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Defective β-Adrenergic Receptor Signaling Precedes the Development of Dilated Cardiomyopathy in Transgenic Mice with Calsequestrin Overexpression*

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Calsequestrin is a high capacity Ca^{2+}-binding protein in the junctional sarcoplasmic reticulum that forms a quaternary complex with junctin, triadin, and the ryanodine receptor. Transgenic mice with cardiac-targeted calsequestrin overexpression show marked suppression of Ca^{2+}-induced Ca^{2+} release, myocyte hypertrophy, and premature death by 16 weeks of age (Jones, L. R., Suzuki, Y. J., Wang, W., Kobayashi, Y. M., Ramesh, V., Franzini-Armstrong, C., Cleemann, L., and Morad, M. (1998) *J. Clin. Invest.* 101, 1385–1393). To investigate whether alterations in intracellular Ca^{2+} trigger changes in the β-adrenergic receptor pathway, we studied calsequestrin overexpressing transgenic mice at 7 and 14 weeks of age. As assessed by echocardiography, calsequestrin mice at 7 weeks showed mild left ventricular enlargement, mild decreased fractional shortening with increased wall thickness. By 14 weeks, the phenotype progressed to marked left ventricular enlargement and severely depressed systolic function. Cardiac catheterization in calsequestrin mice revealed markedly impaired β-adrenergic receptor responsiveness in both 7- and 14- week mice. Biochemical analysis in 7- and 14-week mice showed a significant decrease in total β-adrenergic receptor density, adenylyl cyclase activity, and the percent high affinity agonist binding, which was associated with increased β-adrenergic receptor kinase 1 levels. Taken together, these data indicate that alterations in β-adrenergic receptor signaling precede the development of overt heart failure in this mouse model of progressive cardiomyopathy.

Cardiac hypertrophy represents one of the most important adaptive responses to increase mechanical load on the heart. In an attempt to normalize excessive forces and work performed per contractile unit, myocardial hypertrophy unloads the heart by adding new sarcomeres in order to distribute tension across a greater cellular mass (1). Although the induction of cardiac hypertrophy can be viewed as a corrective response to elevated stress, it is now clear that sustained hypertrophy initiates a myopathic process leading to decompensated heart failure (2). The molecular mechanisms that are responsible for this transition from a compensatory state to one of progressive chamber enlargement and myocardial failure are poorly understood. Derangement in a number of cellular processes have been implicated in this pathological transition including abnormalities in β-adrenergic receptor (βAR)[1] signaling (3), activation of mitogen-activated protein kinase pathways (4), and diminished excitation-contraction coupling due to an altered spatial relationship between the L-type Ca^{2+} channel and the ryanodine receptor (5).

Chronic human heart failure is characterized by marked abnormalities in βAR signaling that result from a number of alterations including a 50% reduction in β_1ARs without change in β_2AR density (6), increased levels of the inhibitory G protein (6), and an increase in myocardial βARK1 activity (7). It remains controversial whether alterations in the βAR system are important in the pathogenesis of the failing heart. In this regard, we have recently reported the striking finding that cardiac overexpression of a βARK inhibitor prevents the development of cardiomyopathy in a genetic model of murine heart failure (8). Thus, abnormal βAR/G protein-coupling early in the development of the failing heart appears to be a critical event in the progressive nature of this disease (8).

Calsequestrin (CSQ) is a 55-kDa high capacity Ca^{+2}-binding protein located in the lumen of the junctional sarcoplasmic reticulum that forms a quaternary complex with junctin, triadin, and the ryanodine receptor, which are all required for the normal regulation of Ca^{2+} release by the ryanodine receptor (9). Recently, we and others have characterized the electrophysiological properties of transgenic mice with impaired Ca^{2+} release achieved through cardiac overexpression of CSQ (10, 11). CSQ-overexpressing transgenic mice showed markedly reduced Ca^{2+}-induced Ca^{2+} release (10, 11) and frequency of Ca^{2+} sparks (10). Interestingly, CSQ overexpression was associated with myocyte hypertrophy, depressed cellular contractility, and induction of a fetal gene program. The purpose of this study was to determine whether defects in βAR signaling are associated with this mouse model of cellular contractile failure so as to better understand the cellular mechanisms

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[1] The abbreviations used are: βAR, β-adrenergic receptor; βARK1, β-adrenergic receptor kinase 1; GRK, G protein-coupled receptor kinase; CSQ, calsequestrin; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; % FS, % fractional shortening; SEPth, septal wall thickness; PWth, posterior wall thickness; LV dP/dtmax and LV dP/dtmin, maximal and minimal first derivative of LV pressure, respectively; mVcfc, heart rate corrected mean velocity of circumferential shortening.

involved in the initiation of pathological hypertrophy and heart failure.

