Roles for αB-crystallin and HSPB2 in protecting the myocardium from ischemia-reperfusion-induced damage in a KO mouse model

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Morrison, Lisa E., Ross J. Whittaker, Robert E. Klepper, Eric F. Wawrousek, and Christopher C. Glembotski. Roles for αB-crystallin and HSPB2 in protecting the myocardium from ischemia-reperfusion-induced damage in a KO mouse model. Am J Physiol Heart Circ Physiol 286: H847–H855, 2004. First published October 30, 2003; 10.1152/ajpheart.00715.2003.—Overexpression studies have shown that the small heat shock proteins (sHSP) protect the myocardium from ischemia-reperfusion (I/R)-induced damage. However, gene deletion studies are necessary to demonstrate whether sHSPs are required for protection. The genes for αB-crystallin (αBC) and HSPB2, two sHSPs that are expressed in high levels in the heart, are in close proximity to one another; as a result, both genes were disrupted in a recently generated knockout (KO) mouse line. The αBC/HSPB2 KO mouse line is currently the only model that features disruption of sHSPs normally expressed in the heart. Accordingly, we examined the cardiac morphology, function, and response to I/R-induced stress in αBC/HSPB2 KO mice. Initial gross, light microscopic and echocardiographic characterization showed that the morphological and functional properties of hearts from adult KO mice were indistinguishable from age-matched wild-type (WT) mice. Electron microscopy showed that, compared with WT mouse hearts, KO mouse heart sarcomeres were relatively normal. Isolated perfused KO mouse hearts displayed normal contractility; however, when compared with WT, after I/R, KO mouse hearts exhibited a twofold reduction in contractile recovery, as well as increased necrosis and apoptosis. Additionally, when compared with WT, αBC/HSPB2 KO mouse hearts exhibited 43% less reduced glutathione, which is known to protect from I/R-induced damage. Thus, whereas neither αBC nor HSPB2 is essential for myocardial development and function under stressfree conditions, one or both are required for maximal functional recovery and protection from I/R-induced necrosis and apoptosis.

small heat shock protein; necrosis; apoptosis; glutathione; knockout mouse

IN MOST ORGS transient ischemia followed by reperfusion (ischemia-reperfusion, I/R) induces tissue damage (38, 51). In the heart, recovery of contractility following transient ischemia is dependent on how quickly reperfusion is resumed. Although reperfusion is necessary for functional recovery, free radicals produced during this time can induce necrosis and apoptosis. Accordingly, preservation of myocardial function following I/R depends on critical adaptive responses, some of which are believed to involve the small heat shock proteins (sHSP).

The mammalian sHSP family includes αB-crystallin (αBC), HSP27,1 HSPB2, HSPB3, HSP20, HSP22, cvHSP, and αA-crystallin, all of which are expressed in the heart, except αA-crystallin, which is expressed primarily in the lens (7, 29, 45, 46). Among the sHSPs expressed in the heart, αBC is present in the highest quantities, where it comprises up to 3% of total myocardial protein (26, 31, 32). Accordingly, numerous studies have focused on possible roles for αBC in the cardiac stress response. Like other sHSPs, αBC is a chaperone and it interacts with various proteins that can foster cell survival, such as cytoskeletal components (6, 13, 50, 52). For example, in cardiac myocytes αBC is localized to the Z disks of sarcomeres, and the amount of Z disk-associated αBC increases during ischemia, as does the level of αBC phosphorylation (16–18). In addition to the localization of αBC to Z disks, αBC phosphorylation is also believed to provide protection from stress-induced myocyte death (35).

The misexpression or mislocalization of αBC is associated with several human diseases, such as the autosomal-dominant desmin-related cardiomyopathy (36, 39, 49). Furthermore, overexpression of αBC in cultured cardiomyocytes and in transgenic mouse hearts reduces I/R-induced cell death (8, 35, 41). These findings indicate that mutated αBC can be deleterious, whereas overexpression of native αBC enhances protection. Also several other studies have shown expression of αBC in the heart to differ as a function of age, implying roles of αBC in myogenic differentiation and cardiac development (5, 20, 31).

