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Critical role of extracellular heat shock cognate protein 70 in the myocardial inflammatory response and cardiac dysfunction after global ischemia-reperfusion

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Abstract

Previous studies showed that Toll-like receptor 4 (TLR4) modulates the myocardial inflammatory response to ischemia-reperfusion injury, and we recently found that cytokines link TLR4 to postischemic cardiac dysfunction. Although TLR4 can be activated in cultured cells by endogenous agents including heat shock protein 70, how it is activated during myocardial ischemia-reperfusion is unknown. In the present study, we examined 1) whether heat shock cognate protein 70 (HSC70), which is constitutively expressed in the myocardium, is released during ischemia-reperfusion; 2) whether extracellular HSC70 induces the myocardial inflammatory response and modulates cardiac function; and 3) whether HSC70 exerts these effects via TLR4. We subjected isolated mouse hearts to global ischemia-reperfusion via the Langendorff technique. Immunoblotting and immunostaining detected the release of HSC70 from the myocardium during reperfusion. Treatment with an antibody specific to HSC70 suppressed myocardial cytokine expression and improved cardiac functional recovery after ischemia-reperfusion. Recombinant HSC70 induced NF- κ B activation and cytokine expression and depressed myocardial contractility in a TLR4-dependent manner. These effects required the substrate-binding domain of HSC70. Fluorescence resonance energy transfer analysis of isolated macrophages demonstrated that extracellular HSC70 interacts with TLR4. Therefore, this study demonstrates for the first time that 1) the myocardium releases HSC70 during ischemia-reperfusion, 2) extracellular HSC70 contributes to the postischemic myocardial inflammatory response and to cardiac dysfunction, 3) HSC70 exerts these effects through a TLR4-dependent mechanism, and 4) the substrate-binding domain of HSC70 is required to induce these effects. Thus extracellular HSC70 plays a critical role in regulating the myocardial innate immune response and cardiac function after ischemia-reperfusion.

Keywords

Toll-like receptor 4; cytokines; nuclear factor- κ B; messenger ribonucleic acid

Cardiac Surgery Often Involves obligatory global myocardial ischemia-reperfusion, which causes a myocardial inflammatory response characterized by cytokine production (16,18). Several proinflammatory cytokines, including TNF- α , IL-1 β , and IL-6, contribute to myocardial injury after ischemia-reperfusion (12,31). Preserving cardiac function after global ischemia-reperfusion therefore requires regulation of the myocardial inflammatory response. However, the signaling mechanisms underlying the myocardial inflammatory response to global ischemia-reperfusion are unclear.

Previous studies implicated Toll-like receptor 4 (TLR4) signaling in the inflammatory response associated with myocardial ischemia-reperfusion injury. In mice treated with the TLR4 antagonist eritoran, myocardial mRNA levels of TNF- α , IL-1 β , and IL-6 were significantly reduced after regional ischemia-reperfusion (26). In a mouse model of isolated global myocardial ischemia-reperfusion, defective TLR4 was associated with reduced myocardial production of TNF- α and IL-1 β peptides and attenuated postischemic cardiac dysfunction (5). These studies link TLR4 signaling to the postischemic myocardial inflammatory response and cardiac dysfunction, but it remains unclear how TLR4 is activated during myocardial ischemia-reperfusion.

TLR4 was first identified as the receptor for Gram-negative bacterial lipopolysaccharide (22), but a number of endogenous factors have since been reported to activate the TLR4 signaling pathway in vitro, including lung surfactant protein-A (13) and heat shock proteins (HSPs; Refs. 4,21). The 70-kDa HSP family includes the inducible HSP70 (also termed HSP72) and the constitutive heat shock cognate protein 70 (HSC70, also termed HSP73 and HSC73). HSP70 and HSC70 are highly homologous, with rat HSC70 and HSP70 ~90% identical (20), but HSC70 is much more abundant in most cell types. Intracellularly, HSP70 and HSC70 function as molecular chaperones, playing important roles in protein folding and transport (27). However, HSPs function not only within the cell to chaperone proteins and protect tissues from stress and injury but also extracellularly to signal cell stress or trauma (4). HSP70 is released into the circulation after coronary artery bypass grafting (11) and acute myocardial infarction (9), and extracellular HSP70 has been shown to induce cytokine production in cultured mononuclear cells (2).