MATERIALS AND METHODS

Experimental Animals—Transgenic mice overexpressing (CSQ) mice were generated as described previously (10). Briefly, full-length canine cardiac CSQ cDNA was fused to the α-myosin heavy chain promoter to drive cardiac-targeted expression. Age-matched transgenic and wild-type littermates of either sex were used. The genotype was determined by polymerase chain reaction as described previously (10). The animals in this study were handled according to approved protocols and the animal welfare regulations of the University of North Carolina at Chapel Hill and the Indiana University.

Transthoracic Echocardiography—Echocardiography was performed in anesthetized mice (Avertin 2.5%, 14 μl/g intraperitoneally) using an ATL HDI 5000 echocardiograph (ATL Ultrasound, Bothell, WA) as described previously (8). Wild-type and CSQ mice at the age of 7 and 14 weeks were evaluated by noninvasive echocardiography followed by invasive hemodynamics. In a separate group of mice, serial echocardiograms were obtained in the same animals over a 5-week period of time with the initial study at 8.4 ± 0.4 weeks of age and a second study performed at 13.3 ± 0.4 weeks of age.

The operator who performed and measured the echocardiograms was blinded to the genotype of the animals. The following parameters were measured: LVEDD; LVESD; % FS, calculated as (LVEDD − LVESD)/LVEDD × 100; SEPth; PWth, mVcfc, calculated as fractional shortening divided by ejection time multiplied by the square root of the R-R interval. Estimated echocardiographic LV mass (in mg) was calculated as $((LVEDD + SEPth + PWth)^3 - LVEDD^3) \times 1.055$, where 1.055 ($mg/mm^3$) is the density of myocardium. Intraobserver and interobserver variation for M-mode measurements was <6% for internal dimensions and <25% for wall thickness.

Cardiac Catheterization—Following echocardiography, hemodynamic evaluation was performed in closed-chest mice as described previously (8, 12). Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) and connected to a rodent ventilator after endotracheal intubation. A 1.4 French high fidelity micromanometer catheter (Millar Instruments) was inserted into the right carotid and advanced retrograde into the LV. Hemodynamic measurements were recorded on an 8-channel chart recorder and in digitized form at 2,000 Hz at base line and 45–60 s after injection of incremental doses of isoproterenol (8). Experiments were then terminated, hearts were rapidly excised, and individual chambers were separated, weighed, and frozen in liquid N_2 for later biochemical analysis. Parameters measured were heart rate, LV systolic pressure, LV end diastolic pressure, LV dP/dtmax, and LV dP/dtmin. Ten sequential beats were averaged for each measurement.

In separate CSQ overexpressing animals at 14 weeks of age, heart rate was matched to the wild-type mice by right atrial pacing. Following anesthesia and instrumentation as above, a bipolar pacing lead made from a pair of fine filament-coated wires was introduced into the right jugular vein and advanced into the right atrium. After a brief period of stabilization, atrial pacing was initiated at a rate of 400/min, and hemodynamic parameters were recorded as described.

Adenylyl Cyclase Activity, βAR Density, and Radioligand Binding—Myocardial sarcolemmal membranes were prepared by homogenizing whole hearts from 7- and 14-week-old mice in ice-cold buffer as described previously (8, 12–14). Total βAR density was determined by incubating 25 μg of cardiac sarcolemmal membranes with a saturating concentration (80 pM) of [^{125}I]cyanopindolol and 20 μM alprenolol to define nonspecific binding (14). Typical nonspecific binding is ≈40% of the total. Competition binding isotherms in sarcolemmal membranes were done in triplicate with 18 varying concentrations of isoproterenol (10^{-13} M to 10^{-4} M). Assays were conducted at 37 °C for 60 min and then filtered over GF/C glass fiber filters (Whatman) that were washed and counted in a γ counter. Competition isotherms were analyzed by nonlinear least square curve fit (GraphPad Prism).

Adenylyl cyclase activity was performed on myocardial sarcolemmal membranes from 7- and 14-week-old mice (8, 12, 14). Membranes (30 to 40 μg of protein) were incubated for 15 min at 37 °C with [α-^{32}P]ATP under basal conditions or indicated agonists, and cAMP was quantified (8, 12, 14).

G Protein-coupled Receptor Kinase (GRK) Activity by Rhodopsin Phosphorylation—GRK activity was measured in myocardial extracts using rhodopsin-enriched rod outer segment membranes as an *in vitro* substrate as described previously (12–14). Concentrated (Centricon, Amicon, Inc.) cytosolic extract (300 μg of protein) was incubated with rhodopsin-enriched rod outer segments in 50 μl of reaction buffer (20 mmol/liter Tris-Cl (pH 8.0), 2 mmol/liter EDTA, 10 mmol/liter $MgCl_2$, 1mmol/liter dithiothreitol), and 0.1 mmol/liter ATP containing [γ-^{32}P]ATP. The reactions were incubated in white light for 20 min and quenched with 300 μl of ice-cold lysis buffer and then centrifuged for 15 min at 13,000 × *g*. Sedimented proteins were resuspended in 20 μl of protein gel loading dye and electrophoresed through SDS, 12% polyacrylamide gels. Phosphorylated rhodopsin was visualized by autoradiography and quantified using a PhoshorImager (Molecular Dynamics).

