Adenylyl Cyclase Increases Survival in Cardiomyopathy

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Background—To test the hypothesis that increased cardiac adenylyl cyclase type VI (AC VI) content, which results in increased cAMP generation, would increase survival in cardiomyopathy, we crossbred mice with Gq-associated cardiomyopathy and those with cardiac-directed expression of AC VI. We also assessed myocardial hypertrophy after prolonged cardiac expression of Gq versus coexpression of Gq and AC VI.

Methods and Results—Three experimental groups, Gq/AC (double positive), Gq, and control (double negative), were studied. Survival was increased by cardiac-directed expression of AC VI (P<0.0001), and Gq/AC mice had survival rates indistinguishable from control mice. Myocardial hypertrophy developed in older Gq mice but was abrogated by cardiac expression of AC VI, as documented by the ratio of ventricular weight to tibial length (Gq, 11.93±0.99 mg/mm, n=11; Gq/AC, 8.00±0.73 mg/mm, n=9; P<0.01) and by left ventricular cardiac myocyte size (Gq, 2800±254 μm², n=4; Gq/AC, 1721±166 μm², n=5; P<0.01). Hearts of Gq mice were dilated, and function was impaired. Concurrent expression of AC reduced end-diastolic diameter (Gq, 4.20±0.15 mm, n=12; Gq/AC, 3.68±0.12 mm, n=7; P<0.05) and increased fractional shortening (Gq, 32±1%, n=12; Gq/AC, 41±2%, n=7; P<0.001). Cardiac myocytes from Gq/AC mice showed increased forskolin-stimulated cAMP production (Gq, 3.8±1.3 fmol/cell, n=5; Gq/AC, 10.7±2.6 fmol/cell, n=6; P<0.02), documenting increased AC function.

Conclusions—Cardiac-directed expression of AC VI restores myocyte AC function, improves heart function, increases cAMP generation, abrogates myocardial hypertrophy, and increases survival in Gq cardiomyopathy. (Circulation. 2002; 105:1989-1994.)

Key Words: receptors, adrenergic, beta ■ heart failure ■ myocytes

A denylyl cyclase (AC) is the effector molecule in β-adrenergic receptor (BAR) signaling, catalyzing conversion of ATP to cAMP.1 Agents that increase intracellular levels of cAMP have been used to treat clinical heart failure,2–4 which is a sound rationale, because failing hearts have reduced amounts of cAMP5 and impaired contractile function. Ironically, results of these clinical trials have been disappointing, perhaps because the agents used (BAR agonists and milrinone) provided sustained increases of intracellular cAMP or have other adverse effects. In contrast, sustained increases in cAMP were not observed in cardiac myocytes overexpressing AC isoform VI (AC VI),6–8 so therapy directed toward restoring cardiac AC function, which is reduced in heart failure, may yield different results than those obtained with agents that produce sustained elevations in intracellular cAMP levels.

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The study of transgenic mice provides a means to explore this concept. In otherwise normal mice, cardiac-directed expression of AC VI resulted in increased cardiac function when stimulated but a lifelong absence of adverse effects.6 Furthermore, cardiac-directed expression of AC VI or AC V in cardiomyopathy has favorable effects on cardiac function when animals are studied at 3 to 4 months of age.7,9 However, the long-term effects of cardiac-directed AC expression in the setting of cardiomyopathy and its impact on survival is completely unexplored. Evaluating long-term effects is important, because cardiac-directed expression of β1AR,10 β2AR,11 and Gs12,13 all associated with initial increases in cardiac function, have deleterious cardiac effects when examined later in life or when coexpressed in cardiomyopathic hearts. These observations10–12 align well with disappointing results from clinical trials using BAR agonists or milrinone.

We propose that increasing the cardiac content of AC VI is fundamentally different than these other strategies and that it will be associated with favorable long-term effects in dilated cardiomyopathy. Cardiac-directed expression of Gq results in left ventricular dilation and dysfunction, decreased cardiac function, and increased mortality.14–16 However, cardiac-directed expression of AC VI results in the opposite, increased survival and left ventricular function.10–12

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responsiveness to catecholamines, and impaired βAR-dependent and AC-dependent cAMP production.\textsuperscript{7,13} We use this model of cardiomyopathy to test the hypothesis that increased cardiac AC\textsubscript{VI} content is associated with long-term improvement in heart function that will provide a survival benefit in cardiomyopathy.