To demonstrate whether sHSPs are required for proper cardiac development and protection from stress, mouse models featuring the targeted disruption of sHSP genes are necessary. Currently, only two sHSP knock-out (KO) mouse lines are available, one features disruption of the αA-crystallin gene (9) and the other features αBC-gene disruption (10). Because αA-crystallin is not expressed in the heart (45), the αBC KO is the only available mouse model relevant for studies of the effects of sHSP gene disruption on cardiac development and function. Subsequent to the generation of the αBC KO mouse, the gene that encodes the sHSP and HSPB2 was found to reside just 863 bp upstream of the αBC-gene oriented in the opposite direction (22, 47). Because of the targeting strategy employed and the close proximity of the αBC and HSPB2 genes, both genes were disrupted in the αBC KO (10). Even though it cannot be discerned whether phenotypic differences between αBC/HSPB2 KO and WT mice are due to the absence of αBC and/or the absence of HSPB2, the αBC/HSPB2 KO mouse line is a valuable model with which to establish the necessities of...
these two sHSPs in cardiac development, structure, and function. Accordingly, we assessed the cardiac phenotype in αB/ HSPB2 KO mice with the goal of evaluating whether the absence of these two sHSPs affects cardiac development, morphology, and/or function under nonstressed and I/R stress conditions.

MATERIALS AND METHODS

Animals Used in Study

All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee at San Diego State University, and they conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Approximately 250 adult mice were used in this study. The KO mice used in this study were originally generated by using a targeting vector that replaced exons one, two, and, most of three, as well as the putative transcriptional regulatory sequences of the αB gene and portions of the linked HSPB2 gene (10). Electroporation of J1 embryonic stem cells (strain: 129S4/SvJae) selection and screening of targeted cells and blastocyst microinjection were performed as previously described (9). Founders were bred into a 129S6/SvEvTac background, and, accordingly, the KO mice used in this study, which were bred as previously reported, are in a mixed background (129S6/ SvEvTac). Bred as previously reported, are in a mixed background (129S6/129S4/SvJae). Control WT mice used in this study were 129S6/SvEvTac, also as previously reported (10).

Histology

Hearts were rinsed free of blood, then fixed in 4% formalin (EMS, Ft. Washington, PA) and embedded in paraffin (University of California, San Diego, Core Facility, San Diego, CA). Five-micrometer sections were stained with either hematoxylin and eosin or Masson’s trichrome (University of California, San Diego).

Echocardiography

TranshERIC two-dimensional guided M-mode echocardiography of ketamine-anesthetized mice was performed as described previously (44, 48) using a Sonos 5500, Agilent echocardiograph and an L15/6-MHz transducer. The parameters measured are shown in Table 2.

Electron Microscopy

Hearts were excised and immediately placed into 0.15 M cacodylate buffer (Sigma, St. Louis, MO) containing 100 mM sucrose and then finely chopped into ~1-mm² pieces. The pieces were incubated in 2% glutaraldehyde-2% formaldehyde (EMS) in 0.15 M cacodylate buffer for 4 h, washed, and fixed again in 1% osmium (EMS) for 2 h. After being rinsed with 0.1 M HEPES, the tissue was dehydrated with acetone buffer for 4 h, washed, and stained with either hematoxylin and eosin or Masson’s stain. Portions of the linked HSPB2 gene (10). Electroporation of J1 embryonic stem cells (strain: 129S4/SvJae) selection and screening of targeted cells and blastocyst microinjection were performed as previously described (9). Founders were bred into a 129S6/SvEvTac background, and, accordingly, the KO mice used in this study, which were bred as previously reported, are in a mixed background (129S6/ SvEvTac). Bred as previously reported, are in a mixed background (129S6/129S4/SvJae). Control WT mice used in this study were 129S6/SvEvTac, also as previously reported (10).