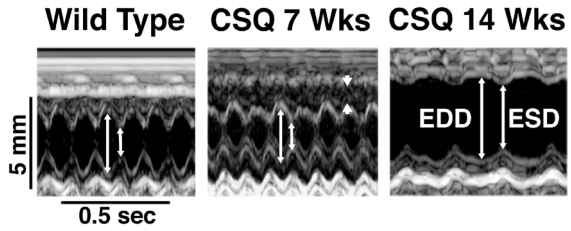

FIG. 1. **Representative M-mode echocardiographic tracings in a wild-type and CSQ mouse.** Transthoracic M-mode echocardiographic tracings in a wild type, a 7-week-old CSQ mouse (*CSQ 7 Wks*), and a 14-week-old CSQ mouse (*CSQ 14 Wks*). The *double-sided arrows* indicate left ventricular internal dimensions. The CSQ mouse at 7 weeks of age has normal dimensions with increased septal (*arrowheads*) and posterior wall thickness. In contrast, the CSQ mouse at 14 weeks of age shows severe chamber enlargement, reduced cardiac performance, and the lack of increased wall thickness. *EDD*, end-diastolic dimension; *ESD*, end-systolic dimension.

Immunoblotting—Immunodetection of myocardial levels of βARK1 was performed on cytosolic extracts following immunoprecipitation using a monoclonal βARK1/2 antibody as described previously (8, 12, 14). The ~80-kilodalton βARK1 protein was visualized with the monoclonal antibody raised against an epitope within the carboxyl terminus of βARK1 and chemiluminescent detection of anti-mouse IgG conjugated with horseradish peroxidase (ECL, Amersham Pharmacia Biotech).

Northern Blot Analysis—Northern blotting was performed on LV tissue obtained from 7- and 14-week CSQ and age-matched wild-type mice as described previously (15). Total RNA was isolated by a modification of the acid guanidine thiocyanate technique (RNeasy; Qiagen, Valencia, CA). RNA (4 μg) was size-fractionated by denaturing gel electrophoresis, transferred to nylon membranes by capillary action, and cross-linked with UV light. Filters were prehybridized at 68 °C for 1 h and then hybridized for 4 h at 68 °C after the addition of ^{32}P cDNA probes labeled using random priming. Post hybridization filters were washed under stringent conditions, and transcripts were detected by autoradiography.

Statistical Analysis—Data are expressed as mean ± S.E. To test for statistical differences in serial echocardiographic data and hemodynamic data between CSQ and wild-type mice, a two way repeated measures analysis of variance was performed. When appropriate, post hoc analysis with regard to differences in mean values between the groups was conducted with a Newman-Keuls test. Statistical significance between wild-type and CSQ mice at either 7 or 14 weeks of age for the biochemical data, chamber weights, and echocardiographic variables was performed using Student's *t* test.

RESULTS

CSQ Overexpression Results in a Cardiac Phenotype of Progressive Chamber Dilatation and Cardiac Failure—Transthoracic echocardiography was used to assess LV chamber size and global cardiac function in transgenic and wild-type mice. As shown in Fig. 1, overexpression of CSQ resulted in an early phenotype of preserved chamber size and increased wall thickness that progressed to one of LV enlargement and severe cardiac dysfunction (Fig. 1). By 16 weeks of age all mice had died. Summary echocardiographic data obtained in 7- and 14-week-old mice are shown in Table I. At 7 weeks, CSQ mice showed mild chamber enlargement, mild reduction in % FS, and increased wall thickness. In contrast, at 14 weeks, severe cardiac enlargement and marked cardiac dysfunction was evident with a reduction in wall thickness back to normal values.

In a separate group of age-matched CSQ and wild-type mice, serial echocardiography was performed. Individual data points

TABLE I

Echocardiographic and physiological parameters in 7- and 14-week-old CSQ-overexpressing mice

BW, body weight; HW, heart weight; LVW, left ventricular weight; TL, tibia length; HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure. Data are expressed as mean ± S.E. Statistical comparison, CSQ *vs.* wild type for either 7- or 14-week mice or CSQ pace up *vs.* CSQ for 14 weeks, see *p* values in footnotes below.

