αB-Crystallin Suppresses Pressure Overload Cardiac Hypertrophy

Asangi R.K. Kumarapeli, Huabo Su, Wei Huang, Mingxin Tang, Hanqiao Zheng, Kathleen M. Horak, Manxiang Li, Xuejun Wang

Abstract—αB-Crystallin (CryAB) is the most abundant small heat shock protein (HSP) constitutively expressed in cardiomyocytes. Gain- and loss-of-function studies demonstrated that CryAB can protect against myocardial ischemia/reperfusion injury. However, the role of CryAB or any HSPs in cardiac responses to mechanical overload is unknown. This study addresses this issue. Nontransgenic mice and mice with cardiomyocyte-restricted transgenic overexpression of CryAB or with germ-line ablation of the CryAB/HSPB2 genes were subjected to transverse aortic constriction or sham surgery. Two weeks later, cardiac responses were analyzed by fetal gene expression profiling, cardiac function analyses, and morphometry. Comparison among the 3 sham surgery groups reveals that CryAB overexpression is benign, whereas the knockout is detrimental to the heart as reflected by cardiac hypertrophy and malfunction at 10 weeks of age. Compared to nontransgenic mice, transgenic mouse hearts showed significantly reduced NFAT transactivation and attenuated cardiac hypertrophic responses to transverse aortic constriction but unchanged cardiac function, whereas NFAT transactivation was significantly increased in cardiac and skeletal muscle of the knockout mice at baseline, and they developed cardiac insufficiency at 2 weeks after transverse aortic constriction. CryAB overexpression in cultured neonatal rat cardiomyocytes significantly attenuated adrenergic stimulation-induced NFAT transactivation and hypertrophic growth. We conclude that CryAB suppresses cardiac hypertrophic responses likely through attenuating NFAT signaling and that CryAB and/or HSPB2 are essential for normal cardiac function. (Circ Res. 2008;103:1473-1482.)

Key Words: heat shock proteins • hypertrophy • nuclear factors of activated T cells (NFAT) • myocyte-enriched calcineurin interacting protein-1 (MCPIP1) • fetal genes

αB-Crystallin (CryAB), also known as heat shock protein (HSP)B5, belongs to the small HSP (sHSP) subfamily. The CryAB gene and another sHSP (HSPB2) gene reside head-to-head in chromosome 9 in mice. Controlled by the shared bidirectional promoter, CryAB and HSPB2 are coexpressed in mammalian hearts.1,2 CryAB is the most abundant sHSP in cardiomyocytes.1 The molecular chaperone properties of CryAB may entail prevention of stress-induced aggregation of denaturing proteins, as well as trapping aggregation-prone proteins in large, soluble, multimeric reservoirs.34

Studies on cardiac role(s) of HSPs including CryAB are focused on their effects on ischemic or oxidative forms of stress.5 Ex vivo perfused hearts from transgenic (TG) mice that ubiquitously overexpress CryAB tolerated ischemia/reperfusion better.6 By contrast, CryAB/HSPB2-null mouse hearts displayed poorer functional recovery, a higher cell death rate,7 increased stiffness, and, hence, poor relaxation of myocardium following ischemia/reperfusion,8 compared with wild-type (WT) controls. CryAB was found to interact with mitochondria.9 Mitochondrial permeability transition and calcium uptake were increased in cardiomyocytes from CryAB/HSPB2-null mice.10 Interestingly, HSP20 was recently shown to attenuate isoproterenol (ISO) infusion induced cardiac remodeling.11 However, the potential role(s) of HSPs in general in cardiac response to mechanical stress has rarely been investigated. As a bona fide sHSP in the heart, CryAB associates with the cytoskeleton and contractile apparatus in cardiac myocytes.12 Both the cytoskeleton and the contractile apparatus play important roles and undergo dramatic remodeling in cardiac response to mechanical overload. Hence, CryAB may modulate this response.

Pressure overload as seen in hypertension and aortic stenosis is an important cause of congestive heart failure. Cardiac hypertrophy is the most powerful cardiac response to increased workload, but increased cardiac mass has been more recently recognized as an independent risk factor for poor prognosis.13 Protein quality control (PQC) is accomplished by the collaboration between molecular chaperones...
and selective proteolysis in the cell. In terminally differentiated cardiomyocytes, PQC is undoubtedly essential for survival and normal function. TG expression of human (cardio)myopathy linked missense (R120G) mutant CryAB compromises PQC in the heart and causes congestive heart failure. Multiple lines of evidence point to an increasingly attractive hypothesis that inadequacy in PQC plays an important role in cardiac remodeling and failure. Using both gain- and loss-of-function approaches, the present study has tested and proven the hypothesis that sHSP CryAB suppresses cardiac hypertrophic responses to pressure overload and delays progression to cardiac failure likely through inhibiting the calcineurin–NFAT signaling pathway.

Materials and Methods
An expanded Materials and Methods section can be found in the online data supplement at http://circres.ahajournals.org.

Experimental Animals
FVB/N TG mice with cardiomyocyte-restricted overexpression of CryAB (TG), NFAT binding site–dependent luciferase reporter (NFAT-Luc) mice, and the CryAB/HSPB2 double knockout mice (KO) were previously characterized. The KO mice used here were derived from 8 generations of back-crossing of the original 129/Svj KO mice into the FVB/N background. Institutional guidelines were followed in the care and use of animals.

Transverse Aortic Constriction
Transverse aortic constriction (TAC) or the sham surgery was performed on 8-week-old male mice, as previously described with minor modifications. A 29-gauge needle (outer diameter, 0.33 mm) was used as the mode of TAC. Sham control mice underwent the same procedure except for aortic constriction.

