; p = 0.55). However, in 7-month-old mice, activation of AC6 expression did not affect the slope of ESPVR (AC6-Off [n = 7]: 4.3 ± 0.4 mm Hg/μl; AC6-On [n =6]: 4.0 ± 0.6 mm Hg/μl; p = 0.69) and +dP/dt (AC6-Off [n = 7]: 5,448 ± 453 mm Hg/s; AC6-On [n = 6]: 7,285 ± 1,073 mm Hg/s; p = 0.12).

LV relaxation. Activation of cardiac-directed AC6 expression in 20-month-old mice decreased LV −dP/dt (p = 0.03) (Fig. 2E). In contrast, AC6 expression in 7-month-old mice did not affect LV −dP/dt (AC6-Off [n = 7]: −5,260 ± 425 mm Hg/s; AC6-On [n = 6]: −6,827 ± 833 mm Hg/s; p = 0.11).

LV hypertrophy. Activation of cardiac-directed AC6 expression in 20-month-old mice did not change LV weight to tibial length ratio (LV/TL) (AC6-Off [n = 10]: 7.2 ± 0.6 mg/mm; AC6-On [n = 9]: 7.5 ± 0.5 mg/mm; p = 0.71) or cross-sectional cardiac myocyte area in LV sections (AC6-Off [n = 6]: 649 ± 35 μm^2; AC6-On [n = 6]: 663 ± 21 μm^2; p = 0.74). AC6-Off and AC6-On mice showed similar mRNA expression of fetal genes, atrial natriuretic factor (AC6-Off [n = 6]: 100 ± 22%; AC6-On [n = 9]: 86 ± 15%; p = 0.61), α-skeletal muscle actin (AC6-Off [n = 6]: 100 ± 20%; AC6-On [n = 9]: 126 ± 24%; p = 0.45), and β-myosin heavy chain (AC6-Off [n = 6]: 100 ± 19%; AC6-On [n = 9]: 84 ± 32%; p = 0.71). In addition, expression of FHL1, a regulator of LV hypertrophy, showed no group difference (AC6-Off [n = 6]: 100 ± 9%; AC6-On

Table 1 **Echocardiography Measurements**

	7-Month-Old Mice			20-Month-Old Mice		
	AC6-Off (n = 7)	AC6-On (n = 6)	p Value	AC6-Off (n = 8)	AC6-On (n = 9)	p Value
HR, beats/min	597 ± 16	607 ± 14	NS	490 ± 20*	515 ± 16*	NS
EDD, mm	3.9 ± 0.5	3.6 ± 0.6	NS	4.0 ± 0.2†	3.8 ± 0.1†	NS
ESD, mm	2.3 ± 0.4	2.0 ± 0.3	NS	2.8 ± 0.1†	2.3 ± 0.2†	<0.05
EF, %	80 ± 2	81 ± 5	NS	59 ± 3‡	70 ± 4†	<0.02
PWTh, mm	0.6 ± 0.02	0.6 ± 0.03	NS	0.9 ± 0.03‡	0.9 ± 0.02‡	NS
IVSTh, mm	0.6 ± 0.01	0.6 ± 0.02	NS	0.9 ± 0.04‡	0.9 ± 0.02‡	NS

Transgenic mice at ages 7 and 20 months were randomized into 2 groups, respectively. Adenylyl cyclase 6 (AC6) expression was activated in one group (AC6-On) but not the other (AC6-Off) for 1 month. Values represent mean ± SE. Probability values shown are from unpaired, 2-tailed Student *t* tests. *p < 0.002 versus same group at 7 months. †Not significantly different versus same group at 7 months. ‡p < 0.001 versus same group at 7 months.

EDD = end-diastolic diameter; EF = ejection fraction; ESD = end-systolic diameter; HR = heart rate; IVSTh = interventricular septum wall thickness; NS = not significant; PWTh = posterior wall thickness.

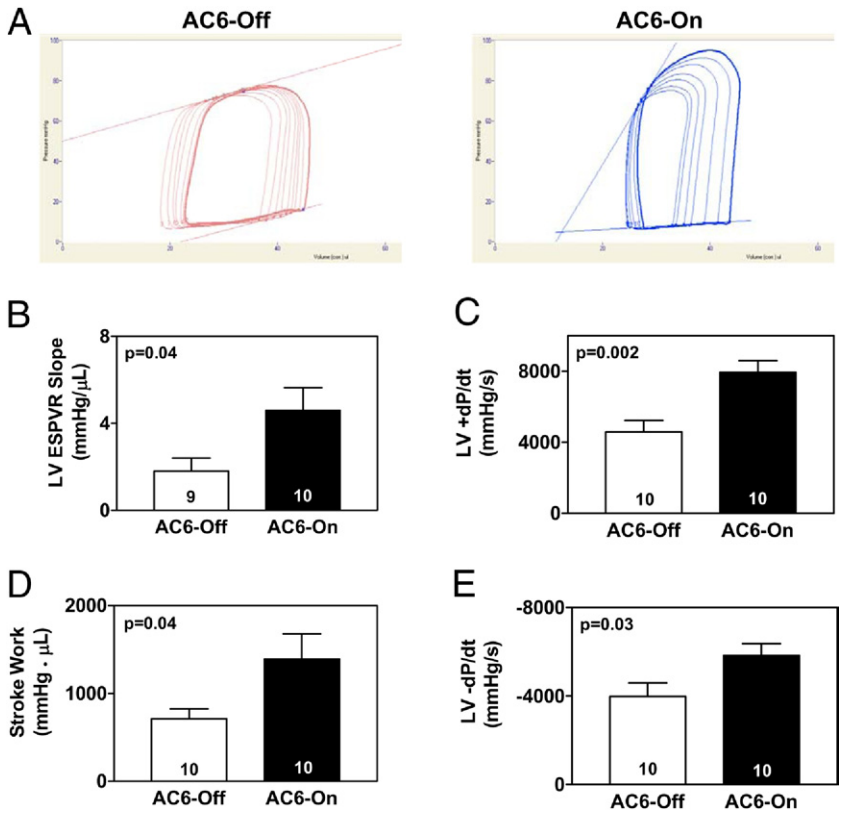

Figure 2 **Cardiac-Directed AC6 Expression Increased LV Function in 20-Month-Old Mice**