Although several studies (8,17,29,35) have examined the expression of HSC70 in the myocardium during ischemia-reperfusion, it is unknown whether the myocardium releases HSC70 during ischemia-reperfusion. Nor is it known whether extracellular HSC70 has any effect on the cellular or tissue inflammatory response. We hypothesized that the myocardium releases HSC70 in response to ischemia-reperfusion, that extracellular HSC70 plays a role in the myocardial inflammatory response and cardiac dysfunction involved in myocardial ischemia-reperfusion, and that these effects are mediated through TLR4.

The purposes of the study were 1) to examine myocardial release of HSC70 during global ischemia-reperfusion, 2) determine whether extracellular HSC70 contributes to the myocardial inflammatory response and cardiac dysfunction after global ischemia-reperfusion, 3) to determine whether HSC70 is sufficient to induce the myocardial inflammatory response and cardiac dysfunction, 4) to examine whether the cardiac effects of extracellular HSC70 are mediated by TLR4, and 5) to determine whether the substrate-binding domain HSC70 is necessary for these effects.

MATERIALS AND METHODS

Animals

Male C3H/HeJ mice (which have a point mutation in TLR4, resulting in a complete loss of signaling function) and C3H/HeN (wild-type control) mice, body weight 23–28 g, were

acclimated in a quarantine room for 2 wk. All experiments were approved by the Animal Care and Research Committee of the University of Colorado Denver, and this investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (National Research Council, revised 1996).

Chemicals and reagents

Recombinant bovine HSC70 (rHSC70), recombinant HSC70 fragment (without the substrate-binding domain), rat monoclonal anti-HSC70, and rabbit polyclonal anti-HSC70 were purchased from Stressgen (Ann Arbor, MI). The recombinant HSC70 was a low-endotoxin preparation, with endotoxin <5.0 pg/ μ g protein (<0.05 EU/ μ g protein) as measured by Limulus assay. In a preliminary study, we used immunoblotting to confirm that both HSC70 antibodies had no cross-reactivity with recombinant HSP70. Polyclonal antibodies against phospho-p38 MAPK, total p38 MAPK, phospho-ERK1/2, total ERK1/2, and TLR4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa 488-tagged wheat germ agglutinin was purchased from Molecular Probes (Eugene, OR). Other reagents were purchased from Sigma Chemical (St. Louis, MO).

Isolated heart perfusion

Isolated hearts were perfused by the isovolumetric Langendorff technique as described previously (1,19). Mice were anesthetized (pentobarbital sodium, 50 mg/kg ip) and heparinized (sodium heparin, 300 U ip). Their hearts were rapidly excised into oxygenated ice-cold perfusion buffer and retrograde-perfused in nonrecirculating mode at a constant pressure of 70 mmHg with Krebs-Henseleit solution, pH 7.4. An ultrathin latex balloon was inserted into the left ventricle, and balloon volume was adjusted to achieve left ventricular end-diastolic pressure of 10–20 mmHg during the initial equilibration. Pacing wires were fixed to the right atrium, and all hearts were paced at 450 beats/min. Hearts were equilibrated for 20 min before all experiments.

For ischemia-reperfusion experiments, hearts were subjected to 20 min of normothermic global ischemia followed by 60 min of reperfusion. During ischemia, hearts were placed in a normal saline-filled organ bath chamber without pacing and the temperature of perfusion buffer in the chamber was maintained at 37°C. Left ventricular developed pressure (LVDP) and dP/dt were continuously recorded with a computerized pressure amplifier/digitizer. For anti-HSC70 experiments, hearts were perfused with rabbit polyclonal anti-HSC70 or control IgG, 0.2 μ g/ml in coronary circulation, for 10 min before ischemia and 30 min immediately after initiation of reperfusion.