Transthoracic Echocardiography
Transthoracic echocardiography (Echo) was performed using a high-resolution Vevo 770 Echo system with a 30-MHz transducer (Visual Sonics, Toronto, Canada).

Left Ventricular Catheterization and Pressure Measurements
Close-chest left ventricular (LV) pressure and its derivatives were recorded using a Powerlab data acquisition system (ADInstruments, Colorado Springs, Colo.) and a high-fidelity 1.4F Millar Mikro-Tip catheter transducer (model SPR-835, Millar Instruments, Tex.) placed into the LV chamber via the right common carotid artery.

RNA Analyses
RNA dot blot analysis and semiquantitative RT-PCR were performed as described.

In Vitro Studies of Cardiomyocyte Hypertrophy
Neonatal rat cardiomyocyte (NRCM) culture and adenoviral infection were performed as described. Recombinant adenoviruses harboring a green fluorescent protein (GFP)-fused NFATc1 (Ad-NFAT-GFP) or a constitutively active form of mouse calcineurin Aα (Ad-CnAΔ) and Ad-WT-CryAB were described previously. Concomitant infections of myocytes with Ad-NFAT-GFP and Ad-WT-CryAB were performed at multiplicities of infection of 100 and 50, respectively. Twenty-four hours after Ad-NFAT-GFP infection, cells were treated with various adrenergic agonists or 100 µmol/L L-ascorbic acid vehicle in serum-free medium for 48 hours. The profile areas of individual cardiomyocytes identified by positive staining of F-actin with Alexa Fluor-568–conjugated phalloidin (Molecular Probes, Eugene, Ore) were measured from digitalized images using the Image-Pro Plus image analysis system (Media Cybernetics, Silver Springs, Md).

Luciferase Reporter Assay
Luciferase activity in NFAT-Luc TG, NFAT-Luc::CryAB double TG, and NFAT-Luc::CryAB/HSPB2-null mice were measured using a Luciferase Assay Kit (Roche Diagnostics Corporation, Indianapolis, Ind) according to the instructions of the manufacturer.

Results
Upregulation of CryAB in the Early Phase of Pressure Overloaded Cardiac Hypertrophy
Our examination revealed that CryAB protein levels were gradually and significantly increased in the LV of WT mice (NTG) during the first 2 weeks after TAC (Figure 1A). To investigate the pathophysiological significance of CryAB in cardiac responses to mechanical overload, we adopted both gain- and loss-of-function approaches. For the gain-of-function, a cardiomyocyte-restricted CryAB overexpression mouse model (TG) was used. For the loss-of-function, the CryAB/HSPB2 double knockout mice (KO) were used. Compared with the NTG TAC group, LV myocardial CryAB remained to be overexpressed in the TG TAC but was absent in the KO TAC mice at 2 weeks after surgery (Figure 1B).

CryAB/HSPB2 KO but Not CryAB Overexpression Produces Cardiomyopathy
To test the effects of CryAB gain- and loss-of-function on cardiac responses to a minor stress condition, we compared
cardiac fetal gene expression, cardiac mass, LV geometry, and cardiac function among the NTG, TG, and KO groups at 2 weeks after the sham surgery (ie, 10 weeks of age). Echo, LV hemodynamics, cardiac mass (Table I in the online data supplement), and the expression of the fetal gene program (Figure 2A and 2B) did not show any statistically significant difference between TG and NTG animals.

However, KO mice showed distinct changes at all the levels examined. Echo revealed a significant increase in LV diastolic posterior wall thickness but no changes in the LV chamber dimension, fractional shortening (FS), and ejection fraction (EF) (supplemental Table I). Consistent with the Echo findings, KO mice displayed a moderate but statistically significant increase in heart weight/body weight ratios (HW/BW) in absence of significant changes in the average body weight compared with the NTG group. There was no evidence of organ congestion at this time point (supplemental Table I). Functionally, although all parameters for LV systolic function or contractile function did not differ among the 3 groups at 10 weeks, LV diastolic function was compromised in the KO mice, as reflected by depressed minimum dP/dt (−dP/dt_{max}) and elevated LV end-diastolic pressure (LVEDP) (supplemental Table I). In a separate cohort, we observed statistically significant decreases in FS and EF in the 18-week-old KO mice at the unstressed baseline condition (supplemental Table II). These findings demonstrate that the absence of CryAB/HSPB2 induces abnormal cardiac growth and defective myocardial relaxation.

The reactivation of the fetal gene program was striking in the LV of the KO mice (Figure 2A and supplemental Figure I). At 10 weeks of age, these mice displayed significant increases in the transcript levels of atrial natriuretic factor (ANF), β-MyHC, skeletal actin (s-Actin), α-MyHC, sarcoplasmic endoplasmic reticulum calcium ATPase 2A (SERCA), PLN, and GAPDH with 32P-labeled transcript-specific oligonucleotide probes. The composed RNA dot blot images are shown in A. Each dot represents an individual animal. After normalization to the corresponding GAPDH signal, the mean intensity value of the NTG sham group was set to 100 arbitrary units (AU). The intensity signal of each individual dot was then normalized to the mean of the NTG sham. Comparison among the 3 sham control groups is presented in supplemental Figure I. ANF, skeletal actin, and β-MyHC transcripts were significantly increased, whereas α-MyHC, SERCA, and PLN were significantly decreased in the NTG TAC (P<0.01). Compared with the NTG TAC group, ANF and β-MyHC upregulation and the downregulation of α-MyHC, SERCA, and PLN were substantially attenuated in the TG group (B), whereas the upregulation of ANF and β-MyHC was significantly enhanced in the KO group (C).

