

Phosphorylation and translocation of heat shock protein 27 and α B-crystallin in human myocardium after cardioplegia and cardiopulmonary bypass

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Supplemental material is available online.

Objectives: Cardiac surgery using cardioplegia and cardiopulmonary bypass subjects myocardium to hypothermic reversible ischemic injury that can impair cardiac function. Research in animal and cell models demonstrates that acute myocardial ischemia/reperfusion injury causes phosphorylation of heat shock protein 27 and α B-crystallin. Phosphorylation of heat shock protein 27 and α B-crystallin is implicated in the regulation of both beneficial and detrimental responses to ischemic injury. The phosphorylation status of these proteins in human myocardium after ischemic insults associated with cardioplegia and cardiopulmonary bypass is unknown.

Methods: Right atrial appendage and chest wall skeletal muscle samples were collected from patients before and after cardioplegia and cardiopulmonary bypass. Cardioplegia and cardiopulmonary bypass-induced changes in phosphorylation and localization of heat shock protein 27 and α B-crystallin were determined using immunoblot and confocal microscopy with total and phospho-specific antibodies.

Results: Cardioplegia and cardiopulmonary bypass increased the phosphorylation of heat shock protein 27 on serine 15, 78, and 82, and α B-crystallin on serine 59 and 45, but not serine 19. The majority of heat shock protein 27 and α B-crystallin localized to I-bands of cardiac myofilaments and shifted to a detergent insoluble fraction after cardioplegia and cardiopulmonary bypass. Cardioplegia and cardiopulmonary bypass-induced phosphorylation of specific heat shock protein 27 and α B-crystallin residues were associated with additional subcellular locations. Increases in phosphorylation of heat shock protein 27 and α B-crystallin were negatively correlated with cardiac function after surgery.

Conclusion: Cardiac surgery using cardioplegia and cardiopulmonary bypass is associated with phosphorylation and myofilament translocation of heat shock protein 27 and α B-crystallin in human myocardium. Phosphorylation of specific heat shock protein 27 and α B-crystallin serine residues is associated with distinct localization. Understanding the human myocardial small heat shock protein response may have significant implications for surgical myocardial protection.

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The majority of coronary artery bypass graft (CABG) and valve repair surgeries use cardioplegia and cardiopulmonary bypass (CP/CPB) to respectively arrest the heart and systemically circulate oxygenated blood. Hypothermic CP solutions provide myocardial protection during prolonged surgically induced global ischemia that would otherwise prove lethal. However, cardioplegic arrest of the heart during surgery results in reversible ischemic injury that manifests as impaired contractility of viable myocardium and reductions as cardiac function (myocardial stunning).^{1,2} Ischemic insults associated with CP/CPB-induced contractile deficits include myocyte hypoxia, intracellular acidosis, increased generation

Abbreviations and Acronyms

CABG	= coronary artery bypass graft
CP	= cardioplegia
CPB	= cardiopulmonary bypass
cryAB	= α B-crystallin
HSP	= heat shock protein
ICU	= intensive care unit
Ser	= serine
sHSP	= small heat shock protein

of reactive oxygen species, and metabolic alterations.³ The small heat shock proteins (sHSP) heat shock protein (HSP)-27 and α B-crystallin (cryAB) are regulated in response to all of these ischemic insults.^{4,6} Furthermore, growing evidence indicates that HSP27 and cryAB may modulate contractile function in response to ischemic insults.⁷⁻⁹

HSP27 and cryAB are abundant in heart and skeletal muscle.¹⁰ These proteins are involved in the beneficial regulation of diverse cellular processes.^{4,11} The function and localization of HSP27 and cryAB are closely linked to its phosphorylation status. In their nonphosphorylated state, HSP27 and cryAB are believed to provide heat tolerance for specific proteins, chaperone and cell signal scaffolding function, and protection against oxidant damage.¹²⁻¹⁴ In their phosphorylated state, HSP27 and cryAB provide enhanced protection to apoptotic stimuli and stabilization of structural elements of the cytoskeleton, including actin, microtubules, titin, and intermediate filaments.^{11,15-17} Non-phosphorylated HSP27 and cryAB exist as cytoplasmic proteins in large hetero-oligomeric complexes. Numerous isolated myocyte and animal models demonstrate that in response to ischemia, HSP27 and cryAB become phosphorylated and move from a diffuse cytoplasmic localization to a striated sarcomeric localization at or near z-lines and intercalated discs.^{5,18} HSP27 and cryAB are each phosphorylated on multiple residues: serine (ser)82, ser78, and ser15 of HSP27 and ser59, ser45, and ser19 of cryAB. The p38-mitogen-activated protein kinase (MAPK)/MAPK activated protein-2 pathway can phosphorylate all 3 residues of HSP27 and ser59 of cryAB.^{19,20} ERK can phosphorylate cryAB on ser45. The kinase responsible for phosphorylation of cryAB on ser19 is unknown.²⁰ Both p38-MAPK and ERK are activated in patients after CP/CPB.²¹ To our knowledge, no studies have compared the ischemia-induced myocyte localization of HSP27 and cryAB phosphorylated on each residue. Previous studies in animal and isolated myocytes assessed ischemia-induced changes in localization of total protein or limited analysis to single phosphorylated residues. The purpose of the following study was to analyze the regulation of HSP27 and cryAB in human-reversible ischemic injury after CP/CPB. This is the first

report to determine CP/CPB-induced changes in the phosphorylation and phospho-specific localization of HSP27 and cryAB.

Materials and Methods**Patient Tissue and Data Collection**

Samples were obtained from patients undergoing cardiac surgery with cardioplegic arrest (CP) and moderately hypothermic (32°C-34°C) CPB for CABG or valve repair/replacement as described previously.²² Briefly, samples of right atrium were isolated using a double purse-string suture method. During the placement of the venous cannula, the first sample of atrial appendage was harvested (pre-CP/CPB). The superior suture was tightened to secure the venous cannula. The inferior suture remained loose to allow this portion of the atrium to be perfused with blood, exposed to CPB and blood CP, and reperfused after removal of the aortic cross-clamp. The second suture was tightened after weaning from bypass and a brief period of reperfusion (~5-15 minutes), and the remaining distal tissue was harvested as the post-CP/CPB sample. Cold-blood CP (4°C) consisted of a 4:1 mixture of oxygenated blood with a hyperkalemic (K⁺ = 25 mmol/L) crystalloid solution. Skeletal muscle samples were discarded tissue from the internal thoracic artery harvest site. Myocardial and skeletal muscle tissue were immediately frozen in liquid nitrogen (n = 15 patients) or fixed in 10% formalin in phosphate-buffered saline (n = 5 patients) for molecular biology or microscopy studies, respectively. There were no detectable differences in any of the parameters studied between patients undergoing valve repair or CABG. For correlation analysis, ejection fraction (EF) was estimated by the anesthesiologist using intraoperative transesophageal echocardiography before and immediately after CP/CPB. The cardiac index was recorded from patients with a Swan-Ganz catheter in place at the time of surgical intensive care unit (ICU) admittance and 4 hours later. The Clinical Research Committee of the Beth Israel Deaconess Medical Center approved this study.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis and Immunoblot Analyses

Sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblot analyses were performed as previously described.²² Briefly, tissue (50-150 mg) was homogenized in radioimmunoprecipitation assay buffer with 50 mmol/L NaF, protease inhibitors (Complete; Boehringer, Mannheim, Germany), and phosphatase inhibitor cocktail (I + II, 1:100, Sigma, St Louis, Mo). After homogenization, lysates were centrifuged at 10,000g for 10 minutes, and a bicinchoninic acid protein assay was performed to allow equal gel loading; 20 to 40 μ g of lysates were loaded on tris-glycine 8% to 16% gradient gels and electrophoresed for approximately 1 hour at 150 volts. Gels were transferred to polyvinylidene difluoride membranes for 1 hour at 100 volts. Gels were blocked in 3% nonfat dry milk in tris-buffered saline (TBS) for 1 hour, followed by incubation in primary antibodies in 3% TBS milk or 3% bovine serum albumin according to the manufacturer's recommendation. Blots were washed 3 times in TBS and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour, washed 3 times in TBS, and detected using chemiluminescent detection (Pierce, Rockford, Ill). Antibodies for immunoblot were as follows: phospho-specific and total HSP27

and cryAB antibodies were from Stressgen (Vancouver, BC), anti-MLC-2a was from Synaptic Systems (Goettingen, Germany), anti-phospho-MAPK and total p38-MAPK were from Cell Signaling (Beverly, Mass), and anti-myotonic dystrophy protein kinase binding protein was from BD Bioscience (San Jose, Calif). Antibodies were used according to the manufacturer's instructions.

Confocal Immunofluorescent Microscopy

Harvested tissue was immediately placed in 10% formalin in phosphate-buffered saline, fixed overnight, embedded in paraffin blocks, and cut into 4- μ m sections. Deparaffinized slides were boiled in 10 mmol/L Na-citrate pH 6.0 for 10 minutes. Slides were blocked with 2% bovine serum albumin in TBS and incubated in 2% bovine serum albumin-TBS with primary antibodies overnight at 4°C. Slides were washed 3 times with TBS and incubated with the appropriate Alexa-fluor secondary antibodies and ToPro-3 (Invitrogen, San Diego, Calif). Slides were washed 4 times with TBS and mounted with fluorescent mounting medium (Vector Laboratories; Burlingame, Calif). Tissue was visualized using a Bio-Rad confocal microscope system (Bio-Rad Laboratories, Hercules, Calif). Tissue labeled with secondary only or primary incubation with normal rabbit immunoglobulin-G or rabbit serum was used for negative control.

Triton Solubility Assay

Tissue was lysed as above with 20 mmol/L Tris, pH 7.4, 0.1% Triton-X100, protease, and phosphatase inhibitors. Lysates were centrifuged at 14,000g at 4°C for 10 minutes. Supernatants were taken as the Triton soluble fraction, and pellets were resuspended in an equal volume of Laemmli (Bio-Rad, Boston Bioproducts, Inc, Boston, Mass) sample buffer, rehomogenized with a Polytron homogenizer (Biospec Products, Inc, Bartlesville, Okla), and centrifuged at 14,000g for 10 minutes at 4°C. Immunoblot and protein concentration assays with appropriate standards were performed as described above.

Statistics

Data are presented as the mean \pm standard error of the mean. A 2-tailed *t* test was used to determine statistical significance. Correlation analysis was performed using linear regression. Statistical tests were performed using SigmaStat (SYSTAT, San Jose, Calif). The authors had full access to the data and take responsibility for its integrity. All authors agree to the article as written.

Results

CP/CPB and p38-MAPK Phosphorylation

CP/CPB in atrial (but not skeletal) muscle induced thr180/tyr182 phosphorylation of p38-MAPK, a potential upstream mediator of HSP27 and cryAB phosphorylation (Figure 1).

CP/CPB-induced HSP27 and cryAB Phosphorylation

CP/CPB induced the phosphorylation of HSP27 (Figure 2, A) on ser15 (Figure 2, B), ser78 (Figure 2, C), and ser82 (Figure 2, D) in atrial, but not skeletal muscle. However, there was significantly more basal ser78 phosphorylation of HSP27 in skeletal muscle than in atrial tissue (Figure 2, C). There were no CP/CPB-induced changes in the expression of total HSP27 in atrial or skeletal muscle (Figure 2, A).

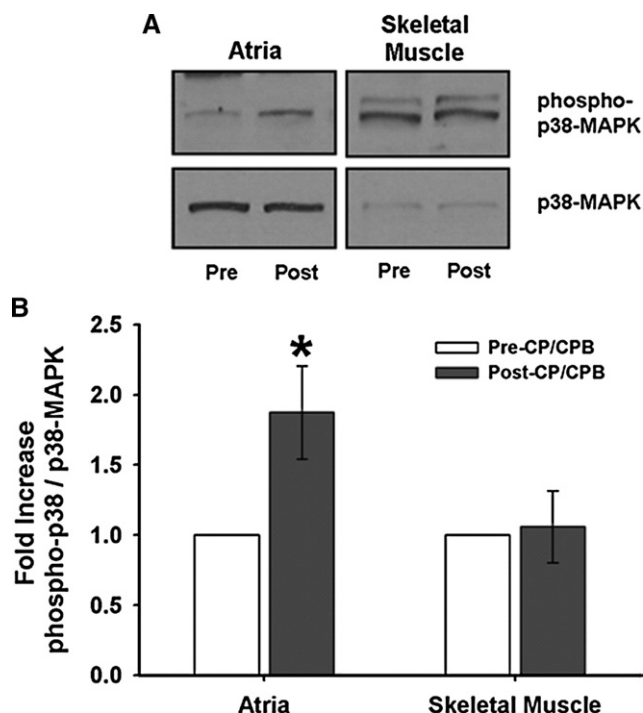


Figure 1. p38 MAPK is phosphorylated after cardioplegic arrest. **A,** Representative blots of phospho-p38MAPK and total p38-MAPK pre-CP/CPB (Pre) and post-CP/CPB (Post) in atria and skeletal muscle. **B,** Quantitative densitometry of the data shown in **A.** Phosphorylation data normalized to the level of total p38-MAPK and presented as the mean fold increase \pm standard error of the mean ($n = 6$). * $P < .05$ versus the respective pre-CP/CPB group. MAPK, Mitogen-activated protein kinase; CP/CPB, cardioplegia/cardiopulmonary bypass.

CP/CPB induced the atrial phosphorylation of cryAB (Figure 3, A) on ser59 (Figure 3, D) and ser45 (Figure 3, C), but not ser19 (Figure 3, B). Ser45 was highly phosphorylated in skeletal muscle both pre- and post-CP/CPB. There were no CP/CPB-induced changes in the expression of total cryAB in atrial or skeletal muscle.