(A) LV pressure-volume loops, generated by inferior vena caval occlusion, are shown. **(B)** Activation of AC6 expression increased the slope of the end-systolic pressure-volume relationship (ESPVR) in AC6-On mice compared with AC6-Off mice. **(C)** AC6-On mice showed increased +dP/dt compared with AC6-Off mice. **(D)** Activation of AC6 expression increased stroke work in hearts from 20-month-old mice. **(E)** There was a substantial increase in −dP/dt in AC6-On mouse hearts. Probability values shown are from the Student *t* test (unpaired, 2-tailed). **Error bars** denote 1 SE; **numbers in bars** indicate group size. Abbreviations as in Figure 1.

[n = 9]: 113 ± 12%; p = 0.47). Similarly, activation of cardiac AC6 expression in 7-month-old mice did not change LV/TL (AC6-Off [n = 7]: 6.4 ± 0.4 mg/mm; AC6-On [n = 6]: 6.2 ± 0.3 mg/mm; p = 0.71) or mRNA expression of fetal genes and FHL1. Although activation of AC6 expression in 7-month-old mice did not change cross-sectional cardiac myocyte area in LV sections (AC6-Off [n = 6]: 489 ± 21 μm^2; AC6-On [n = 6]: 465 ± 27 μm^2; p = 0.50), aging was associated with an increase (7-month-old [n = 6]: 489 ± 21 μm^2; 20-month-old [n = 6]: 649 ± 35 μm^2; p = 0.003).

Calcium uptake. We compared ATP-dependent initial SR calcium uptake in LV homogenates from 20-month-old AC6-Off and AC6-On mice. Activation of AC6 increased the velocity of calcium uptake in the presence of 0.2 μM and 2 μM calcium (Fig. 3A), the approximate intracellular calcium concentration range in cardiac myocytes (27). AC6 expression also increased sarcoplasmic reticulum calcium-ATPase (SERCA2a) affinity for calcium (Fig. 3B). Increased calcium uptake and SERCA2a affinity for calcium would be expected to increase cardiac contractile function as we observed. In contrast, activation of AC6 expression in 7-month-old mice did not change the velocity of calcium uptake (Fig. 3C) and SERCA2a affinity for calcium (Fig. 3D).

In addition, we isolated cardiac myocytes from 23-month-old rats, infected these cells with adenovirus encoding AC6, and measured the intracellular calcium concentration under basal and caffeine-stimulated conditions (Fig. 4A). There was no group difference in basal intracellular calcium concentration in cardiac myocytes (Fig. 4B). However, there was a 1.5-fold increase in the peak amplitude of caffeine-stimulated calcium transients associated with AC6 expression in cardiac myocytes from aging rats (Fig. 4C). These results indicated that AC6 expression increased calcium storage in aging cardiac myocytes.

AC6 expression and cAMP production. In 20-month-old mice, cardiac-directed AC6 transgene expression was associated with a 10-fold increase in LV AC6 protein content (Figs. 5A and 5B). LV expression of other cardiac AC isoforms (AC2, AC3, AC4, AC5, AC7, and AC9) was unchanged in these mice (Fig. 5C). Increased AC6 protein content was associated with a 1.7-fold increase in

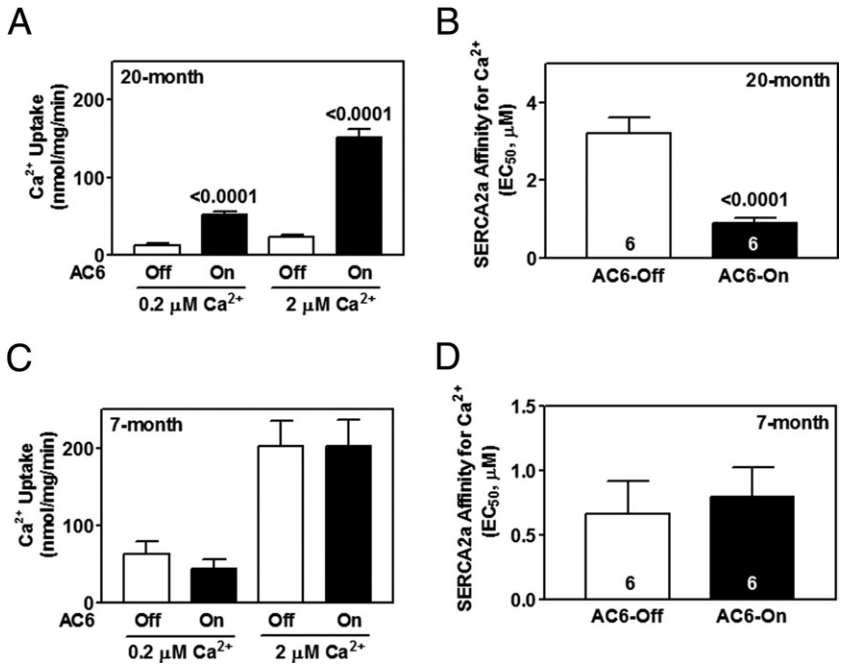

Figure 3 **Activation of Cardiac AC6 Expression Increased Calcium Uptake in 20-Month-Old But Not 7-Month-Old Mice**