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Table I. Summary of gene expression in the LV of NTG, TG, and KO mice at 10 weeks of age.

Figure 2. Reactivation of the fetal gene program. Total RNA isolated from LV myocardium at 2 weeks after TAC or sham surgery was used for quantitative RNA dot blot analysis of the transcript levels of ANF, β-MyHC, skeletal actin (s-Actin), α-MyHC, sarcoplasmic endoplasmic reticulum calcium ATPase 2A (SERCA), PLN, and GAPDH with 32P-labeled transcript-specific oligonucleotide probes. The composed RNA dot blot images are shown in A. Each dot represents an individual animal. After normalization to the corresponding GAPDH signal, the mean intensity value of the NTG sham group was set to 100 arbitrary units (AU). The intensity signal of each individual dot was then normalized to the mean of the NTG sham. Comparison among the 3 sham control groups is presented in supplemental Figure I. ANF, skeletal actin, and β-MyHC transcripts were significantly increased, whereas α-MyHC, SERCA, and PLN were significantly decreased in the NTG TAC (P<0.01). Compared with the NTG TAC group, ANF and β-MyHC upregulation and the downregulation of α-MyHC, SERCA, and PLN were substantially attenuated in the TG group (B), whereas the upregulation of ANF and β-MyHC was significantly enhanced in the KO group (C).
(ANF), β-myosin heavy chain (β-MyHC), and skeletal actin and a tendency for decreases in α-MyHC, SERCA, and phospholamban (PLN). This genetic reprogramming persisted and was aggravated at 20 weeks (supplemental Figure II).

CryAB Overexpression Attenuates the Early Cardiac Response to Pressure Overload

Previous reports and the baseline characterizations performed in this study have shown that the cardiac-restricted CryAB overexpression does not produce an abnormal phenotype. This study delineates whether CryAB overexpression is beneficial or detrimental in a mechanical overload condition. Two weeks after TAC, NTG mice displayed classic pressure-overloaded hypertrophic responses, including fetal gene program reactivation, increases in LV diastolic posterior wall thickness, and increases in HW/BW and ventricular weight/BW ratios (Figures 2 through 4), compared with NTG sham controls. These changes were significantly attenuated in the TG TAC mice, although the LV systolic peak pressure (LVSP) was comparable between the NTG TAC and TG TAC groups (Figure 5A). Compared with NTG TAC mice, gravimetric data showed 15% less ventricular hypertrophy in the TG TAC hearts (Figure 3). The induction of fetal genes by TAC was significantly attenuated in the TG hearts as well. The TG TAC mice showed ANF upregulation, but this was significantly less than that in the NTG TAC hearts. MyHC isoform switch (from α to β) and the significant downregulation of the genes involved in calcium handling (SERCA and PLN) in the LV were observed in NTG TAC mice. However, these changes were significantly less in the TG TAC group (Figure 2A and 2B).

Despite less hypertrophic responses in TG TAC mice, neither Echo nor hemodynamic assessments showed any
A statistically significant difference in LV function between the TG TAC and the NTG TAC groups (Figures 4 and 5). It should be noted that NTG mice had not yet shown significant decreases in major LV function parameters (eg, FS, EF, \( \frac{dP}{dt_{40}} \), \( \frac{dP}{dt_{40}} \)) at 2 weeks after TAC.

**CryAB Overexpression Attenuates Hypertrophic Growth of Cultured Cardiomyocytes**

To test whether the hypertrophy suppression effects of CryAB is cardiomyocyte-autonomous, we induced CryAB overexpression via adenoviral vectors in cultured NRCMs and tested its effects on pharmacologically induced cardiomyocyte hypertrophic growth. Compared with the endogenous CryAB of the control viral (Ad-empty)-infected cells, Ad-CryAB infection at the multiplicities of infection used here overexpressed CryAB protein by a factor of \( \times 3 \) (Figure 6A), which is less than the levels of overexpression in the TG mice (\( \times 5 \) folds). The treatment of norepinephrine (2 \( \mu \)mol/L), phenylephrine (PE) (30 \( \mu \)mol/L), or ISO (2 \( \mu \)mol/L) induced significant increases in the profile area of NRCMs, but the increases were significantly attenuated by CryAB overexpression (Figure 6B and 6C). Consistent with previous reports,\textsuperscript{27,28} the calcineurin inhibitor cyclosporine A (CsA) (500 ng/mL) significantly suppressed norepinephrine-induced NRCM hypertrophy, but the combination of CryAB overexpression and CsA treatment did not show additional suppression (Figure 6B and 6C). Similar results were obtained over PE-induced hypertrophy (data not shown). These data suggest that CryAB suppresses hypertrophy likely through the same pathway as CsA does.

**Absence of CryAB/HSPB2 Is Deleterious in Cardiac Pressure Overload**

Because of the proximity between the CryAB and the HSPB2 genes in mouse genome, HSPB2 is accidentally ablated when targeting the CryAB gene.\textsuperscript{18} The resultant CryAB/HSPB2-null mouse is the only mouse model presently available for CryAB loss-of-function studies. Compared with the NTG TAC group, KO TAC mice showed significantly lower LVSP (Figure 5A) but statistically greater HW/BW and ventricular weight/BW ratios, indicating more hypertrophy (Figure 3A and 3B). Consistently, transcriptional upregulation of ANF and \( \beta \)-MyHC was also significantly greater in the KO TAC mouse hearts (Figure 2C). Compared with the NTG TAC group, Echo showed statistically smaller EF and FS in the KO TAC group (Figure 4B and 4C). Because LVSP significantly differed between KO TAC and NTG TAC groups (Figure 5A), \( +\frac{dP}{dt_{40}} \) and \( -\frac{dP}{dt_{40}} \) were analyzed and compared. The absolute values of LV \( +\frac{dP}{dt_{40}} \) and \( -\frac{dP}{dt_{40}} \) were significantly smaller in the KO TAC group than the NTG TAC group (Figure 5C and 5D). LVEDP was elevated in all 3 TAC groups, but the elevation was greater in the KO TAC