Cardiac Gene Expression Analyses

RNA analyses. Frozen ventricles were pulvarized and sonicated in RNAzol B (Tel-Test; Friendswood, TX) as directed in the manufacturer’s protocol. Two micrograms of RNA from each sample were blotted onto nitrocellulose by using a Bio-Rad DotBlot apparatus. After UV cross-linking, prehybridization was carried out in QuikHyb (Stratagene, Cedar Creek) for 1 h at 60°C. Oligonucleotide probes were labeled using T4 PNK (New England Biolabs) and [α-32P]ATP (Amersham; Arlington Heights, IL). Unincorporated label was removed via G-25 Microspin Columns (Amersham). Hybridization was carried out with ~1 × 10⁸ counts min⁻¹ μg of oligonucleotide probe⁻¹ at 60°C for 1 h in QuikHyb, with 100 μl of sonicated salmon sperm DNA (10 mg/ml). The following oligo probes were used: ANF, 5′-AATGTGACCAAGGTCTGAGCACAAAGGGCTT GMCATTTTUCGACTGCAAG-3; BNP, 5′-CAGTTGAGATAT GTTCACCITGGATAATT-3; β-MHC, 5′-GCTTATTCTCTCCACTA AAAGGGCTTGAAAGGCCTAGCTGAGGCCCT-3; α-skeletal actin, 5′-TGAGCAAAACAGAATGCGGCTTATAAGTGTCAAGTTTCTACCATTTTCCACAGG-3; and GAPDH, 5′- GGACATGTACAGATGTTGAGTTCAGAAG-3.

Membranes were washed and exposed to a PhosphorImager screen. Densitometries were quantified by ImageQuant and normalized to GAPDH levels.

Protein analyses. Frozen ventricles were pulvarized and sonicated in homogenization buffer [50 mM Tris (pH 7.5), 250 mM NaCl, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml p-nitrophenyl phosphate leupeptin, 2 mM EDTA, 3 mM EGTA, 0.1 mM Na orthovanadate, 1 mM, 10 μg/ml aprotinin, and 0.1% Triton X-100]. Aliquots (75 μg of protein) were subjected to 12% SDS-PAGE followed by Western blot analysis using atrial natriuretic factor (ANF) antiserum (Peninsula IHC 9103), B-type natriuretic factor (BNP) antiserum (Peninsula IHC 9085), and anti-GAPDH (RDI-TRK5G4-6C5). Visualization of immune complexes was carried out by enhanced chemiluminescence (NEL Life Science), and immunoreactive bands were quantified by ImageQuant and normalized to GAPDH.

Global I/R. Age-matched (12–14 wk of age) WT and KO mice (n = 12 of each) were treated with heparin (500 U/kg, Sigma) and anesthetized with pentobarbital sodium (150 mg/kg, Sigma) administered intraperitoneally. Hearts were excised and placed into ice-cold modified Krebs-Henseleit buffer (KH, Sigma). Aortas were cannulated and hearts were perfused in Langendorff mode by gravity flow. Hearts were then transferred to an isolated perfused heart apparatus at a constant pressure of 80 mmHg. A water-filled balloon connected to a pressure transducer (Gould Statham P23 ID) was inserted into the left ventricle through the left atrium to monitor left ventricular developed pressure (LVP, mmHg); data, collected using Powerlab, were processed with AD Instruments Chart v4 v4.12. Hearts were submersed in warm KHB (37°C) throughout the perfusion and paced at ~400 Hz using Powerlab Stimulus Isolator (AD Instruments) at 0.5 mA; hearts were not paced during ischemia. Hearts were equilibrated for 30 min before being subjected to 25 min of no-flow ischemia, which was followed by reperfusion for 1.4–5 h, depending on the experimental. Functional recovery was expressed as a percentage of preischemic LVP.

DNA fragmentation. Genomic DNA was isolated from whole hearts that were flash frozen in liquid nitrogen. Hearts were homogenized by hand with a glass homogenizer in digestion buffer [100 mM NaCl, 10 mM Tris (pH 8.0), 2.5 mM EDTA, and 0.5% SDS]. RNaseA was added to a final concentration of 3 mg/ml and incubated at 37°C for 30 min followed by the addition of 0.4 mg/ml Proteinase K and incubated at 55°C for 30 min. A 1/20 volume of 5 M NaCl was added to the samples, which were then centrifuged twice with phenol, once with chloroform-isooamyl alcohol (24:1), and the DNA was precipitated with isopropanol. The samples were centrifuged for 10 min at 4°C, and sedimented DNA was washed with 70% ethanol, air-dried, and dissolved in water. Ten micrograms of DNA per sample were fractionated on a 2% agarose gel.