(A) Cardiac-directed AC6 expression improved sarcoplasmic reticulum (SR) calcium uptake in the presence of 0.2 and 2 μM calcium in LV samples from 20-month-old mice (n = 6 for each group). **(B)** Cardiac-directed AC6 expression increased SR calcium-ATPase2a (SERCA2a) affinity for calcium in LV samples from 20-month-old mice. **(C)** AC6 expression did not affect SR calcium uptake in the presence of 0.2 μM (p = 0.33) and 2 μM calcium (p = 1.00) in LV samples from 7-month-old mice (n = 6 for each group). **(D)** AC6 expression did not affect SERCA2a affinity for calcium in LV samples from 7-month-old mice (p = 0.72). Probability values are from the Student *t* test (unpaired, 2-tailed). **Error bars** denote 1 SE; **numbers in bars** indicate group size. Abbreviations as in Figure 1.

isoproterenol-stimulated cAMP production ($p < 0.01$) and a 3-fold increase in forskolin-stimulated cAMP production ($p < 0.002$) in LV samples from 20-month-old mice, and basal LV cAMP production was not changed (Fig. 5D). Despite unchanged protein expression of PKA catalytic subunit (Fig. 5E), PKA activity was increased after activation of AC6 expression (Fig. 5F), indicating a role of AC6-increased cAMP production in regulating PKA activity in the aging heart.

Protein phosphorylation. We compared PKA-induced phosphorylation of cardiac troponin I (cTnI) and phospholamban (PLN) in LV homogenates from 20-month-old AC6-Off and AC6-On mice. Activation of cardiac AC6 expression in these mice was associated with a 1.9-fold increase in phosphorylation of cTnI at the Ser23/24 sites ($p = 0.01$) (Figs. 6A and 6B). No change was found in total cTnI protein content. There was also a 1.9-fold increase in phosphorylation of PLN at the Ser16 site in LV samples from 20-month-old AC6-On mice compared with that of AC6-Off mice ($p = 0.04$; Figs. 6A and 6C), although total PLN protein content was unaltered. Western blotting showed no group differences in protein content of protein phosphatase 1 (AC6-Off [n = 6]: 1,152 ± 195 densitometric units (du); AC6-On [n = 9]: 1,153 ± 277 du; $p = 1.00$) or protein phosphatase 2A (AC6-Off [n = 6]: 743 ± 104 du; AC6-On [n = 9]: 604 ± 65 du; $p = 0.25$), indicating a selective role of AC6-increased PKA activity in regulating phosphorylation of cTnI and PLN. In addition, we found no group differences in expression of SR calcium pump SERCA2a (Fig. 6D), the calcium-binding protein calsequestrin (Fig. 6E), or Na^+-Ca^{2+} exchanger 1 (Fig. 6F).

Extracellular matrix. Extracellular matrix remodeling, a process that involves changes of collagen synthesis, degradation, and cross-linking, leads to increased number and size of collagen fibers within interstitial spaces. There was substantial collagen deposition in LV sections from 20-month-old mice compared with those from 7-month-old mice (collagen fractional area: 7-month-old [n = 7]: 3.1 ± 1.1%; 20-month-old [n = 6]: 15.4 ± 2.5%; $p < 0.001$); however, activation of cardiac AC6 expression did not change collagen fractional area in 20-month-old mouse hearts (AC6-Off [n = 6]: 15.4 ± 2.5%; AC6-On [n = 9]: 14.4 ± 2.0%; $p = 0.77$).

We compared mRNA content of type I and III collagens, 2 major constituents of the cardiac extracellular matrix, in LV samples from 20-month-old AC6-Off and AC6-On mice. Activation of AC6 expression did not change mRNA expression of collagen Iα1 (AC6-Off [n = 6]: 100 ± 14%; AC6-On [n = 9]: 129 ± 19%; $p = 0.30$) or IIIα1 (AC6-Off [n = 6]: 100 ± 29%; AC6-On [n = 9]: 85 ± 29%; $p = 0.73$). Quantitative reverse transcriptase-

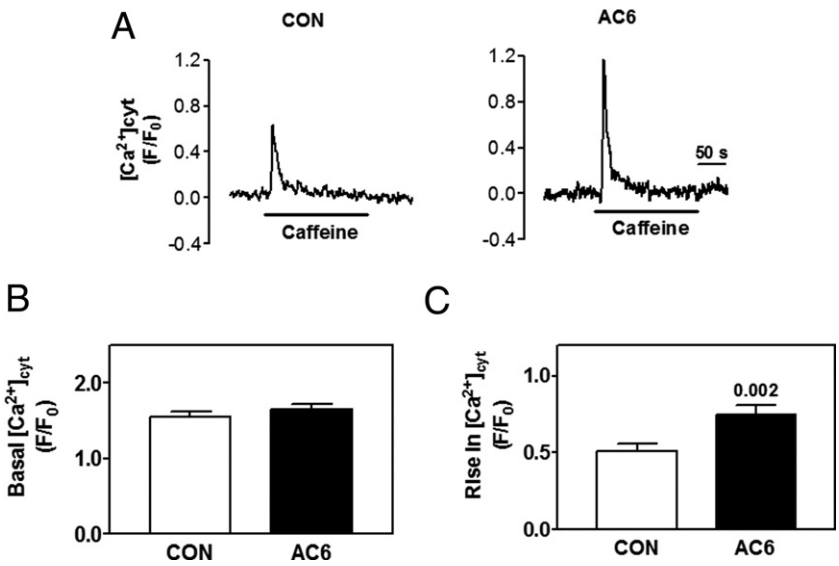

Figure 4 **Increased Calcium Storage in Cardiac Myocytes From 23-Month-Old Rats Following AC6 Gene Transfer**