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**Figure 5.** Changes in LV pressure at 2 weeks after TAC or sham surgery. A, Changes in the LV systolic peak pressure (LVSP). B, Changes in the LVEDP. C and D, Changes in the LV pressure rising \( (+\frac{dP}{dt_{40}}) \) (C) and declining velocities \( (-\frac{dP}{dt_{40}}) \) (D), with LV pressure being 40 mm Hg. \( *P<0.05 \) (A through C), \( *P<0.01 \) (D) compared with the same genotype sham control.
To determine the in vivo potential effect of CryAB on the NFAT signaling pathway, a well-established signaling pathway for pathological cardiac hypertrophy, we conducted both in vivo and cell culture experiments to determine the influence of CryAB on the NFAT signaling pathway, a well-established signaling pathway for pathological cardiac growth. To determine the in vivo potential effect of CryAB loss-of-function on NFAT signaling, NFAT-Luc reporter mice were crossed with the KO mice, and the luciferase activities in ventricular myocardium and in the soleus muscle were measured at 18 weeks. Compared with WT littermates, luciferase activities were significantly increased in both cardiac and skeletal muscle of KO mice (Figure 7A). Consistent with the reporter assays, increases in NFATc4 nuclear to cytoplasmic ratios were detected in the KO hearts (Figure 7B). Moreover, myocyte-enriched calcineurin-interacting protein-1.4 (MCIP1.4) is a bona fide NFAT target gene.29,30 MCIP1.4 transcript levels were significantly higher in KO hearts than the WT controls (Figure 7C). These findings indicate that CryAB and/or HSPB2 suppress NFAT signaling at the baseline condition.

To illustrate further the in vivo effect of CryAB on NFAT signaling, NFAT-Luc reporter mice were crossed with CryAB TG mice, and the resultant littermates were subjected to TAC at 12 weeks of age. NFAT activation at 2 weeks after TAC was significantly attenuated by CryAB overexpression (Figure 7D).

NFAT nuclear translocation is a critical step of NFAT activation. To test whether the in vivo effect of CryAB on NFAT transactivation (Figure 7) is cardiomyocyte-autonomous, we determined the effects of CryAB overexpression on hypertrophy agonist–induced NFAT nuclear translocation and NFAT target gene expression in cultured cardiomyocytes. Consistent with previous reports,31,32 GFP-tagged NFATc1 expressed in cultured NRCMs existed predominantly in the cytoplasm in a serum-free culture condition, but it showed significant nuclear translocation on expression of a constitutively active form of CnAδ or exposure to an α1-adrenergic agonist (PE). Overexpression of CryAB markedly reduced PE-induced nuclear translocation of NFAT-GFP (Figure 8A and 8B). Furthermore, adrenergic stimulation–induced MCIP1.4 expression was markedly attenuated by CryAB overexpression (Figure 8C and 8D).

**Discussion**

PQC in the cell assists proper folding of nascent proteins, keeps normal matured proteins from denaturing and misfolding, and removes terminally misfolded proteins.4 Molecular chaperones play critical roles in each of these processes and thereby protect the cell. Even under physiological conditions, the heart is constantly under tremendous stress. Various insults from pathological conditions, such as hypertension and myocardial ischemia, inevitably increase the stress on cardiomyocytes. The stress conceivably poses a significant challenge to PQC in cardiomyocytes. For example, increases in protein synthesis in cardiomyocytes characteristic of cardiac hypertrophy require PQC to work harder because approximately 30% of newly synthesized polypeptides are degraded before they become mature proteins.33 HSPs are an important family of molecular chaperones and therefore are essential to the cell in dealing with various stress conditions by participation in PQC. However, until the present study, the (patho)physiological significance of HSPs, especially the sHSPs, in cardiac responses to hemodynamic overload had not been demonstrated.
In cardiomyocytes, CryAB is a bona fide constitutively expressed sHSP. Here, we report that cardiac CryAB protein expression can be induced by pressure overload. Moreover, we have investigated the (patho)physiological role of CryAB in early cardiac responses to pressure overload and in the modulation of a pivotal hypertrophic signaling pathway. Our data show that CryAB/HSPB2 deficiency activates the NFAT signaling and induces cardiac hypertrophic responses at the unstressed or minimal stress conditions and exacerbates cardiac malfunction on pressure overload, whereas CryAB overexpression significantly attenuates pressure-overloaded hypertrophic responses and associated NFAT activation in mouse hearts. Our further experiments revealed that CryAB overexpression suppressed adrenergic stimulation–induced nuclear translocation of NFAT and the expression of a bona fide NFAT target gene (Figure 8) and attenuated adrenergic stimulation induced hypertrophy (Figure 6) in cultured cardiomyocytes. CryAB overexpression failed to further suppress hypertrophic growth when the calcineurin–NFAT pathway is blocked by CsA (Figure 6). These new findings demonstrate that CryAB negatively regulates pressure overload cardiac hypertrophic responses likely through inhibiting NFAT signaling in cardiomyocytes.