Myoglobin. Approximately 800 μl of perfusate were collected at various times of perfusion and reperfusion. Two hundred microliters were analyzed at the indicated times (see figures) using the Wyntek Diagnostics Myoglobin/CK-MB apparatus courtesy of Dr. Brad Cunningham, Genzyme Diagnostics.

GSH and GSSG levels. GSH and GSSG levels were determined as previously described (1). Briefly, hearts were excised, homogenized in 5% trichloroacetic acid, and centrifuged at 10,000 g for 10 min at 4°C. To determine total glutathione, 10 μl of supernatant were added to 700 μl of daily buffer (0.248 mg/ml NADPH, 143 mM NaH₂PO₄, 6.3 mM EDTA) and 100 μl of 6 mM 5,5-dithiobis-2-nitro-benzoic acid
prepared in stock buffer (143 mM NaH2PO4, 6.3 mM EDTA) and 190 μl H2O, and the reaction was warmed for 15 min at 30°C followed by the addition of 10 μl GSSG reductase (266 U/ml, Sigma). Reaction progress was followed at 412 nm for 3 min. To determine GSSG, 2 μl of 2-vinylpyridine and 6 μl of triethanolamine were added to 100 μl of supernatant and allowed to stand for 60 min before proceeding as above for the assessment of total glutathione. GSH and GSSG standards were used for calibration.

Statistical analyses. All values are expressed as means ± SE. Statistical significance was assessed with a two-tailed Student’s t-test using SPSS software. Significance was defined by $P < 0.05$, and $P$ values are indicated in the figure legends.

RESULTS

In the study that reported on the generation of the αBC-HSPB2 KO mouse, the lens and several muscle types were morphologically characterized (10); however, a detailed analysis of cardiac morphology and function has not been carried out. Accordingly, we assessed gross and microscopic cardiac morphology and evaluated function, in vivo, using echocardiography, and in vitro, using an isolated-perfused mouse heart model.

Characterization of αBC-HSPB2 KO mice. As part of our initial characterization of the KO mice, a longevity study was carried out. We found that through 10 mo of age there was no difference in the survival rates of WT and KO mice (Fig. 1). However, beginning at 11 mo of age, the KO mice exhibited significantly reduced survival. At 14 mo of age, 90% of the WT mice survived; however, all of the KO mice had died. On postmortem examination, the hearts of KO mice appeared no different from those of WT mice (not shown). Accordingly, the premature death of the αBC-HSPB2 KO mice is most likely associated with malnourishment, as suggested in the initial report on these mice (10), and not cardiac functional deficit.

Physical examination showed that the weights of the KO mouse hearts were the same as those from age- and gender-matched WT mice (Table 1). However, the liver weights and body weights of the KO mice were about 30% lower than WT. This combination of characteristics resulted in similar liver-to-body weight ratios but 38% greater heart-to-body weight ratios in the KO mice than in WT mice (Table 1). Although suggestive of cardiac hypertrophy, the higher heart-to-body weight ratio in the KO mice is actually the result of reduced body weight, which may be the result of malnutrition in the KO mice.

To further assess possible affects of αBC and HSPB2 gene disruption, the levels of expression of several cardiac genes that serve as markers of hypertrophy were analyzed. The mRNA levels for α-skeletal actin and β-myosin heavy chain were the same in the KO and WT mouse hearts; whereas the mRNA levels for ANF and BNP appeared lower in the KO than those of WT mouse hearts, this trend did not reach statistical significance (Fig. 2A). Moreover, Western analysis showed that the levels of ANF and BNP protein were the same in the WT and KO mouse hearts (Fig. 2B). Thus deletion of the αBC and HSPB2 genes does not activate the expression of these hypertrophic marker genes.

Morphology of αBC-HSPB2 KO mouse hearts. To assess whether disruption of the αBC and HSPB2 genes affected cardiac morphology, hearts from 9-mo-old KO and WT mice were processed for histology. Hematoxylin and eosin or trichrome staining revealed no tissue alterations or fibrosis in the KO mouse hearts (Fig. 3, A and B), consistent with previous observations (10).

