αB-Crystallin Improves Murine Cardiac Function and Attenuates Apoptosis in Human Endothelial Cells Exposed to Ischemia-Reperfusion

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Background. **This study investigates the protective effect of exogenous αB-crystallin (CryAB) on myocardial function after ischemia-reperfusion injury.**

Methods. **Mice underwent temporary left anterior descending artery occlusion for 30 minutes. Either CryAB (50 μg) or phosphate-buffered saline (100 μL [n = 6, each group]) were injected in the intramyocardial medial and lateral perinfarct zone 15 minutes before reperfusion. Intraperitoneal injections were administered every other day. Left ventricular ejection fraction was evaluated on postoperative day 40 with magnetic resonance imaging. To investigate the effect of CryAB on apoptosis after hypoxia/reoxygenation in vitro, murine atrial cardiomyocytes (HL-1 cells) or human microvascular endothelial cells (HMEC-1) were incubated with either 50 μg CryAB (500 μg /10 mL) or phosphate-buffered saline in a hypoxia chamber for 6, 12, and 24 hours, followed by 30 minutes of reoxygenation at room air. Apoptosis was then assessed by western blot (Bcl-2, free bax, cleaved caspases-3, 9, PARP) and enzyme-linked immunosorbent assay analyses (cytoplasmic histone-associated DNA fragments and caspase-3 activity).**

Results. **On postoperative day 40, CryAB-treated mice had a 1.8-fold increase in left ventricular ejection fraction versus control mice (27% ± 6% versus 15% ± 4% SD, $p < 0.005$). In vitro, (1) the HL-1 cells showed no significant difference in apoptotic protein expression, cytoplasmic histone-associated DNA fragments, or caspase-3 activity; (2) the HMEC-1 cells had increased but not significant apoptotic protein expression with, however, a significant decrease in cytoplasmic histone-associated DNA fragments (1.5-fold, $p < 0.01$) and caspase-3 activity (2.7-fold, $p < 0.005$).**

Conclusions. **Exogenous CryAB administration significantly improves cardiac function after ischemia-reperfusion injury, in vivo. The protective anti-apoptotic affects of CryAB may target the endothelial cell.**

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A cytosolic protein expressed in a variety of long-lived tissue, αB-crystallin (CryAB), or heat shock protein (HSP) B5, is a member of the family of small heat shock proteins (sHSP). The CryAB protein is the most abundant sHSP expressed in cardiac tissue, comprising approximately 3% total protein in the heart [1]. Although the exact pathophysiologic effects of endogenous CryAB in cardiac tissue continue to be investigated, CryAB prevents apoptosis [2, 3], is involved in cytoskeleton remodeling [4], and provides cardioprotection [5]. Myocardial function after ischemia-reperfusion (I/R) injury is dependent on adaptive responses involving CryAB and other sHSP [5, 6]. Ray and colleagues [6] found that ex vivo heart preparations from CryAB overexpressing transgenic mice had decreased oxidative stress and less cardiomyocyte apoptosis after I/R. Corroborating this finding, hearts from CryAB-HSPB2 knockout mice displayed a twofold reduction in contractility in conjunction with increased apoptosis after I/R in an ex vivo model system [5]. Although exogenous intravenous CryAB has proved useful in an experimental autoimmune encephalomyelitis model [7], the effect of exogenous CryAB on myocardial function after I/R injury in an in vivo model system has not been studied.

In the present study, we investigated the protective effect of exogenous CryAB administration utilizing both an in vivo model (murine left anterior descending artery [LAD] ligation and reperfusion injury) and an in vitro model (hypoxia and reoxygenation). Mouse cardiomyocytes (HL-1) and human microvascular endothelial cells (HMEC-1) were used to investigate the biological role of exogenous CryAB on different cell types. Mouse HL-1 cells are a cardiomyocyte line derived from AT-1 mouse atrial tumor cells that retain morphologic, biochemical, and electrophysiologic properties of differentiated cardiomyocytes, and are widely used for in vitro physiologic models of myocardial I/R injury [8-11]. The HMEC-1 are immortalized cell lines obtained by transformation of

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Abbreviations and Acronyms

CryAB = αB-crystallin
HMEC = human microvascular endothelial cell
HSP = heat shock protein
I/R = ischemia-reperfusion
LAD = left anterior descending artery
LVEF = left ventricular ejection fraction
PBS = phosphate-buffered saline
sHSP = small heat shock protein
TBS = Tris-buffered saline

human primary dermal microvascular endothelial cells with the SV40 large T antigen [12]; they retain endothelial cell phenotype and functional characteristics, and provide an in vitro physiologic model related to microvascular endothelium [13-15]. Our results reveal that exogenous CryAB significantly improves cardiac function in vivo as evaluated by magnetic resonance imaging and demonstrate an anti-apoptotic effect on HMEC-1, not HL-1 cells, in vitro.