CP/CPB-induced HSP27 and cryAB

Myocyte Localization

Before CP/CPB, total HSP27 exhibited patches of diffuse cytoplasmic and perinuclear staining, as well as areas of striated sarcomeric staining (Figure 4, A). There was minimal staining of phosphorylated HSP27 residues (Figure 4, C, E, G). After CP/CPB, total HSP27 (Figure 4, B, B') and HSP27 phosphorylated on ser82 (Figure 4, H, H'), ser78 (Figure 4, F, F'), and ser15 (Figure 4, D, D') exhibited a more distinct striated sarcomeric staining pattern. The staining of total (Figure 4, B', I) and phosphorylated HSP27 (Figure 4, J, K, L) alternated with cardiac MLC-2a, indicating that the majority of HSP27 localized in the I-bands/z-disc

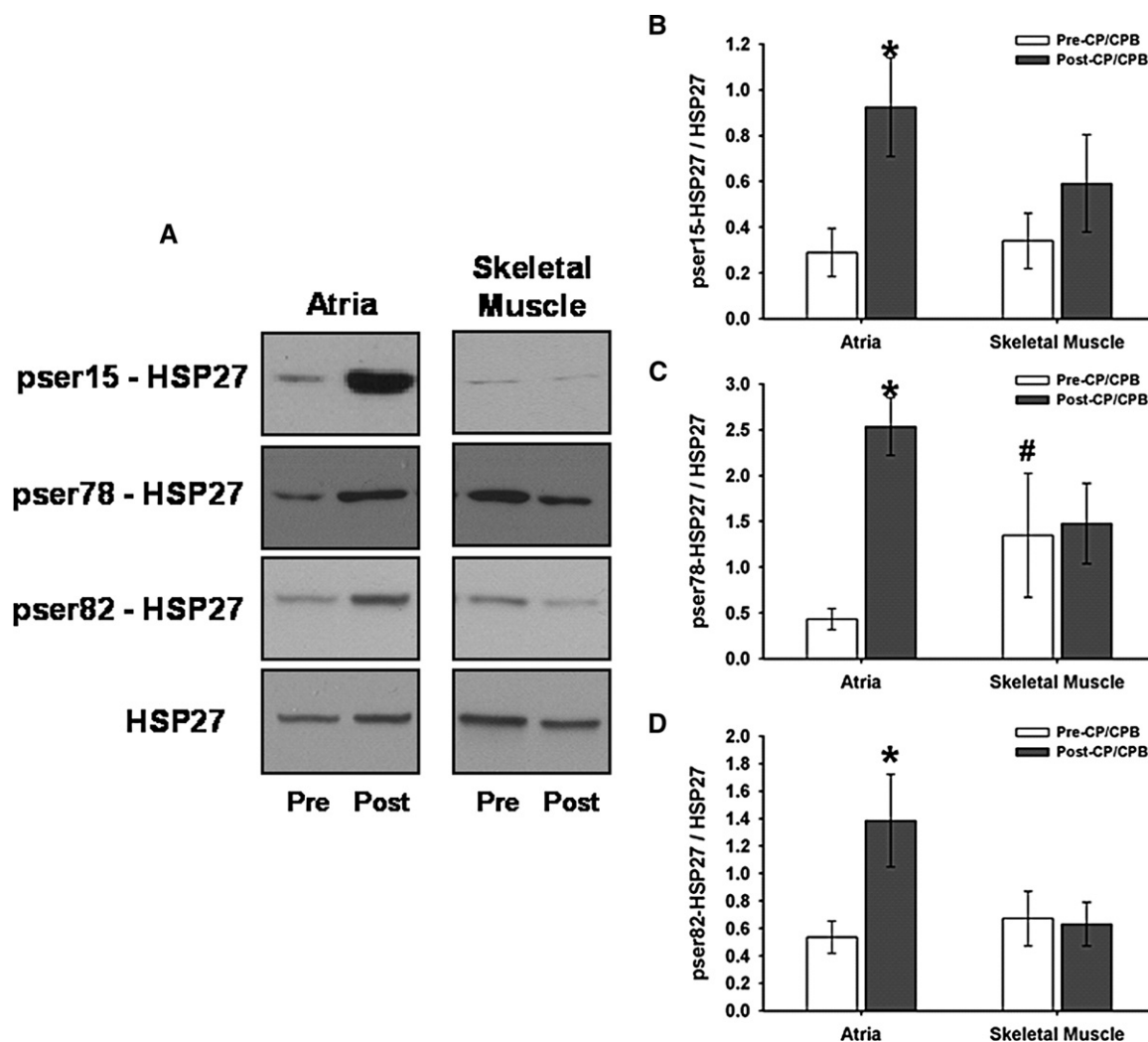


Figure 2. CP/CPB increases phosphorylation of HSP27 in cardiac tissue. **A**, Representative blots of total HSP27 and HSP27 phosphorylated on ser15, ser78, and ser82 in atrial and skeletal muscle pre-CP/CPB (Pre) and post-CP/CPB (Post). **B-D**, Densitometry values of phospho-HSP27 normalized to total HSP27 in atrial and skeletal muscle tissue pre- and post-CP/CPB: pser15-HSP27 (**B**), pser78-HSP27 (**C**), and pser82-HSP27 (**D**), minimum $n = 6$ for each residue. Data presented as the mean \pm standard error of the mean in all graphs. * $P < .05$ versus the respective pre-CP/CPB group. # $P < .05$ versus the atrial pre-CP/CPB value. *pser*, Phosphorylated serine; *HSP*, heat shock protein; *CP/CPB*, cardioplegia/cardiopulmonary bypass.

region of cardiomyocytes post-CP/CPB. In addition to the I-band/z-disc region, HSP27 phosphorylated on ser78 (Figure 4, E, F, F') and ser15 (Figure 4, D, D') localized to intercalated discs and myocyte nuclei, respectively.

Before CP/CPB, total cryAB exhibited diffuse cytoplasmic and membrane staining with areas of striated sarcomeric staining (Figure 5, A). There was minimal staining of ser59 phosphorylated cryAB (Figure 5, G). Ser19 (Figure 5, C) and ser45 (Figure 5, E) phosphorylated cryAB displayed I-band/z-disc and nuclear localization, as well as phospho-specific localization to intercalated discs and myocyte periphery, respectively. After CP/CPB, total (Figure 5, B, B', I)

and ser59 phosphorylated cryAB exhibited distinct I-band localization (Figure 5, H, H', L). Ser45 phosphorylated cryAB displayed increased peripheral/membrane localization (Figure 5, F, F', K vs E). Ser19 phosphorylated cryAB did not differentially localize after CP/CPB (Figure 5, D, D', J vs C).

Small Heat Shock Protein Cytoskeletal Association After Cardioplegia and Cardiopulmonary Bypass

Atrial and skeletal muscle tissue was fractionated into a Triton-X100 soluble fraction (cytoplasmic/membrane) and insoluble fraction (cytoskeleton). CP/CPB induced phos-