Because αBC and HSPB2 both interact with myofilaments (6, 13, 47, 50, 52, 54), it was of interest to examine myofibrilar ultrastructure in the KO mouse hearts. Earlier analysis of tongue and soleus muscle from the KO mice demonstrated cell degeneration and vacuoles with irregular edges (10); however, these features were not apparent in the heart, as determined in the present study. Electron photomicrographs of cardiac tissue revealed that the myofibrils from WT and KO mouse hearts contained regular repeats of sarcomeres delineated by Z disks, and parallel arrangements of myofibrils (Fig. 3, C and D). Thus deletion of the αBC and HSPB2 genes had no apparent affect on myofibrillar structure.

Functional analysis of αBC-HSPB2 KO mouse hearts. To assess the hemodynamic properties of αBC-HSPB2 KO mouse hearts, two-dimensional guided M-mode echocardiography measurements were performed on 6-mo-old mice. All but one of the parameters measured were the same in KO and WT mouse hearts (Table 2). The one statistically different parameter was the left ventricular end-systolic dimension (LVESD), which was lower in the KO than in the WT mouse hearts ($1.8 \pm 0.02$ vs. $2.3 \pm 0.2$ mm, respectively), suggesting enhanced left ventricular contractility in the KO mouse. This possibility is supported by the slightly higher percent fractional shortening in the KO mouse hearts compared with WT, although this difference did not reach statistical significance. Thus deletion of the αBC and HSPB2 genes had little affect on

![Fig. 1. Time course of survival of αBC-HSPB2 knock-out (KO) and wild-type (WT) mice. Equal numbers ($n = 10$) of male and female WT and KO mice were observed for 15 mo. Values are percentage of mice surviving at the times shown. *$P < 0.05$, #$P < 0.0005$, and †$P < 0.0001$ different from WT mice as determined by two-tailed Student’s t-test.](http://ajpheart.physiology.org/)

**Table 1. Physical characteristics of αBC/HSPB2 KO Mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>KO</th>
<th>WT</th>
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<tbody>
<tr>
<td>Heart weight, mg</td>
<td>140.1±7.7</td>
<td>143.6±9.9</td>
</tr>
<tr>
<td>Liver weight, mg</td>
<td>888.8±39.8*</td>
<td>1298.4±78.9</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>26.0±1.7*</td>
<td>36.4±2.4</td>
</tr>
<tr>
<td>Liver weight/body weight, mg/g</td>
<td>34.2±1.5</td>
<td>35.7±7.0</td>
</tr>
<tr>
<td>Heart weight/body weight, mg/g</td>
<td>5.40±0.20*</td>
<td>3.95±0.40</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 10$ mice. Heart, liver, and body weights of 4-mo-old mice were obtained as described in MATERIALS AND METHODS. KO, knock-out mice; WT, wild-type mice. *$P < 0.001$ different from WT mice, as determined by Student’s t-test.
hemodynamic cardiac function as determined by echocardiography.

**Postischemic contractile recovery.** Increased \( \alpha \)-BC expression in transgenic mouse hearts has been shown to enhance functional recovery of isolated perfused hearts following global ischemia (41). Accordingly, we assessed the effects of \( \alpha \)-BC and HSPB2 gene disruption on myocardial function following I/R of isolated perfused mouse hearts. Hearts were subjected to 30 min of equilibration, followed by 25 min of no-flow (global) ischemia and 90 min of reperfusion. As illustrated in Fig. 4, hearts from KO and WT mice exhibited similar LVDP (% maximum LVDP) values during equilibration and ischemia. However, on reperfusion, the KO mouse hearts exhibited lower contractile recovery than controls. This functional difference was evident within 5 min of reperfusion, when the LVDP of the KO mouse hearts was less than half that of WT mouse hearts (7.0 ± 1.8% vs. 18.2 ± 3.5%, respectively, \( P = 0.007 \)). The functional deficit exhibited by KO mouse hearts continued throughout the 90-min reperfusion (25.65 ± 3.54% and 51.42 ± 5.09% for KO and WT, respectively, \( P = 0.0003 \)).