	7 weeks		14 weeks		
	Wild type (n = 11)	CSQ (n = 13)	Wild type (n = 9)	CSQ (n = 13)	CSQ pace up (n = 4)
LVEDD (mm)	3.3 ± 0.8	3.9 ± 0.1[a]	3.7 ± 0.1	5.0 ± 0.1[a]	
LVESD (mm)	1.7 ± 0.7	2.5 ± 0.1[a]	2.0 ± 0.1	3.8 ± 0.2[a]	
FS (%)	47.5 ± 1.9	35.4 ± 1.7[a]	44.8 ± 1.8	22.7 ± 1.5[a]	
SEPth (mm)	0.65 ± 0.03	0.83 ± 0.04[b]	0.74 ± 0.03	0.76 ± 0.04	
mVcfc (circ/s)	2.9 ± 0.2	2.3 ± 0.2[b]	2.6 ± 0.2	1.53 ± 0.1[b]	
BW (g)	18.9 ± 1.6	19.6 ± 1.0	20.1 ± 1.0	20.5 ± 0.5	
HW/BW (mg/g)	5.3 ± 0.1	8.5 ± 0.1[a]	4.8 ± 0.1	8.8 ± 0.8[a]	
LVW/BW (mg/g)	3.7 ± 0.1	6.2 ± 0.1[a]	3.5 ± 0.1	6.5 ± 0.2[a]	
HW/TL (mg/mm)	6.2 ± 0.3	10.4 ± 0.3[a]	5.9 ± 0.2	11.1 ± 0.5[a]	
LVW/TL (mg/mm)	4.4 ± 0.2	7.6 ± 0.2[a]	4.3 ± 0.1	7.7 ± 0.3[a]	
HR (beats/min)	399 ± 14	327 ± 13[a]	401 ± 21	307 ± 25[a]	398 ± 12[c]
LV dP/dtmax (mm Hg/s)	8382 ± 563	4696 ± 299[a]	8169 ± 829	4135 ± 442[a]	2907 ± 398[c]
LV dP/dtmin (mm Hg/s)	−6003 ± 560	−3719 ± 284[a]	−6534 ± 831	−2740 ± 346[a]	−2779 ± 341
LVSP (mm Hg)	90 ± 3	81 ± 3	112 ± 6	90 ± 5[a]	80 ± 4[c]
LVEDP (mm Hg)	0.9 ± 1.0	0.5 ± 1.1	10.7 ± 1.7	11.0 ± 1.5	10.7 ± 1.8

[a] $p < 0.0005$.
[b] $p < 0.05$.
[c] $p < 0.001$.

for 3 echocardiographic parameters are plotted for 12 CSQ mice studied at 8.4 ± 0.4 weeks of age and at a follow-up echocardiographic study 5 weeks later (Fig. 2). On the initial study, 7 of the 12 CSQ mice had normal LV chamber size and systolic function as indicated by the normal LVEDD (Fig. 2*A*) and % FS (Fig. 2*B*). This early phenotype was associated with a significant increase in septal (Fig. 2*C*) and posterior wall thickness (CSQ; 0.97 ± 0.04 *versus* wild type; 0.74 ± 0.03, mm, $p < 0.001$). In contrast, over a period of 5 weeks, nearly all CSQ mice showed an increase in LVEDD (Fig. 2*A*), decrease in % FS (Fig. 2*B*), and reduction in wall thickness (Fig. 2*C*). Thus, cardiac overexpression of CSQ leads to an initial phenotype of cardiac hypertrophy with only a mild reduction in systolic function that progresses to severe cardiac enlargement and marked LV dysfunction with premature death.

In addition to the time-dependent enlargement in chamber size, CSQ-overexpressing mice showed a progressive increase in LV mass as determined by serial echocardiography. Estimated LV mass of CSQ mice at echo #1 was significantly greater than in wild-type mice (CSQ; 169.1 ± 17.9 *versus* wild-type; 90.4 ± 10.7, mg, $p < 0.001$). Over the 5-week study period, little change in estimated LV mass was seen in wild-type animals, whereas a small increase in the estimated LV mass was seen in the CSQ-overexpressing mice at echo #2 (CSQ; 185.2 ± 9.45 *versus* wild-type; 90.5 ± 7.7, mg, $p < 0.001$). These data are consistent with the heart weight data obtained at autopsy following the hemodynamic study in the 7- and 14-week-old mice (Table I).

Contractile Dysfunction and Impaired βAR Responsiveness Associated with CSQ Overexpression—To determine whether *in vivo* βAR responsiveness was altered in mice with CSQ overexpression, cardiac catheterization was performed in 7- and 14-week old CSQ and age-matched wild-type mice. At 7 weeks of age, basal contractility as measured by the first derivative of LV pressure rise (LV dP/dtmax), was significantly reduced in the CSQ mice compared with wild-type mice (Fig. 3*A*, Table I). In response to isoproterenol, 7-week CSQ mice showed a markedly blunted response as compared with the brisk increase in LV dP/dtmax observed in wild-type mice (Fig. 3*A*). Myocardial relaxation (as assessed by LV dP/dtmin) was also significantly impaired under basal conditions and with isoproterenol infusion (Fig. 3*B*). Although LV systolic pressure was only slightly reduced (Fig. 3*C*), heart rate was significantly