(A) Representative calcium transients recorded from cardiac myocytes stimulated with caffeine. Cardiac myocyte infected with adenovirus encoding green fluorescent protein (CON) **(left panel)**; cardiac myocyte infected with adenovirus encoding AC6 **(right panel)**. **(B)** The bar graph shows no group difference in basal intracellular calcium concentration in cardiac myocytes. Data were derived from 67 CON cells and 77 AC6 cells (blinded; p = 0.40). **(C)** AC6 expression increased the peak amplitude of caffeine-stimulated calcium transients recorded from cardiac myocytes. Data were derived from 41 CON cells and 51 AC6 cells (blinded). Probability values shown are from the Student *t* test (unpaired, 2-tailed). **Error bars** denote 1 SE. Abbreviations as in Figure 1.

polymerase chain reaction also showed that AC6 expression did not change the mRNA content of matrix metalloproteinase 2 (MMP2) (AC6-Off [n = 6]: 100 ± 14%; AC6-On [n = 9]: 135 ± 20%; p = 0.22) or MMP8 (AC6-Off [n = 6]: 100 ± 16%; AC6-On [n = 9]: 87 ± 14%; p = 0.55). Similar levels of periostin mRNA, a regulator of cardiac fibrosis, were seen in LV samples from 20-month-old AC6-Off and AC6-On mice (AC6-Off [n = 6]: 100 ± 16%; AC6-On [n = 9]: 121 ± 19%; p = 0.44). There were no group differences in mRNA expression of elastin (AC6-Off [n = 6]: 100 ± 10%; AC6-On [n = 9]: 121 ± 18%; p = 0.69) or fibronectin (AC6-Off [n = 6]: 100 ± 17%; AC6-On [n = 9]: 89 ± 19%; p = 0.39).

Cardiac myocyte apoptosis. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling was used to evaluate apoptosis. In 20-month-old mouse hearts, AC6 expression did not change the apoptosis rate (data not shown). AC6-Off and AC6-On mice showed comparable LV mRNA expression of Bcl2 (AC6-Off [n = 6]: 100 ± 27%; AC6-On [n = 9]: 104 ± 18%; p = 0.89), an important apoptosis inhibitor. Increased AC6 expression in 20-month-old mouse hearts did not change LV caspase 3/7 activity (AC6-Off [n = 6]: 40.3 ± 2.4 U/mg; AC6-On [n = 9]: 37.6 ± 2.0 U/mg; p = 0.41). Finally, no group difference in expression of superoxidase dismutase 2 was seen after activation of AC6 expression in 20-month-old mice (AC6-Off [n = 6]: 100 ± 6%; AC6-On [n = 9]: 93 ± 6%; p = 0.46).

Discussion

The most important finding of this study is that activation of cardiac AC6 expression improved aging-impaired LV contractile function and relaxation. These beneficial effects were associated with increases in cAMP production, PKA activity, and phosphorylation of PLN and cTnI. Moreover, both SR calcium uptake and SR calcium storage in cardiac myocytes were increased following activation of AC6 expression. Improved SR calcium uptake appears to be the mechanism by which AC6 expression evokes these beneficial effects.

Results from our previous studies (17,18,28,29) indicate that increased cardiac AC6 has beneficial effects in the failing heart. However, increased expression of other elements of the βAR signaling pathway (e.g., β_1AR, Gαs, PKA) is associated with pronounced deleterious effects on the heart, including precipitation of heart failure, fibrosis, and increased mortality (30–33). These results indicate that AC6 is unique among βAR signaling elements in regulating cardiac function. In clinical settings, increased age is associated with reduced LV function (1–4), and elderly patients have an increased prevalence of heart failure (7–9). In this study, we tested the hypothesis that activation of AC6 expression increases function of the aging heart. We assessed LV contractile function by 3 different methods: LV ejection fraction, +dP/dt, and slope of ESPVR (34). Each of these measures led to the same conclusion that increased expression of cardiac AC6 improves LV contractile function in the aging heart.