**Methods**

**Transgenic Mice**

Animal use was in accordance with National Institutes of Health and institutional guidelines. Transgenic mice with cardiac-directed AC\textsubscript{VI} expression were generated as previously described.\textsuperscript{5} AC\textsubscript{VI} mice (C57BL/6J) were crossed with mice with cardiac-directed expression of Gq\textsuperscript{15} (Gq-40 mice; FVB/N) provided by G.W. Dorn II (University of Cincinnati School of Medicine, Cincinnati, Ohio). Three lines emanating from this cross were studied: Gq/AC (double positive), Gq alone, and control (double negative), a total of 113 mice. Offspring from the cross of 2 strains may differ in background traits, an effect that would be normally distributed among groups. The blinded study of these 3 groups ensured that effects observed were transgene specific.

**Transgene Expression**

Transgene presence was confirmed using polymerase chain reaction of tail tissue, and expression was assessed using immunoblotting of cardiac homogenates as described previously.\textsuperscript{5,7}

**Echocardiography**

Fourteen-month-old animals were anesthetized with inhaled isoflurane (1.5% in 1 L/min oxygen) and studied as previously described.\textsuperscript{6,7} Data were acquired and analyzed without knowledge of group identity. To obtain an estimate of LV mass in vivo, we applied the following formula: LV Calc=$[(PW+IVS+LVEDD)^3-(LVEDD)^3] \times 1.05$, where LV Calc indicates left ventricular calculated mass, PW indicates end-diastolic posterior wall thickness, IVS indicates end-diastolic interventricular septal wall thickness, and LVEDD indicates left ventricular end-diastolic dimension.

**Survival and Myocardial Hypertrophy**

Three groups of animals (Gq, n=24; Gq/AC, n=12; and control, n=25) were housed until death or killed at 24 months of age. Morphometric measurements were acquired if death occurred <12 hours previously. This occurred in 12 Gq, 2 Gq/AC, and 3 control mice. Seven additional Gq/AC and 4 control mice that survived the 24-month survival study underwent morphometric assessment when killed, so the total number assessed for each group was as follows: Gq: 12; Gq/AC: 9; and control, 7.

We also measured left ventricular myocyte size by isolating cardiac myocytes\textsuperscript{6,7} from 16-month-old mice. Myocytes were arrested in diastole (20 mmol/L KCl) and allowed to attach to laminin-coated 8-well slides for 1 hour (37°C; 7.5% CO\textsubscript{2}), fixed with 4% formaldehyde (4°C for 15 minutes), and stained with Nuclear Red (R&D Systems). Cell area was measured using Image Pro Plus (Media Cybernetics); 132±11 randomly selected cells per heart were analyzed, blinded to group identity.

**Cardiac Myocyte Isolation and cAMP Production**

Cardiac myocytes were isolated from 15-month-old animals, and cAMP levels were determined as previously described.\textsuperscript{5,7}

**Necropsy and Histology**

Necropsy was performed to detect pleural effusions in 58 additional animals (20 Gq, 13 Gq/AC, and 25 controls), and hearts from 12 16-month-old animals (Gq, 4; Gq/AC, 5; and control, 3) underwent histological examination. Heart samples were fixed in formalin, paraffin embedded, sectioned, and counterstained with H&E and with Masson’s trichrome. A cardiac pathologist, blinded to treatment

**Statistical Analysis**

Values represent group mean±SEM. A log-rank test was used to evaluate Kaplan-Meier curves. Differences in AC activity, myocardial hypertrophy, and echocardiography measurements were examined by one-way ANOVA. The primary intergroup comparison (Gq versus Gq/AC) was made with Student’s t test and was performed only when the overall ANOVA showed P<0.05. The null hypothesis was rejected when P<0.05 (2 tails).

**Results**

**Transgenic Mice**

Transgene DNA was present in tail tissue from all animals at 1 month of age and reconfirmed at death using polymerase chain reaction (data not shown). Immunoblotting documented persistent increased expression of both AC\textsubscript{VI} and Gq proteins in heart samples from Gq/AC mice and Gq protein in heart samples from Gq mice (Figure 1). Expression levels of these proteins, even at 22 months of age, was qualitatively similar to what we reported in heart samples obtained at 15 weeks of age,\textsuperscript{6,7} documenting long-term and robust cardiac expression of the 2 transgenes.