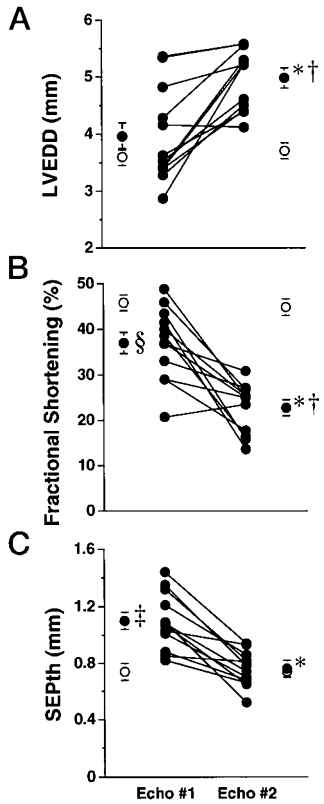

FIG. 2. **Progressive deterioration of cardiac function in CSQ mice as measured by serial echocardiography.** Serial echocardiography was performed in wild type (○, n = 7) and CSQ (●, n = 12) mice at base line (*Echo #1*) and repeated 5 weeks later (*Echo #2*). Data for individual CSQ mice and mean ± S.E. for both groups are shown. *A*, LVEDD; *B*, % fractional shortening; *C*, SEPth, septal wall thickness. Age at first echocardiogram (*Echo #1*): wild-type (8 weeks), CSQ (8.4 ± 0.4 weeks); repeat echocardiogram (*Echo #2*): wild type (13 weeks), CSQ (13.3 ± 0.4 weeks). *, $p < 0.0005$, CSQ Echo #2 *versus* CSQ Echo #1; †, $p < 0.0005$, CSQ Echo #2 *versus* wild-type Echo #2; ‡, $p < 0.0005$, and §, $p < 0.05$, CSQ Echo #1 *versus* wild-type Echo #1.

lower in the CSQ mice at base line and with isoproterenol stimulation (Fig. 3*D*). These data show that even in the absence of overt heart failure, young CSQ mice show marked abnormalities of βAR function *in vivo*. Cardiac catheterization in 14-

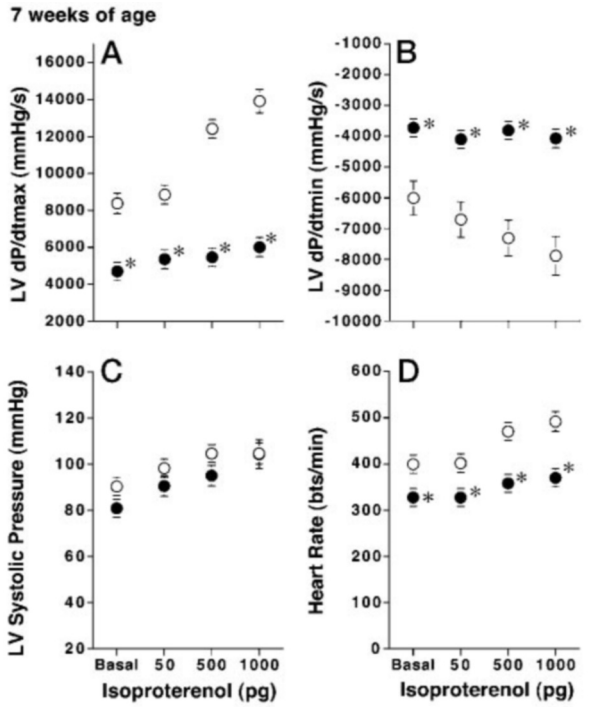

FIG. 3. ***In vivo*** **assessment of LV contractile function in response to βAR stimulation at 7 weeks.** At 7 weeks of age, cardiac catheterization was performed in closed chest-anesthetized mice using a 1.4 French high fidelity micromanometer. Four measured parameters are shown at base line and after progressive infusion of isoproterenol in wild type (○) $n = 9$ and CSQ (●) ($n = 13$) mice. *A*, LV dP/dtmax; *B*, LV dP/dtmin; *C*, LV systolic pressure; *D*, heart rate. *, $p < 0.0001$, CSQ *versus* wild type. The pattern of change between groups was statistically significant for LV dP/dtmax, $p < 0.00001$ (*A*), LV dP/dtmin, $p < 0.0005$ (*B*).