**Analysis of I/R-induced necrosis and apoptosis.** In the heart, I/R results in a combination of necrosis and apoptosis (23); accordingly, apoptosis and necrosis were measured by DNA fragmentation (laddering) and myoglobin release, respectively. Initial control experiments showed that after 25 min of ischemia, DNA laddering was not evident in isolated perfused WT mouse hearts until after 4 h of reperfusion (Fig. 5A). Whereas neither WT nor KO mouse hearts displayed DNA laddering after 5 h of perfusion only (Fig. 5B, lanes 1 and 2), 25 min of ischemia followed by 4 h of reperfusion resulted in more intense laddering in KO (Fig. 5B, lanes 5 and 6) than WT mouse hearts (Fig. 5B, lanes 3 and 4). Myoglobin release was evident only during the first 30 min of reperfusion and was about twofold greater in KO than in WT mouse hearts (Fig. 5C). Based on myoglobin release, it was apparent that necrosis occurred very soon after reperfusion was initiated, reaching maximal values after about 5 min and then returning to control values by 60 min. These results indicate that compared with WT, the KO mouse hearts exhibit greater apoptotic and necrotic tissue damage after I/R.

**Analysis of glutathione levels.** Reactive oxygen species (ROS) are a major cause of I/R-induced myocardial damage. Reduced glutathione (GSH) serves an important role in neutralizing the damaging effects of ROS (27). Small HSPs contribute to reducing damage from ROS by raising the intracellular concentration of GSH compared with oxidized glutathione (GSSG) (34). Accordingly, we assessed GSH and GSSG levels in the hearts of \( \alpha \)-BC-HSPB2 KO mice. When compared with WT, hearts from KO mice exhibited a 43% decrease in total glutathione (Fig. 6A). Additionally, when compared with WT, KO mouse hearts displayed 54% more oxidized glutathione (Fig. 6B). The GSH-to-GSSG ratio in hearts from \( \alpha \)-BC-HSPB2 KO mice was 21.73 ± 0.818, which was decreased by 60% compared with WT, which was 56.79 ± 2.82 (Fig. 6C). These results indicate that the absence of \( \alpha \)-BC and/or HSPB2 in the KO mouse hearts might lead to a reduced GSH-to-GSSG ratio, which is at least partly responsible for the increased damage following I/R.

**DISCUSSION**

In this study we found that mice featuring the disruption of the \( \alpha \)-BC and HSPB2 genes exhibited increased mortality compared with WT mice. It is presently unclear what causes the increased mortality in the KO mice; however, because they exhibit a slightly dystrophic phenotype (10), it is possible that they were less able to acquire food and water. This could also account for the lower body weights of the KO mice. Although we were able to study the KO mice up to about 8 mo of age,
this increased mortality poses some limitation on the types of studies that can be undertaken on the αB- HSPB2 KO mice.

We also found that under nonstressful conditions the αB- HSPB2 KO mice exhibited no discernible defects in cardiac development, morphology, or function. Although the observation that the αB- HSPB2 KO mouse hearts appeared normal was also made in the earlier report that described the generation of these mice (10), it is a surprising result, because αB is expressed in high levels in the embryonic heart and because both αB and HSPB2 are found in abundance in the adult heart (46), implying critical functions for these sHSPs. This apparent lack of phenotype could be because these sHSPs do not play critical roles in cardiac development, structure, and function. However, it is more likely that other sHSPs are sufficiently abundant so that under certain conditions they can compensate for the absence of αB and HSPB2. The high degree of structural similarity among the sHSPs expressed in the heart is consistent with such putative functional overlap (46). In fact, αB and HSPB2 are 32% identical at the amino acid level and 47% identical in the α-crystallin domain. Moreover, whereas it