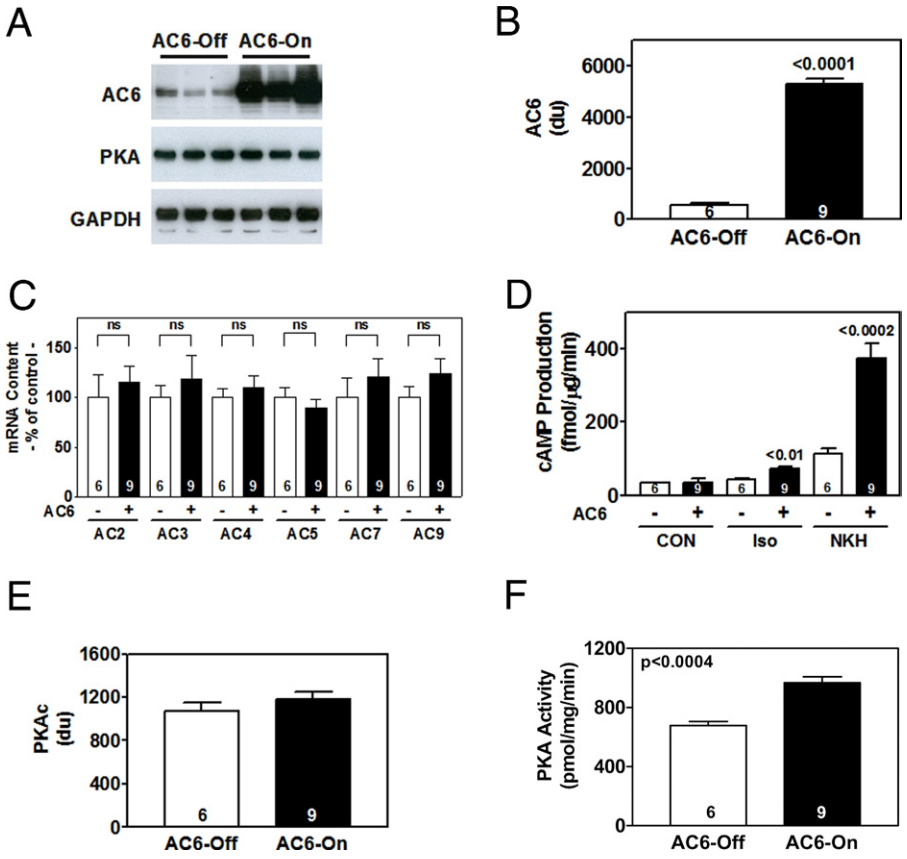

Figure 5 Cardiac-Directed AC6 Expression Activated cAMP Signaling in 20-Month-Old Mouse Hearts

(A) Representative Western blot shows marked increase in AC6 protein content in LV samples from AC6-On mice. **(B)** The bar graph summarizes AC6 content in LV samples from AC6-Off and AC6-On mice. **(C)** AC6 expression did not affect expression of other AC isoforms in the heart. **(D)** AC6 expression was associated with increased LV cAMP generation capacity. **(E)** No group difference was observed in protein content of cAMP-dependent protein kinase (PKA) catalytic subunit ($p = 0.30$). **(F)** AC6-On mice showed increased PKA activity. Probability values are from the Student *t* test (unpaired, 2-tailed). **Error bars** denote 1 SE; **numbers in bars** indicate group size. du = densitometric unit; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; other abbreviations as in Figure 1.

Calcium plays a crucial role in controlling LV contraction and relaxation. During every heartbeat, calcium is taken up and then released from the SR. Aged hearts show dysfunctional SR calcium uptake (35,36), which reflects reduced SR calcium pump SERCA2a content (37,38) and reduced PLN phosphorylation (37–39). In the current study, we found that activation of AC6 expression increased PLN phosphorylation at the Ser16 site in 20-month-old mice, whereas it did not change SERCA2a protein expression (Fig. 6). This change of PLN phosphorylation was associated with increased cAMP-generating capacity and PKA activity. Most importantly, activation of cardiac AC6 expression increased SR calcium uptake and SERCA2a affinity for calcium in these 20-month-old mice (Figs. 3A and 3B). These results indicate that AC6 expression improves calcium uptake by activating a signaling cascade leading to increased PLN phosphorylation in 20-month-old mice. Improvement of calcium uptake may be of mechanistic importance for the observed increased LV function after AC6 expression (Fig. 2), given the fact that deletion of AC6 decreased calcium uptake and impaired LV function (23). This notion is further supported by our findings that activation of AC6 expression did not improve SR calcium uptake and SERCA2a affinity for calcium in 7-month-old mouse hearts (Figs. 3C and 3D), where LV contractile function was unaffected.

Cardiac myocytes isolated from the hearts of 20-month-old mice may not be suitable for accurate studies for calcium transient measurements. For example, despite our substantial experience in isolated cardiac myocytes from mice, we found a >25% cell death rate and spontaneous beating in one-third of the viable cells. Because of these unavoidable problems, we elected to use instead cardiac myocytes isolated from elderly rats for calcium transient measurements. We used rats at the age of 23 months, rather than 20 months, to compensate in part for life span difference between mice and rats. Our data from caffeine-stimulated SR calcium transient measurements provided direct evidence that AC6 expression increased SR storage. But more importantly, we showed that AC6 expression increased SR