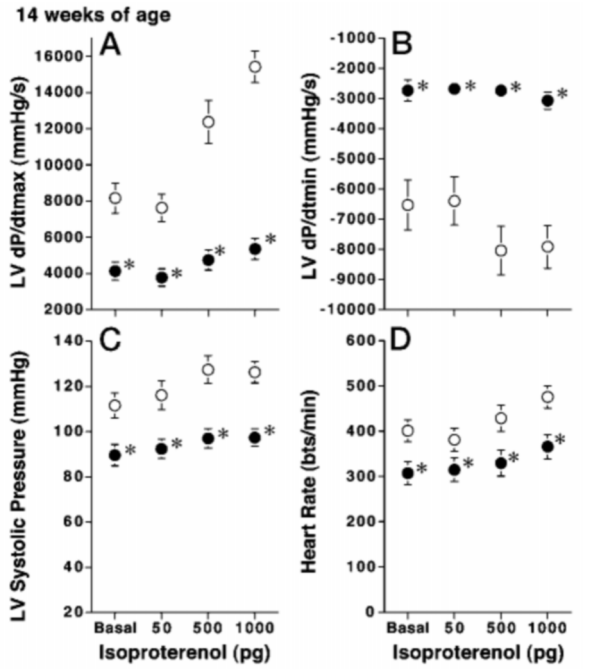

FIG. 4. ***In vivo*** **assessment of LV contractile function in response to βAR stimulation at 14 weeks.** Following the final echocardiogram at 14 weeks of age, cardiac catheterization and was performed as above in wild type (○) $n = 11$ and CSQ (●) ($n = 13$) mice. *A*, LV dP/dtmax; *B*, LV dP/dtmin; *C*, LV systolic pressure; *D*, heart rate. *$p < 0.0001$ CSQ *versus* wild type. The pattern of change between groups was statistically significant for LV dP/dtmax, $p < 0.00001$ (*A*), LV dP/dtmin, $p < 0.005$ (*B*), and heart rate, $p < 0.00001$ (*C*).

week old CSQ mice showed a similar pattern with markedly depressed basal and isoproterenol-stimulated LV dP/dtmax (Fig. 4*A*, Table I), significantly impaired LV dP/dtmin (Fig. 4*B*), reduced LV systolic pressure (Fig. 4*C*), and slower heart rate (Fig. 4*D*) compared with age-matched controls. These *in vivo* data demonstrate that CSQ overexpression is associated with a severe impairment in βAR responsiveness that occurs early in the course of the cardiac phenotype and precede the development of severe LV enlargement and pump failure.

Since heart rate can affect LV contractility as measured by the isovolumic phase index, LV dP/dtmax, *in vivo* hemodynamic parameters were obtained in a separate group of 14-week CSQ animals that underwent right atrial pacing to match heart rate to that of wild-type mice. Increasing heart rate to ≈400 beats/min did not increase LV dP/dtmax in the CSQ mice but rather resulted in a significant decline (Table I).

Overexpression of CSQ Leads to βAR Signaling Defects—To determine whether overexpression of CSQ leads to abnormalities in βAR signaling, we evaluated receptor-effector coupling in sarcolemmal membranes from hearts of CSQ mice removed after the 7- and 14-week hemodynamic study. At both 7 and 14 weeks of age, total βAR density was significantly reduced in CSQ hearts compared with wild-type hearts (Fig. 5*A*). In addition to the decrease in βAR density, the percentage of βARs exhibiting high-affinity binding for isoproterenol was significantly reduced in membranes prepared from both 7- and 14-week CSQ hearts compared with controls (Fig. 5*B*). The significant reduction in the number of high affinity receptors is consistent with a decreased ability of βARs to form the coupled hormone-receptor-G protein high affinity state. These changes occur when receptors are more desensitized (14).

This decrease in βAR density and % high affinity agonist binding was associated with a marked reduction in isoproterenol-stimulated adenylyl cyclase activity in both the 7- and 14-week old mouse hearts, indicating a severe impairment in βAR coupling (Fig. 5*C*). These biochemical data are consistent with the *in vivo* findings and indicate that cardiac overexpression of CSQ leads to profound functional uncoupling of βARs prior to the development of severe pump failure.

Functional βAR uncoupling in human heart failure is associated with increased levels and activity of βARK1 (7, 16), a GRK that phosphorylates and desensitizes βARs. Thus, we measured GRK activity and βARK1 protein levels in cytosolic extracts from hearts of CSQ and wild-type mice. We have previously shown that the cytosolic enzyme βARK1 accounts for nearly all the GRK activity in cytosolic extracts prepared from mouse hearts (12). βARK1 protein levels in myocardial extracts from 7- and 14-week CSQ hearts were determined by protein immunoblotting following immunoprecipitation of soluble heart extracts with a monoclonal antibody to βARK1. As shown in Fig. 6, *A* and *B*, the level of βARK1 protein was higher in the hearts of the CSQ mice at both ages compared with age-matched wild-type mice. Rhodopsin-enriched rod outer segment membranes were used as a substrate for myocardial GRK activity. Consistent with the increase in cytosolic βARK1 protein levels, modest but significant increases in GRK activity was found in cytosolic extracts obtained from 7-week (1.5 fold, $p < 0.05$) and 14-week CSQ mice (1.8-fold, $p < 0.05$) compared with age-matched wild-type mice (Fig. 6*C*). These data demonstrate that the abnormal receptor-effector coupling found early in the progressive cardiac phenotype caused by CSQ overexpression is associated with increased βARK1 levels, which likely contributes to the marked functional uncoupling of βARs when measured both *in vivo* (responsiveness to isoproterenol infusion) and *in vitro* (adenylyl cyclase activity).