Table 2. Echocardiographic assessment of αB/HSPB2 KO mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>KO</th>
<th>WT</th>
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<tr>
<td>LVEDD, mm</td>
<td>3.3±0.2</td>
<td>3.8±0.1</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.8±0.2*</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>PWth, mm</td>
<td>1.0±0.1</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>SEPh, mm</td>
<td>0.9±0.1</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>LVM</td>
<td>0.1±0.01</td>
<td>0.1±0.01</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>374±28</td>
<td>392±32</td>
</tr>
<tr>
<td>ET, ms</td>
<td>64.8±3.3</td>
<td>63.3±4.5</td>
</tr>
<tr>
<td>VCF, circ/s</td>
<td>7.3±0.5</td>
<td>6.6±1.1</td>
</tr>
<tr>
<td>%FS</td>
<td>47±2.4</td>
<td>40±4.0</td>
</tr>
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</table>

Values are means ± SE; n, 6 mice. Echocardiography was performed on 29-wk-old mice as described in MATERIALS AND METHODS. LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; PWth, posterior ventricular wall thickness (diastole); SEPh, septal wall thickness (diastole); LVM, left ventricular mass; HR, heart rate; ET, ejection time; VCF, mean velocity of circumferential fiber shortening; %FS, percent fractional shortening. *P < 0.05 different from WT mice as determined by Student's t-test.
is not known whether HSPB2 is phosphorylated during stress, several studies have demonstrated that, like αBC, HSP27 is phosphorylated in response to stress, and that the phosphorylated forms are cytoprotective (2, 35), which supports potential functional redundancy between αBC and HSP27. However, it should be noted that there is also evidence that the cytoprotective activities of αBC and HSP27 do not correlate with phosphorylation. For example, in glioma cells, expression of αBC where serines-19, -45, and -59 have been replaced by aspartic acid, which mimics phosphorylation at these sites, results in reduced chaperone-like activity (21). Also in isolated rabbit cardiac myocytes, ischemic preconditioning, which protects from cell death, did not result in increased phosphorylation of αBC (3). This is similar to results that have been obtained with HSP27, showing that phosphorylation reduces chaperone-like activity (43), and that unphosphorylated forms of HSP27 protect against cell death in NIH 3T3 cells (33).

In contrast to the findings in unstressed hearts, when exposed to I/R αBC-HSPB2 KO mouse hearts exhibited increased necrosis, apoptosis, and only half the contractile recovery of WT mouse hearts. These results are consistent with roles for αBC and/or HSPB2 in protecting the myocardium from the functional deficits incurred on I/R stress. However, it is formally possible that the levels of some proteins, whose identities are not known, may be altered as a result of the gene disruption in this model and that these alterations might contribute to the observed phenotype. Although the precise mechanisms by which αBC and/or HSPB2 contribute to this protection are not yet known, at least in part, they most likely involve the abilities of these sHSPs to serve as chaperones. For example, αBC binds to and stabilizes the enzymatically inactive form of caspase-3, p24, thereby inhibiting caspase-3-dependent apoptosis (24). It has also been shown that HSPB2 colocalizes with mitochondria (37), indicating that it may protect from apoptosis by inhibiting cytochrome c efflux. Thus αBC and HSPB2 may both inhibit the mitochondrial-dependent death pathways, albeit through different mechanisms.

Another mechanism by which αBC and/or HSPB2 could protect the myocardium involves GSH. Overexpression of...
αB-CRYSTALLIN AND HSPB2 PROTECT HEARTS FROM I/R DAMAGE

Fig. 6. Analysis of GSH and GSSG Levels. A and B: total glutathione and GSSG. Supernatants from age-matched adult WT (n = 3) or KO (n = 3) whole heart homogenates were analyzed for GSH and GSSG levels by following the conversion of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB), at 412 nm, using a GSH-reductase assay in the presence of NADPH to provide an estimate of heart tissue (in benzoic acid (TNB), at 412 nm, using a GSH-reductase assay in the presence of NADPH to provide an estimate of heart tissue (in

αB increases the level of GSH, which leads to protection from apoptosis induced by the ROS that are generated on oxidative stresses, such as reperfusion (12, 40). Thus it is possible that the absence of αB in the αB-HSPB2 KO mice leads to reduced levels of GSH. Consistent with this hypothesis are findings of a recent study showing that compared with hearts from WT mice, hearts from mice deficient in heat shock factor 1, a transcription factor known to be required for high expression of αB and other sHSPs, exhibit significantly lower levels of αB and GSH and a reduced GSH-to-GSSG ratio (53). Although GSH levels in the lenses of the αB-HSPB2 KO mice were found to be decreased by only about 10% compared with WT mice (25), in the present study we found that αB-HSPB2 KO mouse hearts possess about 45% lower levels of GSH and a decreased GSH-to-GSSG ratio compared with WT mouse hearts. Perhaps the absence of αB and/or HSPB2 has a greater impact on GSH levels in the metabolically more active cardiac tissue than in the lens. Nonetheless, the results from our study support the notion that reduced GSH leads to increased susceptibility to I/R-induced myocardial damage in the KO mice.