For HSC70 perfusion experiments, hearts were perfused with HSC70 or an HSC70 fragment, 0.5 μ g/ml in coronary circulation, for 30 min followed by 60-min washout. LVDP and dP/dt were continuously recorded.

Isolation and culture of macrophages

Peritoneal macrophages were collected by lavage from C3H/HeN and C3H/HeJ mice. Animals were anesthetized (pentobarbital sodium, 50 mg/kg iv), and the peritoneal cavity was flushed with cold (4°C) serum-free DMEM/F-12 medium. Macrophages were collected by centrifugation of the medium at 1,100 rpm for 10 min at 4°C (IEC Centra MP4R, International Equipment, Needham Heights, MA). Cells were plated and incubated for 2 h in a CO₂ incubator. Nonadherent cells were removed by three washes with DMEM/F-12 medium. During treatment with HSC70, serum concentration in the medium was reduced to 2%.

Analysis of NF- κ B activity

NF- κ B (p65) DNA-binding activity in the myocardial homogenate was measured by an assay kit (Active Motif, Carlsbad, CA), according to the manufacturer's instructions. The assay kit contains 96-well plates to which oligonucleotides containing the NF- κ B binding site were immobilized. Activated NF- κ B binds to the oligonucleotides and is detected with a specific antibody. The amount of active NF- κ B was determined using a microplate reader (Bio-Rad Laboratories, Hercules, CA).

Quantitative RT-PCR

Total RNA was extracted from tissue homogenate by the RNeasy Mini Kit (Qiagen, Valencia, CA). Purified RNA was used for RT-PCR with the GeneAmp Gold RNA PCR Kit (Applied Biosystems, Foster City, CA). Custom primers for mouse GAPDH, TNF- α , IL-1 β , and IL-6 were designed according to GenBank data. The Rotor-Gene 3000 Real-Time DNA Detection System (Corbett Research, Sydney, Australia) was used for all reactions.

Cytokine assay

Aliquots of tissue homogenate and cell culture supernatant were used to assess cytokine peptide levels using ELISA kits (R&D Systems, Minneapolis, MN). Recombinant peptides were used to construct standard curves. Absorbance of standards and samples was determined spectrophotometrically at 450 nm using a microplate reader (Bio-Rad). Results were plotted against the linear portion of the standard curve.

Immunofluorescent staining and fluorescence resonance energy transfer analysis

Myocardial sections (5- μ m thick) were fixed in 4% paraformaldehyde, incubated with a rabbit polyclonal antibody against HSC70, and then incubated with Cy3-tagged secondary goat anti-rabbit IgG (imaged on the red channel). Nuclei were stained with bis-benzimide (DAPI, imaged on the blue channel), and glycoproteins on cell surfaces were stained with Alexa 488-tagged wheat germ agglutinin (imaged on the green channel). Microscopy was performed with a Leica DMRXA digital microscope (Leica Mikroskopie und Systeme, Wetzlar, Germany).

Fluorescence resonance energy transfer (FRET) is the transfer of fluorescent energy from a donor fluorophore to a nearby acceptor fluorophore, which can be detected by the increased fluorescent emission of the acceptor after photobleaching the donor. Since FRET can occur only when two fluorophores are no more than 2–10 nm apart, a positive FRET signal indicates that the fluorophores, and the attached biomolecules, are interacting with each other (34). We used FRET on macrophages treated with HSC70, using monoclonal anti-HSC70 and polyclonal anti-TLR4 to immunofluorescently stain HSC70 and TLR4, and secondary antibodies with fluorescent tags to label HSC70 red and TLR4 green. In these circumstances, a positive FRET signal indicates that HSC70 and TLR4 are no more than 5 nm apart. Microscopic analysis and photography were performed with a Zeiss Axiovert 100M microscope (Carl Zeiss MicroImaging, Thornwood, NY) and SlideBook software (I. I. I., Denver, CO).