Northern blot analysis shows a marked increase in atrial natriuretic factor mRNA levels in CSQ hearts at both 7 and 14

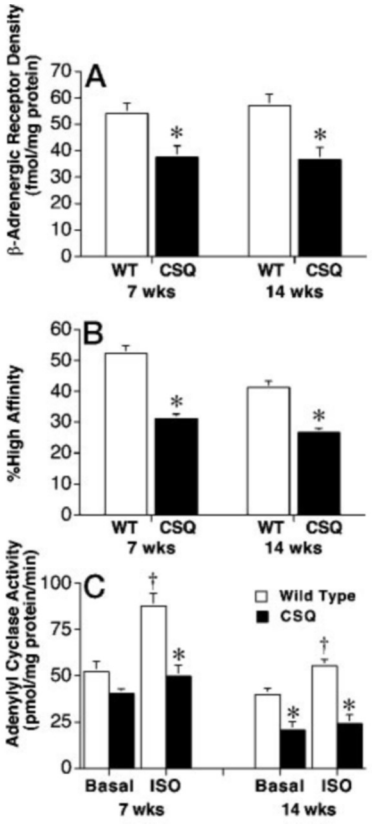

FIG. 5. **Marked βAR down-regulation and receptor uncoupling occurs in 7- and 14-week CSQ mice.** Membrane preparations from wild-type (*WT*, *open bar*) and CSQ (*closed bar*) hearts were used to measure βAR density (*A*), % high affinity agonist binding (*B*), and adenylyl cyclase activity at basal and following isoproterenol ISO stimulation (10^{-4} M) (*C*). *, $p < 0.01$ CSQ *versus* wild-type; †, $p < 0.02$ isoproterenol (*ISO*) wild-type *versus* basal wild type, one factor analysis of variance. NaF stimulation of adenylyl cyclase activity: 7-week (CSQ 331.7 ± 25.2 *versus* wild type 299.2 ± 21.3 pmol/mg protein/min, p = ns); 14-week (CSQ 355.6 ± 53.6 *versus* wild type 234.0 ± 48.0 pmol/mg protein/min, $p < 0.05$), $n = 4–10$ for each group.

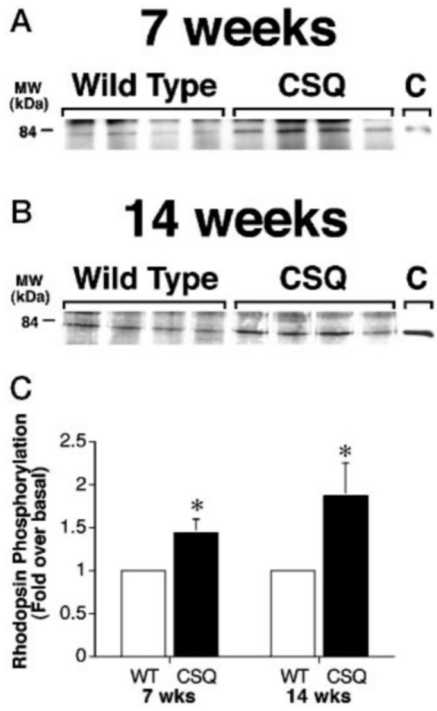

FIG. 6. **βARK1 activity and protein levels were increased in the hearts of CSQ mice.** *A* and *B*, immunodetection of βARK1 in cytosolic extracts from wild-type and CSQ hearts at 7 and 14 weeks of age is shown. An ~80-kDa protein was visualized by Western blotting and chemiluminescence following solubilization of cytosolic extracts and immunoprecipitation. *Lane C*, 25 μg of purified βARK1. *C*, βARK1 activity was measured in cytosolic extracts from wild-type and CSQ hearts by their capacity to phosphorylate the G protein-coupled receptor substrate, rhodopsin. The level of βARK1 activity in CSQ hearts was calculated as ^{32}P incorporation (fmol/min/mg of cytosolic protein) and normalized to values obtained in age-matched wild-type hearts; *$p < 0.05$, $n = 6–8$ for each group. *WT*, wild type.

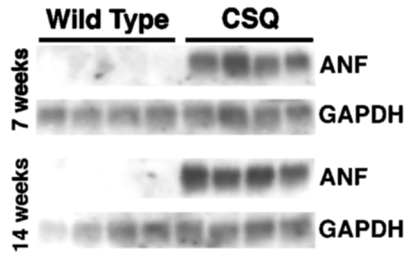

FIG. 7. **Northern blot analysis in 7- and 14-week CSQ mice.** mRNA levels in the left ventricle from 7- and 14-week-old CSQ-overexpressing transgenic mice compared with age-matched wild-type mice. Filters were hybridized with ^{32}P-labeled cDNA probes, and transcripts were detected by autoradiography. Each *lane* represents RNA isolated from a different mouse ventricle. The following cDNA probes were used: *ANF*, atrial natriuretic factor; *βMHC*, *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

weeks compared with age-matched wild-type hearts (Fig. 7). This is consistent with an induction of a fetal gene program observed in response to hypertrophic stimuli (11, 15).