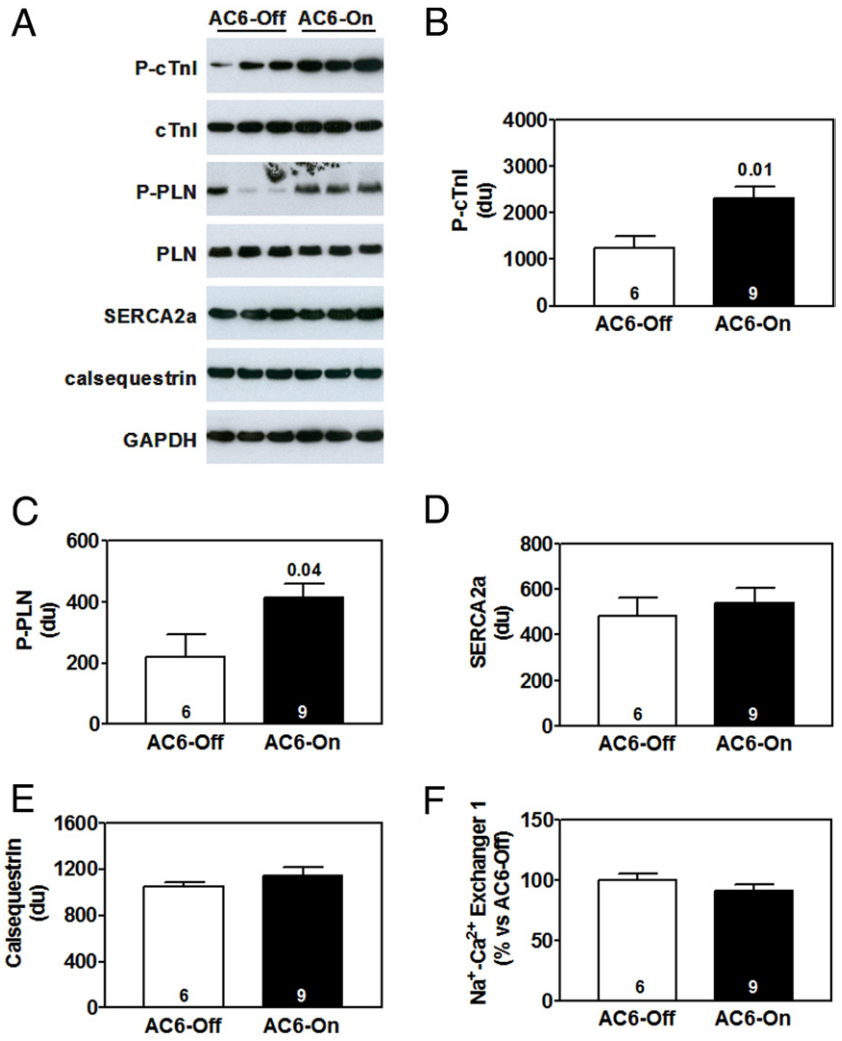

Figure 6 **Cardiac-Directed AC6 Expression Increased Phosphorylation of cTnI and PLN in 20-Month-Old Mouse Hearts**

(A) Representative Western blots show marked increase in protein content of phospho–cardiac troponin I (cTnI) (Ser23/24) and phospho-phospholamban (PLN) (Ser16) in AC6-On mouse hearts. **(B)** The bar graph summarizes protein content of phospho-cTnI from Western blotting. **(C)** The bar graph summarizes protein content of phospho-PLN from Western blotting. **(D)** No group difference was observed in protein content of SERCA2a ($p = 0.61$). **(E)** No group difference was seen in protein content of calsequestrin ($p = 0.37$). **(F)** AC6 expression did not change mRNA expression of Na^{+}-Ca^{2+} exchanger 1 ($p = 0.27$). Probability values shown are from the Student *t* test. **Error bars** denote 1 SE; **numbers in bars** indicate group size. Abbreviations as in Figures 1, 3, and 5.

calcium uptake using LV samples directly from our 20-month-old mice.

Cardiac troponin I is a sarcomeric protein directly regulating the LV function in concert with intracellular calcium signals. In addition to decreased PLN phosphorylation (at the Ser16 site), decreased cTnI phosphorylation (at the Ser23/24 sites) is also a seminal alteration underlying impaired LV function of failing human hearts (40,41). The relative contributions of phosphorylation of cTnI versus PLN are not clear (42). Nevertheless, cTnI phosphorylation increases maximal tension development, cross-bridge kinetics, and systolic power production (42,43). Expression of pseudophosphorylated cTnI at the Ser23/24 sites increased LV contractility in mice (44). Moreover, improved LV function by cardiac AC6 expression is linked to increased cTnI phosphorylation at the Ser23/24 sites in failing hearts (18). Importantly, cardiac aging is associated with decreased cTnI phosphorylation (45), suggesting that cTnI phosphorylation is also a contributing factor for reduced LV function in aging hearts. In the present study, we showed that increased LV function was correlated with increased cTnI phosphorylation after activation of AC6 expression in aging hearts (Fig. 6). These results suggest that AC6 expression, in addition to its effects on SR calcium uptake, directly regulates the property of thin filament in the contractile apparatus.

The extracellular matrix, composed mainly of fibrillar collagens (46), provides an architectural scaffold for cardiac cells and contributes to the maintenance of LV function

(47,48). In this study, we confirmed that cardiac aging is associated with increased collagen deposition. However, activation of AC6 expression in 20-month-old mice did not change LV collagen deposition, indicating that the improved LV function does not likely reflect a change in LV collagen content. Additional support for this notion is our finding that AC6 expression did not change expression of type I and III collagens, the major cardiac collagen isoforms contributing 75% to 80% (type I) and 15% to 20% (type III) of total collagen content (49). Expression of MMPs and periostin was not changed by activation of AC6 expression, although aging is associated with increased expression of these important extracellular matrix proteins (50,51). In addition, our data showed that increased cardiac AC6 expression in 20-month-old mice was not associated with changes in apoptosis or reactive oxygen species. Taken together, our results suggest that activation of AC6 expression improves LV function through increased SR calcium uptake.

Whether LV hypertrophy in aged mice affected our results is an important consideration. Although posterolateral and septal wall thicknesses at the mid-papillary muscle level were increased (0.3 mm) in aged mice (Table 1), the echocardiographic estimate of LV mass, which considers more than a single plane, showed no difference between mice age 20 months versus 7 months (data not shown). Cardiac myocyte cross-sectional area was increased, but the 13% increase in LV/TL did not reach statistical significance. The disparity between cross-sectional area and LV mass at necropsy vis-à-vis LV hypertrophy in 7- versus 20-month-old mice may reflect LV hypertrophy associated with concomitant aging-related increases in cardiac myocyte apoptosis (52) or the limitations of assessing cardiac myocyte cross-sectional area in hearts not obtained after diastolic arrest. Similarly, others have documented that, in aged rats, despite increased myocyte cross-sectional area (52), LV mass and LV/TL were not increased (53). Our study showed that activation of cardiac AC6 expression, which had no independent effect on LV hypertrophy, improved aging-impaired LV contractile function and relaxation. Improved SR calcium uptake appears to be of mechanistic importance for these beneficial effects.