To determine the necessity of CryAB/HSPB2 for the heart to respond to stress, we subjected the KO mice to sham and TAC surgery. Compared with WT mice (NTG), KO mice responded considerably differently to these procedures. At 2 weeks after the sham surgery, which is a relatively milder stress condition, KO mice displayed marked reactivation of the fetal gene program (Figure 2A and supplemental Figure I); concentric cardiac hypertrophy, as evidenced by increased LV wall thickness and HW/BW ratio; and LV diastolic malfunction, as indicated by changes in minimum dP/dt and LVEDP (supplemental Table I). These data suggest CryAB and/or HSPB2 are required to maintain normal cardiac function in response to a general stress.

This is somewhat surprising because it was reported that young CryAB/HSPB2 KO mice under an unstressed condition do not show discernible cardiac abnormalities in expression of the fetal gene program, myocardial histology, and echocardiography. Although increased HW/BW ratio was observed in 4-month-old CryAB/HSPB2 KO mice by Morri-
son et al., it was attributed to a decrease in BW. We did not observe a statistically significant difference in BW between NTG and KO sham groups. This is consistent with previous reports showing normal growth curves in the KO mice until 30 to 40 weeks of age. It is noted that the previously reported study characterized mixed-sex mice in a 129/Svj isogenic background, whereas the present study used all male and FVB inbred mice.

Compared with the NTG TAC group, the KO TAC group showed greater reactivation of ANF and β-MyHC (Figure 2), greater cardiac hypertrophy (Figures 3 and 4A), lower LVSP and dP/dt (Figure 5), lower EF and FS (Figure 4), and higher LVEDP (Figure 5B) and lung weight/BW ratio (Figure 3C). These indicate that the absence of CryAB/HSPB2 renders cardiac responses to TAC-induced LV pressure overload more pathological. Taken together, the loss-of-function studies demonstrated that CryAB and/or HSPB2 are essential to maintaining normal cardiac function in a hemodynamic overload condition.

In the present study, a gradual but significant upregulation of CryAB protein was observed in WT mouse hearts under pressure overload (Figure 1). This upregulation is likely a compensatory response of the heart to deal with increased PQC burden posted by pressure overload cardiac hypertrophy. However, the reactive CryAB increase does not appear to be adequate because constitutively forced overexpression of CryAB, as shown in the TG group, significantly attenuated TAC-induced NFAT transactivation (Figure 7D), reactivation of the fetal gene program (Figure 2A), and cardiac hypertrophy (Figures 3 and 4A). Notably, both Echo and hemodynamics demonstrated that the attenuation of cardiac hypertrophy by CryAB overexpression did not compromise cardiac function under the pressure overload condition. This is consistent with recent reports. This also suggests that at least a fraction of the hypertrophic response might be caused by the hypertrophic growth per se and is dispensable if the increased burden on PQC is diluted by molecular chaperones.

Notably, using a well-established NFAT reporter assay, as well as monitoring NFAT nuclear translocation and the expression of a bona fide NFAT target gene MCIP1.4 (Figure 7A through 7C), we have found, for the first time, that NFAT is activated in the heart and skeletal muscle of CryAB/HSPB2-null mice. This is consistent with the development of cardiac hypertrophy and malfunction observed in the present study (supplemental Table II) and the previously reported skeletal myopathy in the null mice under the baseline condition. Because both CryAB and HSPB2 are ablated in the KO mice, we cannot pinpoint which sHSP mediates the observed function based solely on the data from the KO mice. However, the highly complementary in vivo and in vitro effects of CryAB gain-of-function on both hypertrophic responses and NFAT activation indicate that loss of CryAB is responsible, at least in part, for the phenotypes that we observed in the KO mice. Therefore, the evidence strongly supports that CryAB inhibits the NFAT signaling and suppresses
cardiac hypertrophic responses. The further mechanism underlying this inhibition remains to be delineated. It was recently shown that Mrj, a member of the HSP40 family, interacts with class II histone deacetylases and NFAT to repress NFAT transactivation.\(^1\)\(^2\) Hence, CryAB might directly interact with NFAT and prevent its nuclear translocation. From the PQC point of view, CryAB, which has previously been shown to inhibit aggregation of abnormal proteins,\(^3\) may protect cardiomyocytes from being damaged by misfolded or damaged proteins under both physiological and pathological conditions. Therefore, the observed effect of CryAB on NFAT could also be secondary to its protection against stress.

The role(s) of HSPs in the cardiac hypertrophic response to pressure overload has not been described, whereas hypertension and cardiac hypertrophy are important antecedent factors for the development of congestive heart failure. Both the upregulation of CryAB in familial hypertrophic cardiomyopathy and the downregulation of CryAB in failing human hearts have been reported.\(^\)\(^1\)\(^ \)\(^2\) Therefore, by investigating gain-of-function of CryAB and loss-of-function of CryAB/HSPB2, this study has significantly expanded our understanding of the pathophysiological significance of sHSPs and thereby the importance of PQC in the heart. The findings will help elucidate the potential therapeutic benefits of sHSP in heart disease.

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Disclosures

None.

References


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Materials and Methods

Experimental animals

Line 11 CryAB transgenic mice (TG) were created and have been maintained in the FVB/N inbred background and the initial characterization of this TG line was reported.\(^1\) Generation of CryAB/HSPB2 double knockout mice (KO) in a 129/Svj genetic background was previously described.\(^2\) CryAB/HSPB2 KO mice (line 460) used in this study were derived from 8 generations of back-crossing of the 129/Svj CryAB/HSPB2 KO mice into the FVB/N background. Ntg littermates (NTG) from line 11 served as the controls for both TG and age-matched KO groups. Only male mice at 8 weeks of age were used. The transgenic mouse model carrying an NFAT binding site-dependent luciferase reporter (NFAT-Luc) generously donated by Dr. Jeffery Molkentin (Children’s Hospital Medical Center, Cincinnati, Ohio) has been previously described.\(^3\) Institutional guidelines were followed in the care and use of the mice.