**Echocardiography**

LV fractional shortening was reduced and end-diastolic diameter increased in Gq mice (Table 1). Concurrent expression of AC\textsubscript{VI} (Gq/AC) was associated with increased fractional shortening and reduced chamber dilation (Table 1). Gq mice had reduced heart rates, as previously reported\textsuperscript{7,13}; concurrent AC\textsubscript{VI} expression (Gq/AC) increased heart rate. The velocity of circumferential fiber shortening was impaired in Gq mice but was increased by concurrent expression of AC\textsubscript{VI}. LV Calc, an in vivo echocardiographic estimate of LV mass, indicated that Gq mice had increased LV mass, an effect that was attenuated by concurrent AC\textsubscript{VI} expression. These data document a favorable long-term effect on cardiac structure and function associated with AC\textsubscript{VI} expression in cardiomyopathy.

**Survival**

Over the 2-year study, survival was 0% in Gq, 67% in Gq/AC, and 78% in control mice (Figure 2). Kaplan-Meier...
analysis indicated increased survival associated with expression of cardiac ACVI in a background of cardiomyopathy (P < 0.0001). There was no difference in mortality between Gq/AC and control mice. These data indicate a pronounced favorable effect on survival associated with long-term cardiac-directed ACVI expression in cardiomyopathy.

Myocardial Hypertrophy

Mice with cardiac-directed expression of Gq had increased whole-heart wet weight and wet and dry ventricular weight (Table 2). By comparison, whole-heart weight and wet and dry ventricular weights of hearts from Gq/AC mice were similar to hearts obtained from control animals. These data document the presence of myocardial hypertrophy in Gq mice and its abrogation by simultaneous ACVI expression. Ratios of wet ventricular weight to tibial length are shown in Figure 3. Mice with cardiac-directed expression of Gq had significant increases in these ratios compared with Gq/AC mice. Figure 3 also shows representative hearts from Gq/AC and Gq mice. These hearts were obtained at 11 months of age, the time when mortality increases in Gq mice. Western blots of ventricular homogenates from these hearts confirmed expression of ACVI and Gq protein (data not shown). To determine whether persistent increases in cardiac content of ACVI protein were associated with altered AC function, we isolated cardiac myocytes from Gq/AC mice, but these differences were not statistically significant (Figure 4). Stimulated cAMP levels were substantially reduced in cardiac myocytes isolated from hearts of 16-month-old animals. Cardiac myocytes from Gq animals were substantially increased in size compared with controls (Figure 3). Concurrent expression of ACVI abrogated myocyte hypertrophy so that cardiac myocyte size was normal. These data (Table 2 and Figure 3) show that cardiac-directed Gq is associated with substantial myocardial hypertrophy in later adult life, a hypertrophy that is abrogated by concurrent expression of cardiac ACVI.

Cardiac Myocyte cAMP Levels

To determine whether persistent increases in cardiac content of ACVI protein were associated with altered AC function, we isolated cardiac myocytes from 15-month-old animals in each group. Basal cAMP levels tended to be lower in cardiac myocytes from Gq animals and tended to be increased in cardiac myocytes from Gq/AC mice, but these differences were not statistically significant (Figure 4). Stimulated cAMP levels were substantially reduced in cardiac myocytes from Gq mice but increased in cardiac myocytes from Gq/AC mice. Results were similar whether data were normalized to cell number (Figure 4) or to protein content (data not shown). Thus, even in older Gq/AC mice, increased cardiac AC function was observed.

Necropsy and Histology

The incidence of substantial pleural effusions was 30% in Gq animals; pleural effusions were not present in Gq/AC or control animals (P = 0.001). Histological examination showed no evidence of inflammation. Very mild perivascular fibrosis was observed in 3 of the 12 animals (1 control, 1 Gq, and 1 Gq/AC). Two of these animals also showed very mild interstitial fibrosis (1 control and 1 Gq/AC), These findings were interpreted by the pathologist as very mild changes associated with advanced age.