Gene manipulation has recently been promoted to probe disease mechanism and test treatments designed to attenuate the progression of aging-related diseases. Transgenic lines with increased or decreased expression of a candidate gene typically are used, and if deterioration of heart function is prevented, the candidate gene (or deletion of this gene) is said to have "rescued" the heart. However, a superior strategy, and one used in the present study, is to activate gene expression only when the adverse effect of aging is present. We accomplished this by activating AC6 expression in the setting of declining heart function in aged mice. Although additional studies will be required to determine whether activation of cardiac AC6 expression reduces mortality, our current data suggest a therapeutic potential for increased AC6 expression in aging hearts.

Conclusions

Activation of cardiac-directed AC6 expression improves LV function in aged hearts. This is associated with increased cAMP production and PKA activity and phosphorylation of PLN and cTnI. Improved SR calcium uptake in cardiac myocytes appears to be of mechanistic importance for these beneficial effects.

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REFERENCES

1. Gerstenblith G. Echocardiographic assessment of a normal adult aging population. Circulation 1977;56:273–8.
2. Fraticelli A, Josephson R, Danziger R, Lakatta EG, Spurgeon H. Morphological and contractile characteristics of rat cardiac myocytes from maturation to senescence. Am J Physiol Heart Circ Physiol 1989;257:H259–65.
3. Chiamvimonvat N. Diastolic dysfunction and the aging heart. J Mol Cell Cardiol 2002;34:607–10.
4. Dai DF, Santana LF, Vermulst M, et al. Overexpression of catalase targeted to mitochondria attenuates murine cardiac aging. Circulation 2009;119:2789–97.
5. White M, Roden R, Minobe W, et al. Age-related changes in beta-adrenergic neuroeffector systems in the human heart. Circulation 1994;90:1225–38.
6. Lakatta EG, Sollott SJ. The "heartbreak" of older age. Mol Interv 2002;2:431–46.
7. Lakatta EG. Age-associated cardiovascular changes in health: impact on cardiovascular disease in older persons. Heart Failure Rev 2002;7:29–49.
8. Lakatta EG, Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: part II: the aging heart in health: links to heart disease. Circulation 2003;107:346–54.
9. Marian AJ. Beta-adrenergic receptors signaling and heart failure in mice, rabbits and humans. J Mol Cell Cardiol 2006;41:11–3.
10. Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. Nature 2002;415:206–12.
11. Feldman AM. Adenylyl cyclase: a new target for heart failure therapeutics. Circulation 2002;105:1876–8.
12. Katsushika S, Chen L, Kawabe J, et al. Cloning and characterization of a sixth adenylyl cyclase isoform: types V and VI constitute a subgroup within the mammalian adenylyl cyclase family. Proc Natl Acad Sci U S A 1992;89:8774–8.
13. Rowe JW, Troen BR. Sympathetic nervous system and aging in man. Endocr Rev 1980;1:167–79.
14. Narayanan N, Tucker L. Autonomic interactions in the aging heart: age-associated decrease in muscarinic cholinergic receptor mediated inhibition of beta-adrenergic activation of adenylate cyclase. Mech Ageing Dev 1986;34:249–59.
15. Shu Y, Scarpace PJ. Forskolin binding sites and G-protein immunoreactivity in rat hearts during aging. J Cardiovasc Pharmacol 1994;23:188–93.
16. Tobise K, Ishikawa Y, Holmer SR, et al. Changes in type VI adenylyl cyclase expression correlate with a decreased capacity for cAMP generation in the aging ventricle. Circ Res 1994;74:596–603.
17. Roth DM, Gao MH, Lai NC, et al. Cardiac-directed adenylyl cyclase expression improves heart function in murine cardiomyopathy. Circulation 1999;99:3099–102.
18. Lai NC, Tang T, Gao MH, et al. Activation of cardiac adenylyl cyclase expression increases function of the failing ischemic heart in mice. J Am Coll Cardiol 2008;51:1490–7.