In addition to binding to proteins and organelles involved in apoptosis, αB and HSPB2 also bind to the cytoskeleton, including sarcomeric I bands, which reside adjacent to Z disks in cardiac and skeletal muscle (31, 54). This Z disk localization, which increases following I/R, has led to speculation that through their chaperone functions, αB and HSPB2 might stabilize sarcomeres during stress (4, 16, 17). Although the mechanism by which this localization is enhanced on I/R remains unclear, recent studies showing that αB can bind to the portion of titin that resides in the I band adjacent to the Z disk (16) suggest a possible means for targeting αB to this portion of the sarcomere.

Several recent findings have demonstrated that the Z disk serves as a major location of signal transduction machinery in cardiac myocytes (15), making this an excellent target for the cytoprotection-oriented chaperone function of αB, HSPB2, and other sHSPs. For example, the Z disk serves as the interface between the sarcomeres and the cytoskeleton, and the localization of the Z disk near the sarcolemma, along with the connection between the Z disk and the basement membrane via costameres, are ideal for transmitting signals from outside/in, and inside/out. Among signaling machinery localized to the Z disk is the recently described cardiac stretch sensor (28). Thus the localization of αB and HSPB2 to Z disks positions these abundant chaperones in an ideal environment to contribute to the maintenance of signals that foster myocardial cell survival. As such, αB and HSPB2 may play central roles in maintaining the structural integrity of various signaling complexes at the Z disk.

Although αB and HSPB2 share many structural features and the Z disk localization, there are numerous differences in the characteristics of these two sHSPs. For example, HSPB2 binds to myotonic dystrophy protein kinase (DMPK), whereas αB does not; by binding to DMPK, HSPB2 reduces the enzyme’s susceptibility to thermodenaturation while increasing its kinase activity (47). The DMPK is the locus of mutations that cause myotonic dystrophy (11). Accordingly, it is thought that by stabilizing DMPK, HSPB2 contributes to maintaining proper skeletal muscle development, structure, and function (47). Thus it is possible that certain aspects of the αB-HSPB2 KO mouse phenotype (e.g., skeletal muscle atrophy) are due to reduced DMPK activity resulting from the absence of HSPB2. Moreover, although HSPB2 is able to form oligomers with HSPB3, it cannot bind to αB, HSP27, or HSP20 (46), which form oligomers with one another; these oligomers are thought to be involved in their chaperone functions, and whereas αB and HSP27 bind to actin filaments in C2C12 cells, HSP20 does not exhibit such filamentous localization (46). It is interesting to note that based on developmental profiles of αB expression, several studies have suggested that it may be required for myogenic differentiation and cardiac morphogenesis (14, 19). Although HSP20 does not appear to interact with actin filaments because it localizes to Z disks and
its expression is tightly regulated during the myogenic program, some investigators believe it may also be critical for cardiac morphogenesis (46). However, the apparently normal hearts in the αBC-HSPB2 KO mice indicate that neither of these sHSPs is required for cardiac morphogenesis.

In conclusion, the present study has characterized the cardiac phenotype of mice in which both the αBC and HSPB2 genes have been disrupted. Because both genes are disrupted in the αBC-HSPB2 KO mice, and because there are no mouse lines featuring disruption of either gene alone, or, for that matter, disruption of any other sHSP known to be expressed in the heart, it is impossible to conclude at this time whether either or both of these sHSPs is/are responsible for the observed cardiac effects. Nonetheless, the αBC-HSPB2 KO mouse model has served as a valuable tool that has allowed us to determine that at least one of these two sHSPs is required for maximal protection against the damage and functional deficits incurred by I/R.

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