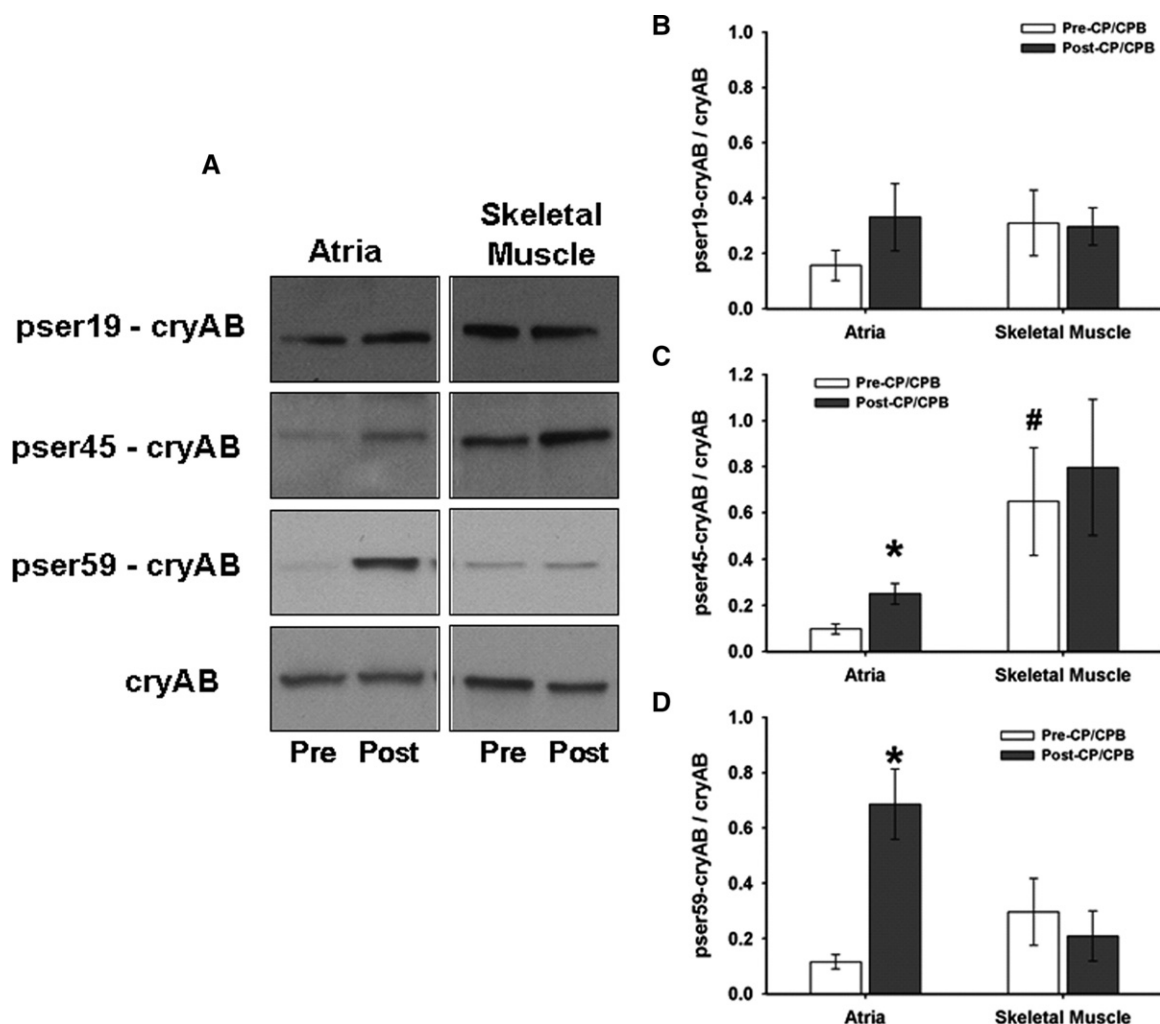


Figure 3. CP/CPB increases phosphorylation of cryAB in cardiac tissue. A, Representative blots of total cryAB and cryAB phosphorylated on ser19, ser45, and ser59 in atria and skeletal muscle pre- and post-CP/CPB. B-D, Densitometry values of phospho-cryAB normalized to total cryAB in atrial and skeletal muscle tissue pre- and post-CP/CPB: pser19-cryAB (B), pser45-cryAB (C), and pser59-cryAB (D), minimum $n = 6$ for each residue. Data presented as the mean \pm standard error of the mean in all graphs. * $P < .05$ versus the respective pre-CP/CPB group. # $P < .05$ versus the atrial pre-CP/CPB value. *pser*, Phosphorylated serine; *cryAB*, α B-crystallin; *CP/CPB*, cardioplegia/cardiopulmonary bypass.

phorylation of ser82 that was associated with movement of total HSP27 from a triton soluble to insoluble fraction (Figure 6, A). Similar to HSP27, CP/CPB-induced phosphorylation of ser59 was associated with the movement of total cryAB to the triton insoluble fraction (Figure 6, B). There was no translocation of either protein in skeletal muscle (data not shown). In addition, the highly related sHSP, myotonic dystrophy kinase binding protein, was predominantly soluble both pre- and post-CP/CPB (Figure 6, C).

Increased Phosphorylation of Heat Shock Protein 27 Is Correlated to Depressed Cardiac Function After Surgery

The fold increase in phosphorylation of HSP27 and cryAB (phospho-ser82 and ser59 only) pre- and post-CP/CPB was plotted against the percent change pre- versus post-surgery EF, cardiac index at ICU admittance, and 4-hour ICU cardiac index (Table 1). Linear regression analysis demonstrated a significant correlation between the cardiac index at ICU admittance and fold increase pser82-HSP27 and