Transverse aortic constriction (TAC)

TAC was performed on mice under aseptic conditions as previously described with modifications.\(^4\) After inducing anesthesia using 2% Isoflurane, the animal was intubated via the mouth and mechanically ventilated with a rodent ventilator (MiniVent Type 845; Harvard Apparatus, MD). Anesthesia was maintained with 1.2-1.5% isoflurane in 100% oxygen with a tidal volume of 200μl and a frequency of 120 strokes/min. Through a midline partial sternotomy approach, the aortic arch was isolated between the innominate and the left common carotid arteries and ligated with a 7-0 nylon suture (Ethicon, NJ) against a 29G needle (OD 0.33mm). The needle was immediately withdrawn after the ligation. Sham control mice underwent the same procedure except for aortic constriction. After surgery, the chest wall was closed with 4-0
silk suture, and the skin was sealed. Pain control was achieved by subcutaneous administration of Buprenorphine and fluid support given intraperitoneally. The animal was gradually weaned from the ventilator and placed on a warm surface during recovery. Terminal experiments were performed at 2 weeks post-TAC.

**Transthoracic echocardiography**

Pre- and post-operative echocardiography (Echo) was performed using a high resolution Vevo 770™ Echo system with a 30MHz transducer and 12.7mm focal length (Visual Sonics, Toronto, Canada). Mice were kept under light anesthesia with Isoflurane in room air supplemented with 100% oxygen. A 2D guided M-mode was acquired through the anterior and posterior walls at the papillary muscle level at a frame rate of 140Hz as previously described.\(^5\)

**Left ventricular (LV) catheterization and pressure measurement**

At the terminal experiment, mice were intubated and mechanically ventilated. The right common carotid artery was isolated and cannulated with a high-fidelity 1.4F Millar Mikro-Tip® catheter transducer (model SPR-835, Millar Instruments, TX) and advanced to the LV chamber. After stabilizing for 10 minutes, LV pressure (LVP) and its first derivatives (dP/dt) were recorded using a Powerlab data acquisition system (ADInstruments, Colorado Springs, CO). Heart rate (HR), systolic and diastolic LVP, and LV end-diastolic pressure (LVEDP) were all measured directly from the LVP waveforms. The LV function was evaluated by the maximal positive and negative dP/dt (+dP/dt\(_{\text{max}}\) and -dP/dt\(_{\text{max}}\)) and the dP/dt at a LVP of 40mmHg (+dP/dt\(_{40}\) and –dP/dt\(_{40}\)).
RNA dot blot analysis

Total RNA was isolated from the LV of 3 individual mice of each group using Tri-Reagent (Molecular Research Center Inc, OH). For RNA dot blot, 4μg of total RNA was loaded on to a nitrocellulose membrane. After cross-linking, the membrane was hybridized with P³²-labeled transcript-specific oligonucleotide probes of the hypertrophic gene panel and detected with the Personal Molecular Imager FX (BioRad) and quantified with the associated Quantity-One software as described.⁵

Semi-quantitative reverse-transcription (RT-) PCR  Isolation of total RNA and RT-PCR were performed as described⁶. The primer sequences for GAPDH are the same as reported⁷. The sequences of the primers specific for the transcript of mouse myocyte-enriched calcineurin interacting protein-1.4 (MCIP1.4) are: forward: 5’-GAGCGAGTCGTTGCTAAGC-3’ and reverse: 5’-TTTGGCCCTGGTCTCAG-3’; Rat MCIP1.4 forward: 5’-AAAGCAGAATGCA TTTAGGG-3’ and reverse: 5’-TTTGGCCCTGGTCTCAG-3’. GAPDH is amplified as the internal control for potential variations in RNA sampling and the efficiency of reverse transcription. The numbers of PCR cycles were optimized for each transcript to avoid potential PCR saturation.

Preparation of cytoplasmic and nuclear protein extracts

Cytoplasmic and nuclear protein fractions were prepared according to the instructions of NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology, Rockford, IL). Briefly, frozen left ventricle myocardium was homogenized in ice-cold PBS at 4°C. The homogenates were centrifuged at 500 x g for 3 min and the supernatants were removed. To
further break down the cells, the pellets were resuspended in 300 μl of CER-I, vigorously vortexed and added 16.5 μl of CER-II. After centrifugation at 16,000 x g for 5 min, the supernatants (cytoplasmic fraction) were collected. The insoluble fraction containing nuclei was resuspended in 0.15 ml of ice-cold NER and subjected to 4 rounds of vortex (15 sec/round) and incubating on ice (10 min). The resultant nuclear lysates were then centrifuged at 16,000 x g for 10 min and the supernatants (nuclear fraction) were collected.

**Determination of NFAT nuclear to cytoplasmic ratio**

The cytoplasmic and nuclear protein extracts were fractionated with 8% SDS-PAGE and transferred to PVDF membrane. NFAT is immunoprobed using an antibody against NFATc4 (Santa Cruz, CA) and the Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ). The images were collected using BioRad Versadoc 4000 (BioRad) and analyzed with the Quantity One software.