Discussion

We examined the long-term effect of cardiac-directed expression of ACVI in the context of Gq-associated cardiomyopathy.
Promoting production of cardiac cAMP in the setting of heart failure presently is not widely embraced. Clinical trials have shown poor outcomes with agents that are associated with sustained elevations in intracellular cAMP in cardiac myocytes, and provocation of ventricular arrhythmias is thought to be a source of these disappointing clinical outcomes. Similarly, transgenic mice with cardiac-directed expression of more proximal elements of the βAR pathway, including β2AR, high levels of β2AR, or Gs, develop cardiac abnormalities that progress to heart failure. Cardiac-directed expression of β2AR is deleterious in several murine models of cardiac dysfunction, including Gq cardiomyopathy.

In contrast, long-term cardiac-directed expression of ACVI increases cardiac function in response to adrenergic stimulation, with no evidence of deleterious effects even 17 months later and, as we have shown in the present study, is associated with increases in heart function, AC function, and survival in Gq cardiomyopathy. Why cardiac-directed expression of β2AR or Gs provides such different results than ACVI in the setting of murine cardiomyopathy may reflect key differences in the general roles played by these distinct transmembrane signaling elements. The degree or nature of cardiac cAMP production provided by Gs and βAR (sustained) compared with AC (not sustained) may be a pivotal difference. Alternatively, the deleterious effects of cardiodirected Gs and βAR expression in heart failure may not be related directly to βAR stimulation and cAMP production. Similarly, increased expression of cardiac ACVI may have favorable effects independent of cAMP generation. For example, AC serves as an effector for several transmembrane receptors, the activation of which may also have protective effects.

Abrogation of Hypertrophy

An unexpected finding of the present study was that increased cardiac ACVI abrogated Gq-associated myocardial hypertrophy. Second, cardiac-directed expression of ACVI abrogates myocardial hypertrophy in older mice with cardiomyopathy. Third, long-term cardiac-directed expression of ACVI resulted in increased AC function and associated with increases in heart function, AC function, and survival in Gq cardiomyopathy. Why cardiac-directed expression of β2AR is deleterious in several murine models of cardiac dysfunction, including Gq cardiomyopathy, may reflect key differences in the general roles played by these distinct transmembrane signaling elements. The degree or nature of cardiac cAMP production provided by Gs and βAR (sustained) compared with AC (not sustained) may be a pivotal difference. Alternatively, the deleterious effects of cardiac-directed Gs and βAR expression in heart failure may not be related directly to βAR stimulation and cAMP production.

Increased Survival

Our study is the first to address the effect of increased expression of cardiac AC on survival in cardiomyopathy. Gq cardiomyopathy is associated with ventricular dilation and hypertrophy, depressed cardiac function, and impaired adrenergic signaling. In the present study we found that long-term cardiac-directed expression of ACVI increased survival in Gq cardiomyopathy. The cause of premature death in Gq mice was not established. However, Gq animals showed obvious signs of heart failure, including cardiac enlargement, the presence of pleural effusions, and the appearance of breathlessness, signs not present in the other 2 groups.

Figure 3. Assessment of hypertrophy. A, Representative hearts from Gq/AC and Gq mice are shown. These hearts were obtained at 11 months of age, the time when mortality increases in Gq mice. These mice were killed electively for this photograph. Western blots of ventricular homogenates from these hearts confirmed increased expression of ACVI and Gq proteins in the Gq/AC mouse and Gq protein in the Gq mouse (data not shown). B, Representative cardiac myocytes isolated from Gq/AC and Gq mice are shown. These myocytes were obtained at 15 months of age. Mice were killed electively for this photograph. Myocyte size was enlarged in hearts from Gq animals but was reduced to normal size by concurrent expression of cardiac ACVI (see also panel D). Magnification ×160. C, Wet ventricular-tibial length (Wet Vent/TL) ratios confirmed prevention of myocardial hypertrophy in Gq/AC vs Gq animals. *P<0.01 Gq/AC versus Gq. Bars indicate group mean; error bar, SEM; and numbers below bars, group size. D, Left ventricular cardiomyocyte size was measured directly and confirmed increased cardiac myocyte size (hypertrophy) in cardiac myocytes from Gq animals, an effect that was abrogated by concurrent expression of cardiac ACVI (see also panel D). Magnification ×400. P<0.001 Gq/AC versus Gq. Bars indicate group mean; error bar, SEM; and numbers below bars, group size.