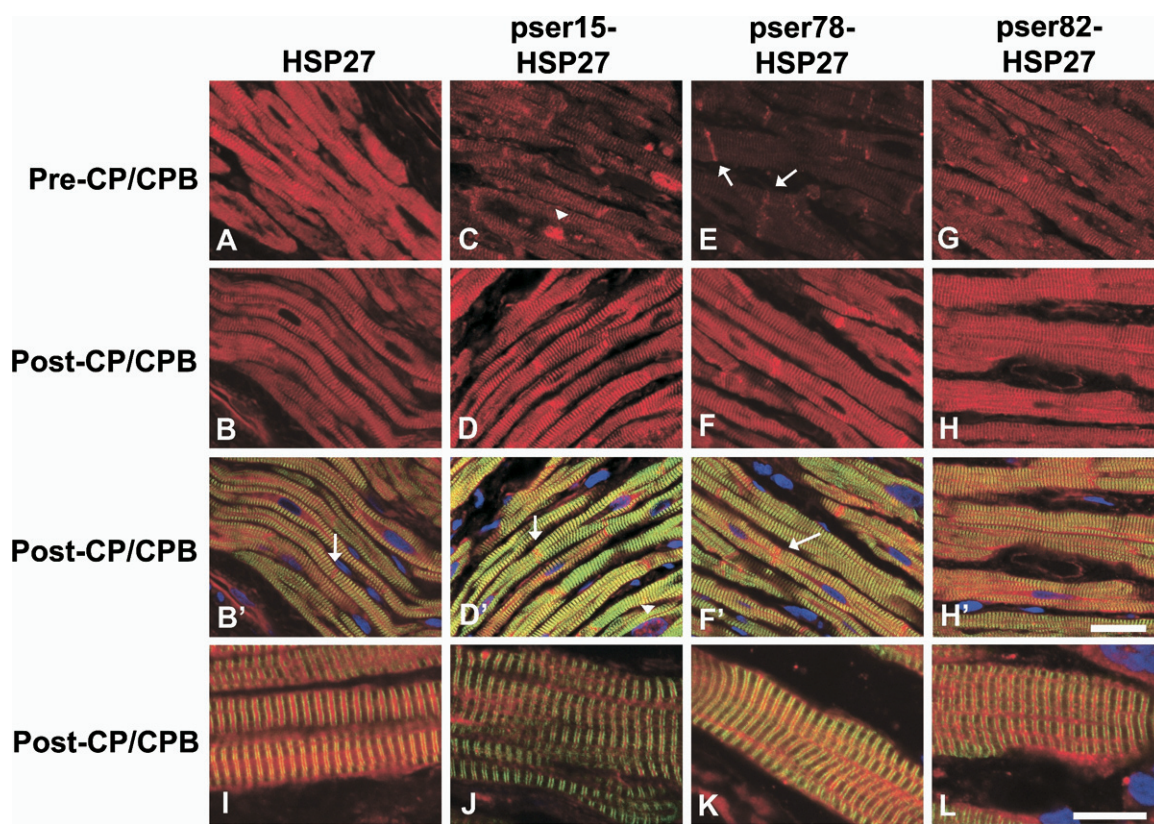


Figure 4. CP/CPB-induces sarcomeric translocation of HSP27. Human atrial sections pre- (A, C, E, G) and post-CP/CPB (B, B', D, D', F, F', H, H', I-L) were labeled with antibodies to HSP27 (red: A, B, B', I), pser15-HSP27 (red: C, D, D', J), pser78-HSP27 (red: E, F, F', K), or pser82-HSP27 (red: G, H, H', L). Sections were co-labeled with the atrial myocyte marker MLC-2a (green) and nuclei stain ToPro-3 (Invitrogen, San Diego, Calif) (blue) (B', D', F', H', I-L). Intercalated disc localization (arrows); nuclear localization (arrowheads). Representative micrographs are shown, minimum n = 3. Bar (H') = 20 μ m (A-H'). Bar (L) = 10 μ m (I-L). *pSer*, Phosphorylated serine; *HSP*, heat shock protein; *CP/CPB*, cardioplegia/cardiopulmonary bypass.

the cardiac index 4 hours post-surgery and pser59-cryAB. There were no other significant correlations.

Discussion

This is the first report of HSP27 and cryAB-specific phosphorylation and phospho-specific changes in localization resulting from acute, reversible cardiac ischemic injury in humans. The CP-induced cardiac effects indicate a response to ischemic insults that are associated with cardioplegic arrest and cessation of blood flow and not a generalized inflammatory stimulus resulting from CPB alone, because HSP27 and cryAB phosphorylation did not change in the skeletal muscle tissue of the same patients. Ischemic injury in rodent skeletal muscle has been shown to induce similar changes in sHSP localization.⁵ Cardioplegic arrest of the heart is highly protective to cardiac tissue subjected to ischemic insults. The main protective benefit of CP is mediated through myocardial hypothermia and diastolic arrest

that preserves myocardial energy reserves. Although protective, CP does not fully protect against the prolonged ischemic insults of myocyte hypoxia, acidosis, oxidant-dependent damage, metabolic and structural alterations, and reduced cardiac function.^{1-3,23} This is similar to alterations in animal models of acute and/or reversible ischemic injury.

The significance of phosphorylation of HSP27 and cryAB for cardiac function is controversial. Increased phosphorylation is believed to provide enhanced protection from apoptosis¹⁵ and enhanced protection/stability of cytoskeletal proteins in the sarcomere, including actin, titin, and intermediate filaments.^{11,16,17} However, a number of recent animal studies have indicated that nonphosphorylated cryAB and HSP27 may positively regulate contractile function. Previous reports demonstrate that almost all patients exhibit some measurable impairment of cardiac contractility post-CP/CPB without detectable decreases in myocardial viability.^{1,2} Prolonged stunning and the associated low-

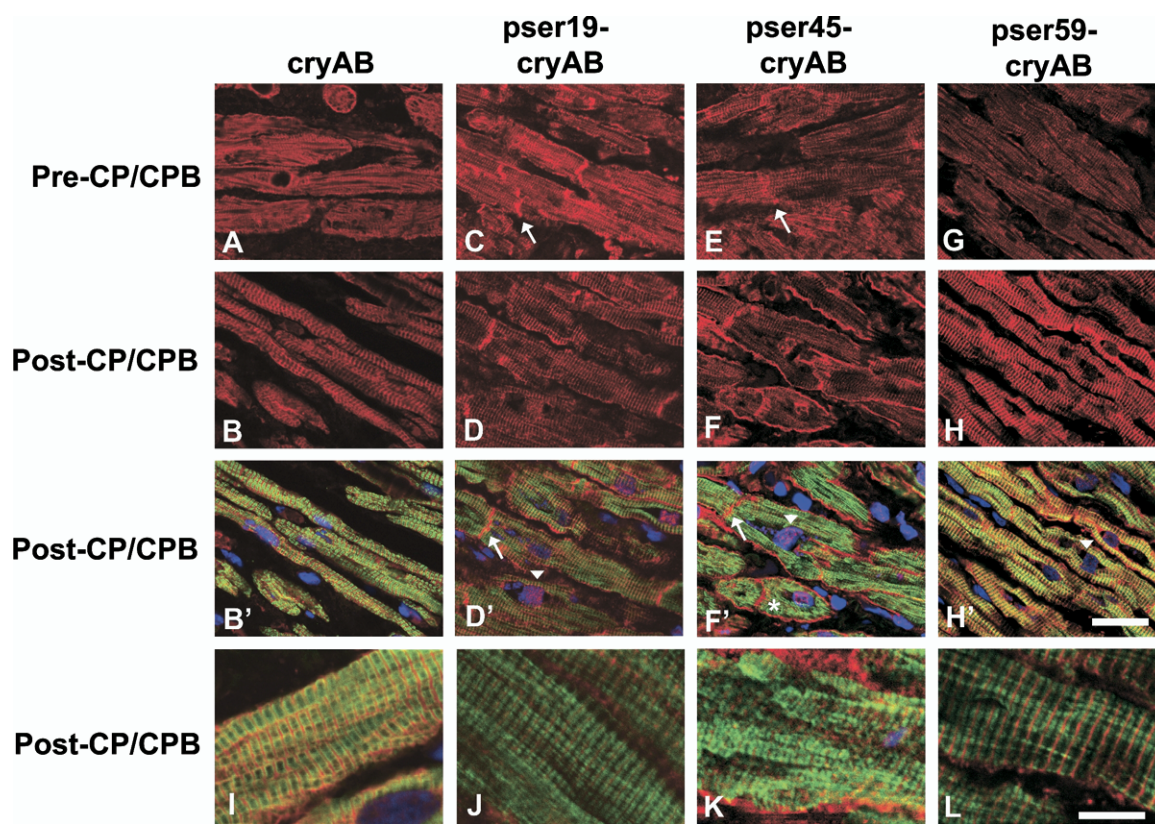


Figure 5. CP/CPB induces sarcomeric translocation of cryAB. Human atrial sections pre- (A, C, E, G) and post-CP/CPB (B, B', D, D', F, F', H, H', I-L) were labeled with antibodies to cryAB (red: A, B, B', I), pser19-cryAB (red: C, D, D', J), pser45-cryAB (red: E, F, F', K), or pser59-cryAB (red: G, H, H', L). Sections were co-labeled with the atrial myocyte marker MLC-2a (green) and the nuclei stain ToPro-3 (blue) (B', D', F', H', I-L). Intercalated disc localization (arrows) and nuclear localization (arrowheads). Representative micrographs are shown, minimum n = 3. Bar (H') = 20 μ m (A-H'). Bar (L) = 10 μ m (I-L). cryAB, α B-crystallin; pSer, phosphorylated serine; CP/CPB, cardioplegia/cardiopulmonary bypass.