**Neonatal rat cardiomyocyte (NRCM) culture, adenoviral infection, and profile area**

NRCM culture was performed as described. Recombinant adenoviruses for overexpression of a GFP-fused NFATc1 (Ad-NFAT-GFP) or a Ca$^{2+}$-independent, constitutively active, truncated form of mouse calcineurin Aα (Ad-CnAΔ) were generous gifts from Dr. Qiangrong Liang of Sanford Research/USD (Sioux Falls, SD). Ad-WT-CryAB was described previously. Adenoviral infection of NRCMs was performed at the indicated multiplicity of infection (MOI) in serum-free DMEM for 2 hours at 37°C in a humidified, 5% CO$_2$ incubator. Concomitant infections of myocytes with Ad-NFAT-GFP and Ad-WT-CryAB were performed at MOIs of 100 and 50, respectively. An empty adenoviral vector (Ad-Empty, MOI50) was used as the
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Kumarapeli et al., AlphaB-Crystallin Suppresses Pressure…

control for Ad-WT-CryAB. As a positive control, Ad-CnAΔ (MOI50) was used.12 Twenty-four hours after Ad-NFAT-GFP infection, cells were treated with 30μM PE or 100μM of L-ascorbic acid (AA) vehicle in serum-free medium for 48 hours. The profile areas of individual cardiomyocytes identified by positive staining with Alexa Fluor-568 conjugated phalloidin were measured from digitalized images using the Image-Pro Plus image analysis system (Media Cybernetics, Silver Springs, MD).

**NFAT nuclear translocation assessment**

Cells in chamber slides were fixed with 4% paraformaldehyde. Alexa Fluor-568 conjugated phalloidin (Molecular Probes, Eugene, OR) and DAPI were used to stain myocyte F-actin and the nucleus, respectively. An inverted epi-fluorescence microscope (model IX71, Olympus, Melville, NY) was used to visualize the stained cardiomyocytes and their GFP distribution. The images were captured and digitized using an Olympus DP70 camera and associated software. NRCMs were identified by phalloidin positive staining (red fluorescence). NFAT-GFP expression is identified by green fluorescence. To calculate the percentage of NRCMs that have NFAT nuclear translocation, 10 microscopic fields (~500 NFAT-GFP expressing NRCMs) per group were examined for the distribution of NFAT-GFP. A cell with nuclear GFP signal equal to or higher than cytoplasmic GFP was considered positive for NFAT nuclear translocation.

**Luciferase reporter assay**

NFAT-Luc reporter activity in NFAT-Luc transgenic, NFAT-Luc::CryAB double transgenic, and NFAT-Luc::CryAB/HSPB2-null mice, were measured using a Luciferase Assay Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA), according to the manufacturer’s instructions.
Left ventricular and skeletal muscle tissues were broken by rapid freezing in liquid nitrogen and followed by crushing the tissues with a pestle. The samples were resuspended in 1X lysis buffer (provided by Luciferase Assay Kit). The samples were incubated at 20°C for 15 minutes and then centrifuged at 15,000×g for 5 minutes. The supernatant was transferred to microcentrifuge tubes and the luciferase activity was measured using a luminometer (Berthold Detection Systems, Oak Ridge, TN, USA).

**Statistical analysis**

All quantitative data was analyzed by Student’s t-test and one factor or multiple factor analysis of variance (ANOVA) using SigmaStat 3.0 software (Systat, Point Richmond, CA), where applicable. The Holm-Sidak test was used for post-hoc comparisons. Unless indicated otherwise, all quantitative data are presented as mean±SD. A probability less than 5% ($P<0.05$) was considered to be statistically significant.
Supplement Material

Kumarapeli et al., AlphaB-Crystallin Suppresses Pressure...

References


Supplemental Data

Online Table I. Baseline Characterization of CryAB TG and CryAB/HSPB2 KO Mice at 10 Weeks of Age

<table>
<thead>
<tr>
<th></th>
<th>NTG</th>
<th>TG</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>490±46</td>
<td>459±45</td>
<td>462±32</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.77±0.18</td>
<td>3.56±0.18</td>
<td>3.57±0.26</td>
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<td>LVIDs (mm)</td>
<td>2.37±0.19</td>
<td>2.18±0.18</td>
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<td>LVPWd (mm)</td>
<td>0.76±0.04</td>
<td>0.73±0.09</td>
<td>0.92±0.04*</td>
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<td>FS (%)</td>
<td>37.1±2.7</td>
<td>38.6±3.7</td>
<td>36.1±3.4</td>
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<td>EF (%)</td>
<td>67.8±3.5</td>
<td>69.8±4.6</td>
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<td><strong>LV pressure</strong></td>
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<tr>
<td>LVSP (mmHg)</td>
<td>90.7±4.6</td>
<td>85.4±12.4</td>
<td>90.3±12.3</td>
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<td>LVEDP (mmHg)</td>
<td>5.5±1.0</td>
<td>4.7±2.4</td>
<td>18.3±2.9*</td>
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<td>+dP/dt\text{max} (mmHg/s)</td>
<td>8620±1406</td>
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<td>-dP/dt\text{max} (mmHg/s)</td>
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<td>6196±1065†</td>
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<td><strong>Gravimetry</strong></td>
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<tr>
<td>Body weight (BW, g)</td>
<td>27.5±2.5</td>
<td>30.4±3.3</td>
<td>25.3±2.2</td>
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<tr>
<td>Heart weight (HW, g)</td>
<td>123.7±8.4</td>
<td>132.5±7.8</td>
<td>131.7±13</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>4.52±0.4</td>
<td>4.38±0.2</td>
<td>5.24±0.7†</td>
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<tr>
<td>Lung/BW (mg/g)</td>
<td>5.4±0.6</td>
<td>5.3±0.5</td>
<td>5.4±0.5</td>
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<tr>
<td>Liver/BW (mg/g)</td>
<td>46.0±3.0</td>
<td>42.6±2.2</td>
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<td>Kidney/BW (mg/g)</td>
<td>13.8±0.2</td>
<td>14.7±0.9</td>
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Compared with NTG, †: p<0.05; *: p<0.01.
Online Table II. Baseline Characterization of CryAB/HSPB2 KO Mice at 18 Weeks of Age