Figure 4. Cardiac myocyte cAMP production. A, Basal cAMP production tended to be lower in cardiac myocytes from Gq animals and higher in cardiac myocytes from Gq/AC mice, although these differences were not statistically significant. B, Stimulated cAMP production (forskolin, 10 μmol/L) was reduced in cardiac myocytes from Gq mice but was substantially increased in cardiac myocytes from Gq/AC mice. These data document increased cardiac AC function when ACVI is expressed in a cardiomyopathic background. Bars indicate mean values; error bar, 1 SEM. P value from ANOVA; *P<0.02 (Gq/AC versus Gq). Number per group indicated below each bar.
dial hypertrophy. Gq signaling plays a role in specific types of myocardial hypertrophy. Cardiac-directed expression of a constitutively active Gq mutant results in ventricular dilation and hypertrophy. Cardiac-directed expression of nonconstitutively active Gq after pregnancy or high-level expression results in ventricular dilation and hypertrophy. Mice with cardiac-directed Gq expression (Gq-40), when crossed into C57BL/6J mice, showed ventricular dilation and reduced left ventricular function but no evidence of myocardial hypertrophy at 15 weeks of age. In the present study we find that, by 8 months of age, hypertrophy has developed in this line. This may reflect activation of hypertrophic stimuli brought on by progressive heart failure or it may represent a delayed effect of Gq.

The mechanism by which cardiac-directed ACVI expression abrogates hypertrophy in Gq cardiomyopathy is unknown. Cultured neonatal rat cardiac myocytes undergo hypertrophy when incubated with phenylephrine, a Gq-dependent event that is blocked by previous transfection with adenovirus encoding ACVI (unpublished data, 2001), suggesting that ACVI may prevent Gq-mediated myocardial hypertrophy. Increased intracellular cAMP and subsequent protein kinase A activation can attenuate phospholipase C signaling and block growth factor receptor signaling to mitogen-activated protein kinases, pathways known to play roles in Gq-dependent myocardial hypertrophy. Alternatively, increased cardiac ACVI in Gq cardiomyopathy favorably affects cardiac function and thereby would be expected to reduce activation of the renin-angiotensin-aldosterone axis and its promotion of myocardial hypertrophy.

Long-Term Expression and Function

This is the first documentation of long-term expression and function of transgenic AC in the setting of murine cardiomyopathy. We crossbred mice with 20-fold increase in cardiac ACVI content with mice with 4- to 5-fold increase in cardiac Gq content (Gq 40), a line previously shown to have reduced cardiac AC content and impaired cardiac AC function. We previously documented in mice that were 15 weeks old that increasing cardiac ACVI improved left ventricular contractile function and increased both βAR- and forskolin-stimulated cAMP production in Gq cardiomyopathy. However, initial favorable effects often result in deleterious outcomes in later life, particularly with transgenic manipulation of adrenergic signaling elements. Therefore, it was important to determine what the long-term effects of increased cardiac ACVI expression would be, particularly in the context of cardiomyopathy.

To study this important issue, we examined LV function by echocardiography in 14-month-old mice and found reduced chamber dilation and increased systolic function in Gq/AC mice compared with Gq mice. Cardiac membranes from 15-month-old mice showed increased ACVI transgene protein levels similar to what was seen at 15 weeks. Finally, isolated cardiac myocytes from 16-month-old animals showed restoration of normal AC function as assessed by forskolin stimulation. We previously reported that βAR-stimulated cAMP production is increased in cardiac myocytes from Gq/AC mice. These data, taken together, support the conclusion that increased cardiac ACVI content has a salutary effect in cardiomyopathy.

Limitations

Although Gq-associated cardiomyopathy shares similarities to clinical heart failure, extrapolating our findings to clinical settings should be done cautiously. For example, a favorable effect would be unlikely in heart failure unassociated with adrenergic desensitization. The crossbreeding paradigm results in continuous expression of 2 transgenes during growth and development, hindering determination of a specific treatment effect, a limitation that will require regulated transgene expression or exogenous transgene delivery in the setting of heart failure to resolve.

Conclusions

Cardiac-directed expression of ACVI restores cardiac myocyte AC function, improves heart function, abrogates myocardial hypertrophy, and increases survival in Gq cardiomyopathy. It will be of interest to determine whether ACVI expression can prevent hypertrophy in other settings.

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