output syndrome greatly increase the risk of postsurgical mortality. In addition, these patients require prolonged ICU recovery and inotropic support. Depressed cardiac output is associated with impairments in both ventricular and/or atrial contractility.²³ The fact that CP/CPB-induced phosphorylation of HSP27 and cryAB causes their translocation to cytoskeletal constituents of the sarcomere suggests that HSP27 and cryAB may disrupt mechanics of the contractile apparatus. However, current data in the literature suggest that phosphorylation is not necessarily a direct negative influence, but that corresponding decreases in nonphosphorylated HSP27 and cryAB negatively impact contractile activity. The following evidence is consistent with this view. First, ischemia-induced activation of p38-MAPK (an upstream kinase of both HSP27 and cryAB) is associated with depressed cardiomyocyte contractile function.^{7,24} Second, overexpression of either wild-type or a nonphosphorylatable mutant of HSP27 partially blocks ischemia-induced

contractile deficits in mouse hearts,⁸ yet nonphosphorylatable HSP27 mutants do not block endogenous HSP27 phosphorylation²⁵ (our unpublished observations). Third, transgenic mice with a double-knockout of cryAB and the related sHSP, myotonic dystrophy protein kinase binding protein, have impaired contractile activity after ischemic injury.⁹ Therefore, ischemic insults (including CP/CPB) that induce phosphorylation of HSP27 and cryAB, and the subsequent depletion of the nonphosphorylated sHSP pool, may play a role in myocardial contractile deficits or stunning. However, manipulation of sHSP phosphorylation for improved myocardial protection should be approached with caution, because sHSP phosphorylation is associated with the aforementioned beneficial effects in ischemic injury.

The idea of sHSP phosphorylation negatively impacting cardiac contractility is supported by the negative correlation between the degree of surgery-induced HSP27 phosphorylation and the postsurgery cardiac index (Table 1). It is

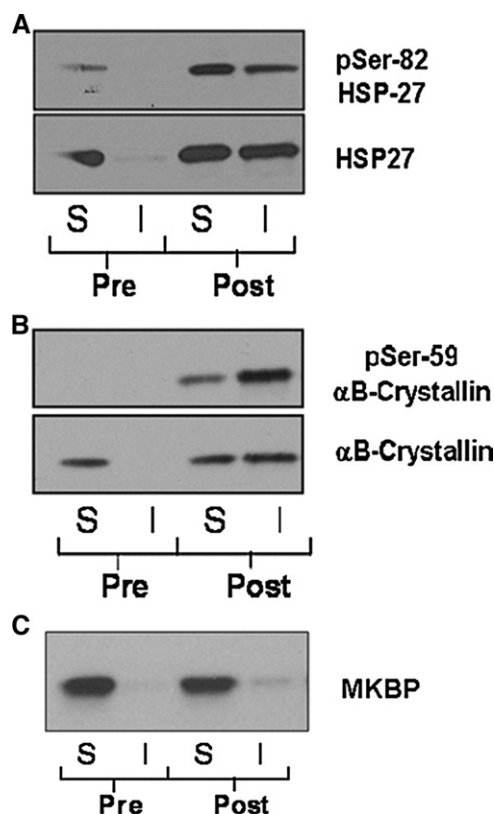


Figure 6. Cardioplegic arrest induces cytoskeletal translocation of HSP27 and cryAB. Human atrial samples were fractionated into Triton-soluble and insoluble tissue lysates. Phosphorylation of HSP-27 (A) and cryAB (B) (*upper*) post-CP/CPB was associated with movement of a significant fraction of total HSP27 and cryAB to the Triton X-100-insoluble protein pool (*lower*). (C) There were no CP/CPB-induced solubility changes of the highly related myotonic dystrophy protein kinase binding protein. Representative blots shown from 1 patient, total $n = 4$. *S*, Soluble; *I*, insoluble; *pSer*, phosphorylated serine; *HSP*, heat shock protein; *MKBP*, myotonic dystrophy protein kinase binding protein.

interesting that ser59 phosphorylated cryAB did not correlate with the cardiac index at ICU arrival but did at the 4-hour time point, potentially reflecting prolonged modifications of cryAB after CP/CPB. However, a lack of correlation with cryAB phosphorylation at earlier time points may be due to the inherent variability in assessing the fold change phosphorylation of cryAB, because the basal phosphorylation was low (Figure 3). The lack of correlation of either protein with the change in EF most likely reflects the wide EF variability ($\% \Delta EF -0.7 \pm 15.7$ standard deviation). This inherent variability stems from large positive increases in EF in specific patients after surgery as the result of restoration of blood flow to chronic ischemic myocardium and/or pharmacologic manipulation. Although supportive, these correlative data should be interpreted with

TABLE 1. Increased phosphorylation of heat shock protein-27 negatively correlates with cardiac function after cardioplegia/cardiopulmonary bypass

	Correlation coefficient	P value
pser82-HSP27		
% ΔEF	0.198	.5
CI-ICU	-0.684	.01
CI-ICU 4 h	-0.408	.19
pser59-cryAB		
% ΔEF	-0.451	.11
CI-ICU	-0.442	.15
CI-ICU 4 h	-0.630	.03

Linear regression values for CP/CPB-induced fold increase in pser82-HSP27 and pser59-cryAB as determined by Western blot analysis versus percent change pre- versus post-ejection fraction, CI at ICU admittance, and CI 4 hours after admittance ($n = 12$ for all CI correlations; $n = 14$ for all EF correlations). *EF*, Ejection fraction; *CI*, cardiac index; *ICU*, intensive care unit; *pser*, phosphorylated serine; *HSP*, heat shock protein; *cryAB*, αB -crystallin.

caution because the cardiac function parameters assessed depend on numerous variables that can be affected by cardiac surgery, patient variability, and pharmacologic support.

The differential phosphorylation and localization of HSP27 and cryAB suggest phosphorylation by distinct upstream kinases. The p38-MAPK/MAPK activated protein-2 signaling cascade can phosphorylate HSP27 on all 3 residues and cryAB on ser59.^{19,20} Ischemia and related insults, such as hypoxia and acidosis, can activate p38-MAPK. We show that CP/CPB leads to increases in p38-MAPK phosphorylation. This result is in agreement with previous reports of CP/CPB-induced activation of MAPK.²¹ The one phosphorylatable residue that did not exhibit the majority of its localization to I-bands was ser45 of cryAB. Ito and colleagues²⁰ demonstrated that ERK phosphorylates cryAB on ser45. ERK is also activated in human myocardium after CP/CPB.²¹ Together, these results indicate that CP/CPB induces activation of multiple signaling pathways that coordinate sHSP phosphorylation and localization in response to ischemic insults.