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<thead>
<tr>
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<th>NTG</th>
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</thead>
<tbody>
<tr>
<td><strong>Echocardiography</strong></td>
<td>N=11</td>
<td>N=11</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>473±31</td>
<td>472±26</td>
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<tr>
<td>LVIDd (mm)</td>
<td>3.81±0.23</td>
<td>3.90±0.23</td>
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<tr>
<td>LVIDs (mm)</td>
<td>2.43±0.19</td>
<td>2.66±0.20</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.75±0.08</td>
<td>0.85±0.06*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>36.4±2.7</td>
<td>31.9±1.61*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>66.7±3.5</td>
<td>60.7±2.4*</td>
</tr>
<tr>
<td><strong>LV pressure</strong></td>
<td>N=5</td>
<td>N=6</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>540±93</td>
<td>541±75</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>96.5±4.8</td>
<td>92.7±8.3</td>
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<tr>
<td>LVEDP (mmHg)</td>
<td>6.5±3.0</td>
<td>18.8±9.2†</td>
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<td>+dP/dt&lt;sub&gt;max&lt;/sub&gt; (mmHg/s)</td>
<td>11632±2254</td>
<td>9041±1160</td>
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<td>-dP/dt&lt;sub&gt;max&lt;/sub&gt; (mmHg/s)</td>
<td>10630±1586</td>
<td>7878±1455†</td>
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<td>Tau (ms)</td>
<td>7.3±2.3</td>
<td>11.7±2.6†</td>
</tr>
<tr>
<td><strong>Gravimetry</strong></td>
<td>N=8</td>
<td>N=10</td>
</tr>
<tr>
<td>Body weight (BW, g)</td>
<td>27.2±4.2</td>
<td>26.6±3.9</td>
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<tr>
<td>Heart weight (HW, g)</td>
<td>113.1±13.9</td>
<td>122.8±17.9</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>4.19±0.35</td>
<td>4.62±0.29†</td>
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<tr>
<td>Lung/BW (mg/g)</td>
<td>5.58±0.59</td>
<td>5.40±0.39</td>
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Compared with NTG, †: p<0.05; *: p<0.01.
Online Figure Legends

**Online Figure I. A summary of the comparison of the fetal gene expression among the sham surgery control groups.** Total RNA was isolated from LV myocardium at 2 wk after TAC or sham surgery. Quantitative RNA dot blot analysis was utilized to assess the transcript levels of atrial natriuretic factor (ANF), β-myosin heavy chain (β-MyHC), skeletal α-actin (s-Actin), α-MyHC, sarcoplasmic endoplasmic reticulum calcium ATPase 2A (SERCA), phospholamban (PLN), and GAPDH using $^{32}$P-labeled transcript-specific oligonucleotide probes. The bound radioactive signal was captured by a phosphor-imaging screen and detected and quantified using an FX Personal Molecular Imager (BioRad). The composed RNA dot blot images are shown in Figure 2A of main text. Each dot represents an individual animal. After normalizing to the corresponding GAPDH signal, the mean intensity value of the NTG sham group was set at 100 arbitrary units (AU). The intensity signal of each individual dot was then normalized to the mean of the NTG sham. The resultant intensity of each of the 3 animals from the same group was used to derive the mean and standard deviation (error bar) and presented in subsequent panels. Compared with the NTG sham group, no changes were detected in the TG sham group but significant increases in ANF, β-MyHC, and s-Actin and decreases in PLN were detected in the KO sham group. ¶: $P<0.05$, *: $P<0.001$, vs NTG and TG; One way ANOVA.

**Online Figure II. Reactivation of the fetal gene program in CryAB/HSPB2 null (KO) mouse hearts at 18 wks of age.** Total RNA was isolated from the LV myocardium. Quantitative RNA dot blot analysis was performed to assess the transcript levels of atrial natriuretic factor A (ANF), β-myosin heavy chain (β-MyHC), skeletal α-actin (s-Actin), α-MyHC, sarcoplasmic endoplasmic reticulum calcium ATPase 2A (SERCA), phospholamban (PLN), and GAPDH using $^{32}$P-labeled transcript-specific oligonucleotide probes. The bound radioactive signal was captured by a phosphor-imaging screen and detected and quantified using an FX Personal Molecular Imager (BioRad). The composed RNA dot blot images are shown in panel A. Each dot represents an individual animal. After normalizing to the corresponding GAPDH signal, the mean intensity value of the wild type (WT) group was arbitrarily set at 100 arbitrary units (AU). The intensity signal of each individual dot is then normalized to the mean of the WT. The resultant AU value of each of the 3 animals from the same group was used to
derive the mean and standard deviation (error bar) and presented in panel B. Compared with the WT, *: $P<0.05$, **: $P<0.001$; unpaired t-test were used.
Online Figure II

A

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>KO</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
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<tr>
<td>ANF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-MyHC</td>
<td></td>
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<tr>
<td>s-Actin</td>
<td></td>
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<tr>
<td>α-MyHC</td>
<td></td>
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<tr>
<td>SERCA</td>
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<tr>
<td>PLN</td>
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</table>

B

- **A**: Western blot analysis showing protein expression levels in WT and KO groups.
- **B**: Bar graph displaying transcript levels (AU) for different genes in WT and DKO groups. Significance levels indicated by asterisks.

- ANF: ***
- s-Actin: *
- β-MyHC: *
- α-MyHC: **
- SERCA: **
- PLN: **