19. Mann DL, Bristow MR. Mechanisms and models in heart failure: the biomechanical model and beyond. Circulation 2005;111:2837–49.
20. Tang T, Gao MH, Roth DM, Guo T, Hammond HK. Adenylyl cyclase type VI corrects cardiac sarcoplasmic reticulum calcium uptake defects in cardiomyopathy. Am J Physiol Heart Circ Physiol 2004; 287:H1906–12.
21. Fishman GI. Timing is everything in life: conditional transgene expression in the cardiovascular system. Circ Res 1998;82:837–44.
22. Gao MH, Bayat H, Roth DM, et al. Controlled expression of cardiac-directed adenylylcyclase type VI provides increased contractile function. Cardiovasc Res 2002;56:197–204.
23. Tang T, Gao MH, Lai NC, et al. Adenylyl cyclase type 6 deletion decreases left ventricular function via impaired calcium handling. Circulation 2008;117:61–9.
24. Tang T, Lai NC, Hammond HK, et al. Adenylyl cyclase 6 deletion reduces LV hypertrophy, dilation, dysfunction and fibrosis in pressure-overloaded female mice. J Am Coll Cardiol 2010;55:1476–86.
25. Tang T, Lai NC, Roth DM, et al. Adenylyl cyclase type V deletion increases basal left ventricular function and reduces left ventricular contractile responsiveness to beta-adrenergic stimulation. Basic Res Cardiol 2006;101:117–26.
26. O'Connor SW, Scarpace PJ, Abrass IB. Age-associated decrease of adenylate cyclase activity in rat myocardium. Mech Ageing Dev 1981;16:91–5.
27. Bers DM. Calcium fluxes involved in control of cardiac myocyte contraction. Circ Res 2000;87:275–81.
28. Lai NC, Roth DM, Gao MH, et al. Intracoronary adenovirus encoding adenylyl cyclase VI increases left ventricular function in heart failure. Circulation 2004;110:330–6.
29. Roth DM, Bayat H, Drumm JD, et al. Adenylyl cyclase increases survival in cardiomyopathy. Circulation 2002;105:1989–94.
30. Gaussin V, Tomlinson JE, Depre C, et al. Common genomic response in different mouse models of beta-adrenergic-induced cardiomyopathy. Circulation 2003;108:2926–33.
31. Antos CL, Frey N, Marx SO, et al. Dilated cardiomyopathy and sudden death resulting from constitutive activation of protein kinase A. Circ Res 2001;89:997–1004.
32. 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;96:7059–64.
33. Iwase M, Uechi M, Vatner DE, et al. Cardiomyopathy induced by cardiac Gsα overexpression. Am J Physiol 1997;272:H585–9.
34. Hess OM, Carroll JD. Clinical assessment of heart failure. In: Libby P, Bonow RO, Mann DL, Zipes DP, editors. Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine. 8th edition. Philadelphia, PA: Saunders, 2008;561–81.
35. Orchard CH, Lakatta EG. Intracellular calcium transients and developed tensions in rat heart muscle. A mechanism for the negative interval-strength relationship. J Gen Physiol 1985;86:637–51.
36. Froehlich JP, Lakatta EG, Beard E, Spurgeon HA, Weisfeldt ML, Gerstenblith G. Studies of sarcoplasmic reticulum function and contraction duration in young and aged rat myocardium. J Mol Coll Cardiol 1978;10:427–38.
37. Taffet GE, Tate CA. CaATPase content is lower in cardiac sarcoplasmic reticulum isolated from old rats. Am J Physiol Heart Circ Physiol 1993;264:H1609–14.
38. Xu A, Narayanan N. Effects of aging on sarcoplasmic reticulum Ca^{2+}-cycling proteins and their phosphorylation in rat myocardium. Am J Physiol Heart Circ Physiol 1998;275:H2087–94.
39. Jiang MT, Narayanan N. Effects of aging on phospholamban phosphorylation and calcium transport in rat cardiac sarcoplasmic reticulum. Mech Ageing Dev 1990;54:87–101.
40. Messer AE, Jacques AM, Marston SB. Troponin phosphorylation and regulatory function in human heart muscle: dephosphorylation of Ser23/24 on troponin I could account for the contractile defect in end-stage heart failure. J Mol Cell Cardiol 2007;42:247–59.
41. MacLennan DH, Kranias EG. Phospholamban: a crucial regulator of cardiac contractility. Nat Rev Mol Cell Biol 2003;4:566–77.
42. Metzger JM, Westfall MV. Covalent and noncovalent modification of thin filament action: the essential role of troponin in cardiac muscle regulation. Circ Res 2004;94:146–58.
43. Solaro RJ. Modulation of cardiac myofilament activity by protein phosphorylation. In: Fozzarad H, Solaro RJ, editors. Handbook of Physiology, Volume 1, the Heart. New York, NY: Oxford University Press, 2002;264–300.
44. Takimoto E, Soergel DG, Janssen PM, Stull LB, Kass DA, Murphy AM. Frequency- and afterload-dependent cardiac modulation in vivo by troponin I with constitutively active protein kinase A phosphorylation sites. Circ Res 2004;94:496–504.
45. Maejima Y, Adachi S, Ito H, Hirao K, Isobe M. Induction of premature senescence in cardiomyocytes by doxorubicin as a novel mechanism of myocardial damage. Aging Cell 2008;7:125–36.
46. Jugdutt BI. Extracellular matrix and cardiac remodeling. In: Villarreal F, editor. Interstitial Fibrosis in Heart Failure. New York, NY: Springer, 2004;23–55.
47. Lindsey ML, Borg TK. Understanding the role of the extracellular matrix in cardiovascular development and disease: where do we go from here? J Mol Cell Cardiol 2010;48:431–2.
48. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002;3:349–63.
49. Legrice I, Pope A, Smaill B. The architecture of the heart: myocyte organization and the cardiac extracellular matrix. In: Villarreal F, editor. Interstitial Fibrosis in Heart Failure. New York, NY: Springer, 2004;3–21.
50. Lindsey ML, Goshorn DK, Squires CE, et al. Age-dependent changes in myocardial matrix metalloproteinase/tissue inhibitor of metalloproteinase profiles and fibroblast function. Cardiovasc Res 2005;66: 410–9.
51. Borg TK, Markwald R. Periostin: more than just an adhesion molecule. Circ Res 2007;101:230–1.
52. Anversa P, Hiler B, Ricci R, Guideri G, Olivetti G. Myocyte cell loss and myocyte hypertrophy in the aging rat heart. J Am Coll Cardiol 1986;8:1441–8.
53. Raya TE, Gaballa M, Anderson P, Goldman S. Left ventricular function and remodeling after myocardial infarction in aging rats. Am J Physiol Heart Circ Physiol 1997;273:H2652–8.

Key Words: adenylyl cyclase ■ calcium uptake ■ cardiac aging ■ extracellular matrix remodeling ■ left ventricular function ■ transgenic mouse.