The consequences of phospho-specific localization of HSP27 are unclear. HSP27 binding in the I-band is in agreement with previous reports of HSP27 association with actin and intermediate filaments in sarcomeric z/discs/I-bands. In vitro association of HSP27 with actin limits acidosis-induced aggregation of actin filaments.²⁶ Intracellular acidosis is a recognized consequence of cardioplegic arrest in human myocardium; therefore, phosphorylation-associated movement of HSP27 may protect sarcomeric and z-disc structural integrity after ischemic insults. The significance of HSP27 exclusively phosphorylated on ser78 in intercalated discs is unclear; this may be the result of unique kinases present in the highly

specialized structure or increased affinity of ser78 phosphorylated HSP27 for proteins found exclusively in the disc. The pattern of phospho-specific localization of all HSP-27 phospho-residues indicates that phosphorylation on ser82 may be a dominant localization signal compared with ser15 and ser78. This argument is supported by the result that HSP27 phosphorylated on ser82 is only found on the z-discs/I-bands, whereas HSP27 phosphorylated on ser78 and ser15 is found in multiple locations. In addition, translocation to a Triton insoluble fraction was not present in skeletal muscle, which had a high level of basal pser78-HSP27. Alternatively, phosphorylation of ser15 or ser78 alone may respectively enable localization to nuclei or intercalated discs.

The CP/CPB-induced phosphorylation of specific residues of cryAB was associated with differential localization. CP/CPB-induced phosphorylation of cryAB on ser59 coincided with intense staining of the cardiac z-discs/I-bands. This result agrees with previous findings in animals showing that, in response to ischemic injury, cryAB binds and stabilizes sarcomeric structural proteins and intermediate filaments, including desmin and titin.^{16,17} Ser45 phosphorylation of cryAB was associated with localization to the cell periphery/membrane, indicating that ser45 phosphorylation may regulate binding to distinct targets. Similar to ser78 phosphorylated HSP27, ser19 phosphorylated cryAB displayed prominent localization to intercalated discs. Similar to the argument above, these data are consistent with the fact that ser59 phosphorylation may represent a dominant localization signal in cardiac myocytes, because the majority of ser59 is found in I-bands and little pser59-cryAB is found in the other prominent cryAB locations (intercalated discs, membrane, and nuclei). In addition, all phosphorylated forms of cryAB displayed punctate localization in cardiomyocyte nuclei. It was recently demonstrated that ser45 phosphorylated cryAB may be involved in nuclear intermediate filament binding in HeLa cells and that phosphorylation of ser59 is required for cryAB transport into the nucleus.²⁷

In addition, both HSP27 and cryAB displayed similar localization patterns both before and after surgery (Figure E1). The results indicate that HSP27 and cryAB physically interact in vivo, and that this association is unaffected by ischemic insults after CP/CPB. However, given the relative amount of HSP27 and cryAB co-immunoprecipitated with the respective antibodies, only a subset of the available HSP27 and cryAB interacts in a protein complex at a given time.

Limitations

Human myocardial responses to ischemic insults associated with cardiac surgery were determined in atrial tissue samples. Atrial and ventricular tissue exhibit many similarities. However, there are documented biochemical differences

between the 2 tissues and atrial versus ventricular differences in intraoperative specific stimuli during CP/CPB (ie, less atrial vs ventricular protection). Previous work in our laboratory demonstrated similar MAPK signaling phenomenon in atrial and ventricular tissue in pigs undergoing CP/CPB.²⁸ In addition, the results presented here agree with the response of sHSP in ventricular tissue from animal models of ischemic injury. However, the unavailability of the more relevant human ventricular tissue represents a limitation of this study. Finally, it is difficult to discern the relative contribution of ischemia-induced insults versus effects caused by cardioplegic arrest alone. The response of HSP27 and cryAB most likely reflects ischemic insults to the myocardium (hypoxia, acidosis, and oxidant damage) during CP/CPB and not direct effects of CP perfusion, which protect against lethal ischemic injury.

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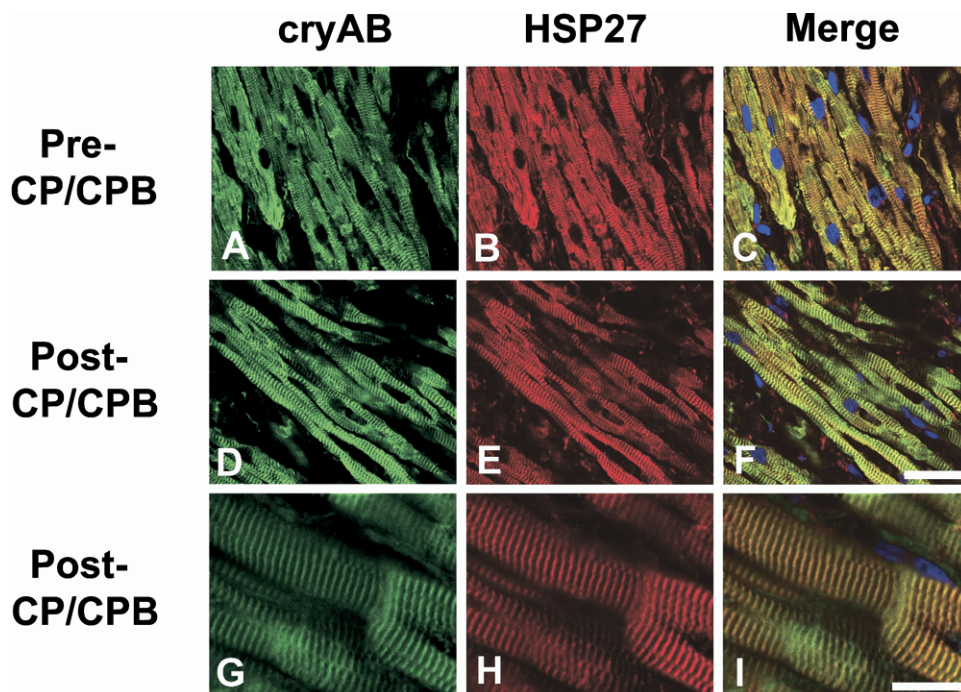
Appendix E1

HSP27 and cryAB colocalize and interact in human myocardium in vivo. Both pre- and post-CP/CPB HSP27 (Figure E1, B, E, H) and cryAB (Figure E1, A, D, G) were similarly localized in atrial myocytes (Figure E1, C, F, I, yellow). The association of HSP27 and cryAB was verified by immunoprecipitation (Figure E1, J). Atrial tissue lysates pre- and post-CP/CPB were immunoprecipitated with antibodies specific to HSP27 (lanes 5 [pre] and 6 [post]) or cryAB (lanes 9 [pre] and 10 [post]). Immunoblot with total HSP27 demonstrated effective immunoprecipitation of HSP27 (Figure E1, J, lanes 5 and 6) and cryAB coimmunoprecipitation both pre- and post-CP/CPB (Figure E1 J, lane 10 vs lane 9). Similarly, immunoblot with total HSP27 demonstrated effective immunoprecipitation of cryAB (Figure E1, J, lanes 9 and 10) and HSP27 coimmunoprecipitation both pre- and post-CP/CPB (Figure E1 J, lane 5 vs lane 6).

Increased phosphorylation of HSP27 and cryAB correlates with depressed postsurgery cardiac function. Individual linear regression graphs on which Table 1 is based compare the CP/CPB-induced fold increase in pser82-HSP27 and pser59-cryAB with the cardiac index at ICU arrival (Figure E2, A, B) and 4 hours later

(Figure E2, C, D), and the percent change in EF (post-EF to pre-EF/pre-EF * 100) (Figure E2, E, F). Linear regression analysis demonstrated a significant correlation between the cardiac index at ICU admittance and fold increase in pser82-HSP27 ($r = -0.684$, $P = .01$) and the cardiac index 4 hours postsurgery, and in pser59-cryAB ($r = -0.630$, and $P = .03$). There were no other significant correlations.