Material and Methods

Animals

Six- to 8-week-old male C57BL/6 (H-2^b) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed at the Stanford University Medical Center Animal Care Facility. Animal care and interventions were provided in accordance with the Laboratory Animal Welfare Act, and all animals received humane care and treatment in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, publication 78-23, revised 1978) and the Stanford University School of Medicine guidelines and policies for the use of laboratory animals for research and teaching.

Cloning, Expression, and Purification of T7-Human CryAB

The full-length clone of human αB-Crystallin (Accession #: NM001885) was received from Open Biosystems. The Eco R1, an ATG site, a HindII, and a stop site were introduced into the gene as the clones were expanded. The resulting CryAB polymerase chain reaction fragment was ligated into the EcoR1-HindIII restriction site of pET21b (+) (Novagen; Merck KGaA, Darmstadt, Germany), in frame with the amino terminal T7-tag. One-shot TOP10 cells (Invitrogen, Carlsbad, CA) were transformed with the resulting plasmid. Several of the resulting colonies were selected and expanded, and the insertion was verified by restriction digest with EcoR1 and HindIII, and sequencing. Selected colonies were grown in 250 mL of lysogeny broth with carbenicillin, induced with isopropyl β-D-1-thiogalactopyranoside, and bacteria were isolated 4 to 12 hours later. The supernatant was loaded directly onto an anti-T7 affinity column and eluted with glycine buffer, pH 3.0. The eluant was neutralized with 1M Tris, pH 8.0, and gel filtered on Sephacryl S-300 in PBS. Gel slices from a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis were reduced and alkylated with iodoacetamide, the protein was cleaved with trypsin, and the resultant peptides, eluted, separated on a C-18 reverse phase column, and characterized by matrix-assisted laser desorption/ionization mass spectrometry.

Experimental Protocols

The C57BL/6 mice were anesthetized in an isoflurane (2%) inhalational chamber and endotracheally intubated with a 20G angiocatheter (Ethicon Endo-Surgery, Cincinnati, OH). Ventilation was maintained with a Harvard rodent ventilator (Harvard Apparatus, Holliston, MA). Ischemia was created by 30 minutes' ligation of the mid LAD through a left thoracotomy. After 15 minutes of ischemia, mice were randomly allocated to two groups: (1) LAD ischemia with 100 μL phosphate-buffered saline (PBS) injection intramyocardially (PBS group, n = 6); or (2) LAD ischemia with 50 μg (500 μg/mL) CryAB injection intramyocardially (CryAB group, n = 6). Two 50-μL injections using a 30G needle connected to a microsyringe containing either PBS or CryAB were administered 15 minutes before reperfusion in the medial and lateral aspects of the ischemic area. An additional 15 minutes of ischemia then occurred. After a total of 30 minutes' ischemia, the slipknot was released and the myocardium was reperfused. After chest tube placement, the chest was closed in three layers with 5-0 Vicryl (Ethicon, Somerville, NJ) suture. Similar to the strategy previously reported in our experimental autoimmune encephalomyelitis model [7], exogenous CryAB (50 μg) was injected intraperitoneally every other day for 40 days. Control mice received PBS intraperitoneally for 40 days.

Magnetic Resonance Imaging

Mice were anesthetized with 2% isoflurane with 1 L/min oxygen and placed in the supine position on postoperative day 40. A small-animal electrocardiography and respiratory gating system (Small Animal Instruments, Stony Brook, NY) were used to acquire images at the end of each QRS interval and end respiration. All imaging was performed on a Sigma 3.0 T Excite HD scanner (GE Health Systems, Milwaukee, WI) with a customized small-animal surface coil.

Gated gradient echo fast spoiled grass sequences were used to acquire sequential short-axis slices spaced 1 mm apart from apex to base of the mouse heart. For each sequence, 20 cine frames encompassing one cardiac cycle were obtained with the following sequence parameters: echo time (TE) = 4.6 ms, number of excitations = 2, field of view = 50 × 50 mm, matrix = 256 × 256, and flip angle = 60 degrees.

A contouring program, Fujin Plus 08 version 3 (Tokyo, Japan) was used to trace the endocardial border of the left ventricle myocardium for each slice of the heart over the entire cardiac cycle to determine left ventricular ejection fraction (LVEF), and left ventricular end-diastolic and end-systolic volumes.