DISCUSSION

The present study demonstrates that cardiac-targeted overexpression of CSQ results in a cardiac phenotype initially characterized by marked LV hypertrophy and mild decrease in cardiac systolic function, which then rapidly deteriorates to LV enlargement, severe cardiac dysfunction, and reduction in myocardial wall thickness. This phenotype is associated with a number of abnormalities in βAR signaling, which occur prior to the development of overt heart failure as documented by a decrease in total βAR density and % high affinity agonist binding, reduced basal and isoproterenol-stimulated adenylyl cyclase activity, blunted *in vivo* βAR responsiveness, and the increase in βARK1 levels.

CSQ is a Ca^{2+} storage protein located in the junctional sarcoplasmic reticulum lumen, which serves as the source of the contractile Ca^{2+} released in response to opening of the ryanodine receptor after activation of the L-type Ca^{2+} channel in the plasma membrane. Recent electrophysiologic studies in the CSQ-overexpressing mice has revealed that CSQ is not only a storage protein but also is involved in the regulation of intracellular Ca^{2+} homeostasis in heart (10). The data from this study suggests that chronic suppression of Ca^{2+} release (caused by overexpression of CSQ) may initiate a cascade of molecular events that activates the hypertrophic program but ultimately leads to progressive cardiac decompensation. Interestingly, in this study we show that one of the signaling pathways to become affected early in the time course of this phenotype is the βAR pathway. In addition to the significant reduction in βAR density and % high affinity binding in the hearts of CSQ mice, a marked functional uncoupling of βARs was shown by the severely depressed response to isoproterenol *in vivo* (LV dP/dtmax) and *in vitro* (adenylyl cyclase activity). A possible mechanism that contributes to the uncoupling of βARs in this model is the significant elevation in βARK1 levels. Thus, our results further point to a possible role for βAR desensitization in the pathogenesis of the failing heart (8).

Cardiac hypertrophy is an adaptive response to increased mechanical stress on the heart characterized by the activation of a distinct program of gene expression leading to an increase in myocyte size, expansion of contractile elements, and induction of an embryonic gene program (17). This early hypertrophic adaptation is frequently associated with alterations in the intracellular Ca^{2+} transient (5). The data from our study suggest that abnormalities in intracellular Ca^{2+} homeostasis can primarily activate the hypertrophic program possibly related to the increase in mechanical stress on the cell as a consequence of a chronic reduction in force-generating capacity. It is interesting that in many of the CSQ mice studied, the initial phase was one of increased wall thickness, normal chamber dimensions, and preserved systolic function (Fig. 2). Since most CSQ mice at 7 weeks did not show LV enlargement, the marked increase in LV/ body weight is consistent with an early phenotype of concentric LV hypertrophy that then progresses to one of dilated cardiomyopathy. The increase in atrial natriuretic factor gene expression in 7-week CSQ mice, which persists into the dilated phase at 14 weeks (Fig. 7), is consistent with the induction of an embryonic gene program and with data from another model of CSQ overexpression (11).

Recent evidence suggests that signals transduced through G protein-coupled receptors are critical in the initiation of the hypertrophic program. These receptor-coupled signals may participate in the progressive deterioration in cardiac function that follows with chronic stress (18). In this regard, we have recently shown an obligatory role for the heterotrimeric guanine nucleotide-binding protein, G_q, in the initiation of ventricular hypertrophy *in vivo* (19). Whether G_q-coupled receptor signaling pathways are activated in the hearts of transgenic mice with CSQ overexpression and progressive cardiomyopathy remains to be tested. Nonetheless it is interesting that CSQ mice have, as the initial phase, LV hypertrophy, supporting the concept that alterations in intracellular Ca^{2+} homeostasis are important in triggering the hypertrophic gene program (20).

Although other mouse models of cardiomyopathy have been developed (18, 20–24), the CSQ overexpression mouse is unique for the following reasons. First, the primary abnormality is in intracellular Ca^{2+} regulation (10), which has previously been shown to be altered in heart failure (25). Second, the initial phase is concentric LV hypertrophy and preserved systolic function, which then leads to LV enlargement and decompensation.

In summary, this model of cardiac-targeted overexpression of CSQ should provide a unique opportunity to test hypotheses related to the transition from cardiac hypertrophy to heart failure. Using a strategy that combines the mating of gene-targeted mouse models with comprehensive physiological analysis of the ensuing cardiac phenotype will allow the direct testing of the molecular framework involved in the development of cardiac hypertrophy and heart failure.

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