Tissue was homogenized and centrifuged as described above. Atrial samples were diluted to 500 μL at a protein concentration of 1 mg/mL in radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors. Lysates were pre-cleared with 20 μL of Protein A/G beads (Santa Cruz Biotechnology: Santa Cruz, Calif) for 30 minutes at 4°C. Lysates were centrifuged at 2000 rpm for 2 minutes. Supernatants were incubated with 10 μg monoclonal HSP27, cryAB, or normal mouse immunoglobulin-G and 20 μL of Protein A/G beads for 2 hours at 4°C. Beads were washed 5 times in radioimmunoprecipitation assay + inhibitors and boiled in 40 μL of Laemmli (Bio-Rad) sample buffer. Immunoprecipitates were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis and immunoblotted with rabbit anti-HSP27 and cryAB as described in “Materials and Methods.”



J

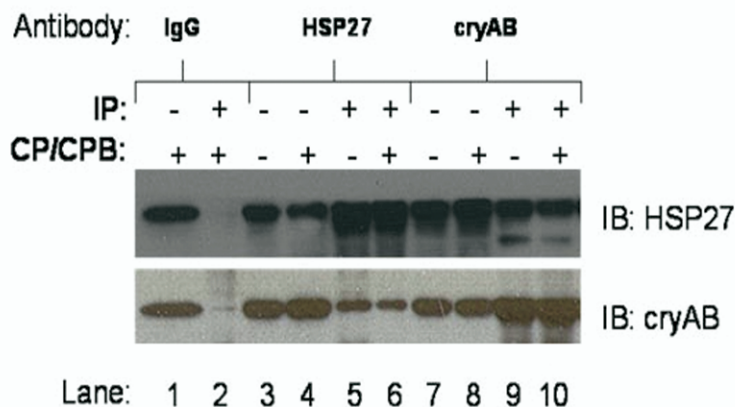


Figure E1. HSP27 and cryAB colocalize and interact pre- and post-CP/CPB. Human atrial sections pre- (A-C) and post-CP/CPB (D-I) were labeled with antibodies to cryAB (*green*: A, C, D, F, G, I) or HSP27 (*red*: B, E, F, H, I) and the nuclei stain ToPro-3 (*blue*) (C, F, H). Representative micrographs are shown (n = 3). Bar (F) = 20 μ m (A-F). Bar (G) = 10 μ m (G-I). J, Human atrial radioimmunoprecipitation assay lysate pre- (lanes 3, 5, 7, and 9) and post-CP/CPB (lanes 1, 2, 4, 6, 8, and 10) was immunoprecipitated for HSP27 (lanes 5 and 6, IP fraction; lanes 3 and 4, post-IP radioimmunoprecipitation assay fraction), cryAB (lanes 9 and 10, IP; lanes 7 and 8, post-IP radioimmunoprecipitation assay fraction), or nonspecific mouse immunoglobulin-G (lane 2, IP fraction; lane 1, post-IP fraction, post-CP/CPB only). Duplicate gels were immunoblotted for HSP27 (*upper*) and cryAB (*lower*). Blots are representative of 3 independent experiments. *cryAB*, α B-crystallin; *HSP*, heat shock protein; *CP/CPB*, cardioplegia/cardiopulmonary bypass; *Ig*, immunoglobulin; *IP*, immunoprecipitation.

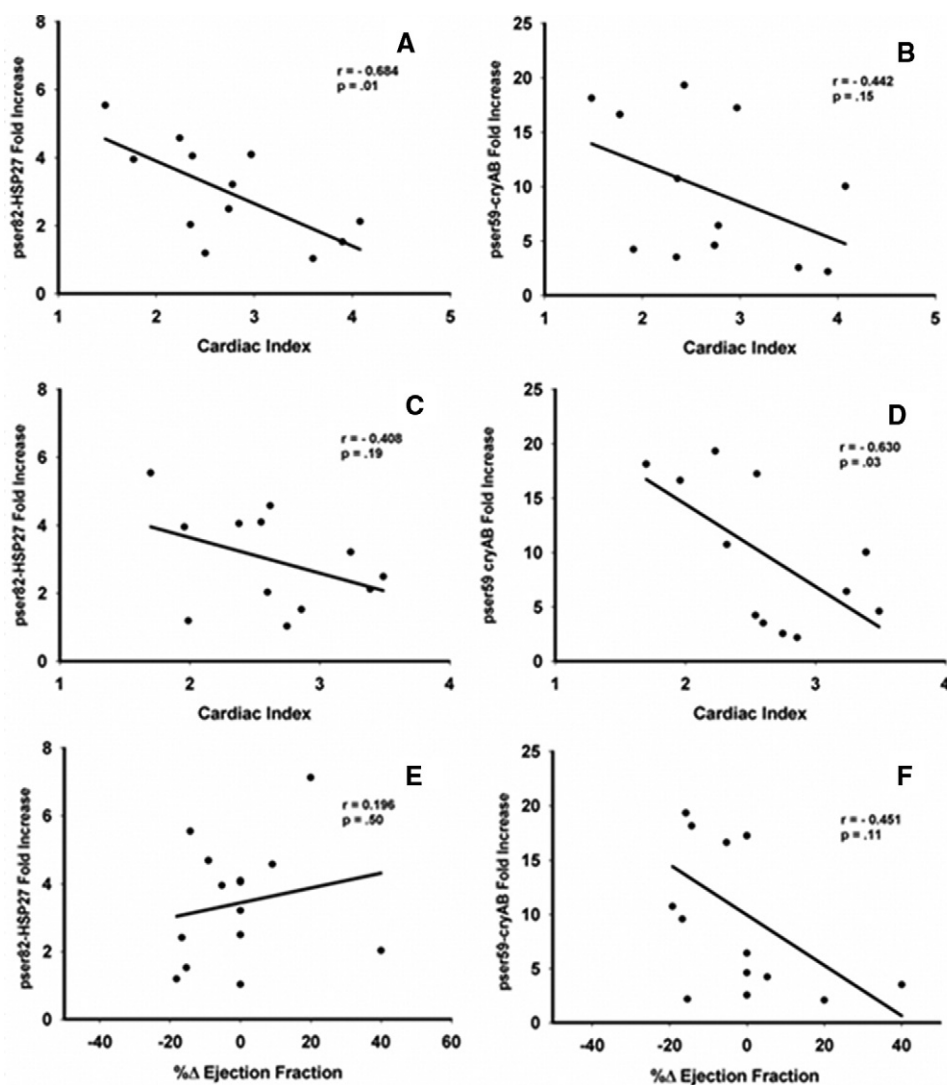


Figure E2. Increased phosphorylation of HSP27 and cryAB correlates with depressed post-surgery cardiac function. Fold increase phosphorylation of HSP27 and cryAB (phospho-ser82 (A, C, E) and ser59 (B, D, F) pre- and post-CP/CPB were plotted against cardiac index at ICU admittance (A, B), the 4-hour postsurgery ICU cardiac index (C, D), and percent change presurgery versus postsurgery EF (E, F) (n = 12 for A-D; n = 14 for E and F). *psr*, Phosphorylated serine; *HSP*, heat shock protein.