Immunoblotting

Immunoblotting was used to analyze HSC70, phosphorylated-p38 MAPK, phosphorylated-ERK1/2, total p38 MAPK, and total ERK1/2. Myocardial tissue was homogenized in cell lysis buffer containing PBS, 1% Triton X-100, and a protease inhibitor cocktail. Protein samples were separated on gradient (4–20%) mini-gels (Bio-Rad) and transferred onto membranes. After being blocked with 5% nonfat dry milk, membranes were incubated with primary antibodies diluted to 1 μ g/ml in PBS containing 0.05% Tween 20 and 5% dry milk

(TPBS) at room temperature for 2 h. After being washed, membranes were incubated with peroxidase-linked secondary antibodies (diluted 1:5,000 to 10,000 with TPBS and 5% dry milk) at room temperature for 1 h. After further washes, ECL solution was added for 1 min at room temperature and membranes were exposed on X-ray film.

Statistical analysis

Data are means $\pm$ SE. Statistical analyses were performed using ANOVA with a post hoc Bonferroni-Dunn test. Statistical significance was accepted within a 95% confidence limit.

RESULTS

The myocardium releases HSC70 during ischemia-reperfusion

We used immunoblotting to probe for HSP70 and HSC70 in coronary effluent from the hearts of naïve animals at three time points: before ischemia, in the first 5 min of reperfusion and before the end of reperfusion. While HSP70 was barely detectable at any time point (not shown), HSC70 was detected in coronary effluent at both the beginning and end of reperfusion (Fig. 1A). It seems that ischemia induces a sustained release of HSC70 from the myocardium. Interestingly, two additional HSC70-immunoreactive bands with lower molecular size were visible in early reperfusion samples. Immunofluorescent staining detected HSC70 within cells before ischemia-reperfusion but in the extracellular space of the myocardium after ischemia-reperfusion (Fig. 1A). Together these data demonstrate that ischemia-reperfusion causes the release of myocardial HSC70.

Extracellular HSC70 is involved in the postischemic myocardial inflammatory response and in cardiac dysfunction

To determine the role of extracellular HSC70 in the postischemic myocardial inflammatory response and in cardiac dysfunction, we assessed the effect of polyclonal anti-HSC70 on myocardial cytokine expression and cardiac contractile function after ischemia-reperfusion. Using immunoblotting, we confirmed that this antibody had no cross-reactivity with recombinant HSP70. As presented in Fig. 1B, anti-HSC70 treatment improved postischemic cardiac functional recovery, while nonimmune IgG had no effect. At the end of reperfusion, LVDP recovered to $70.4 \pm 5.8\%$ of baseline levels ($P < 0.05$) in the anti-HSC70 group compared with 37.0 ± 6.8 and $42.0 \pm 5.2\%$ in ischemia-reperfusion controls and the nonimmune IgG group, respectively. Similarly, $dP/dt_{\max}$ was higher in the anti-HSC70 group at the end of reperfusion.

Treatment with anti-HSC70 reduced expression of the myocardial cytokines TNF- α , IL-1 β , and IL-6 after ischemia-reperfusion. mRNA levels of TNF- α , IL-1 β , and IL-6 were lower at the end of reperfusion in the anti-HSC70 group than in ischemia-reperfusion controls and the nonimmune IgG group (Fig. 1C). Myocardial peptide levels of TNF- α , IL-1 β , and IL-6 were 65, 68, and 61% lower, respectively (all $P < 0.05$) in hearts treated with anti-HSC70 than in untreated hearts (ischemia-reperfusion controls). These results demonstrate that neutralization of extracellular HSC70 reduces the myocardial inflammatory response and improves cardiac functional recovery after global ischemia-reperfusion. Thus extracellular HSC70 plays a critical role in the myocardial inflammatory response and in cardiac dysfunction after global ischemia-reperfusion.

Recombinant HSC70 induces the inflammatory response and depresses contractility in isolated hearts through TLR4

To examine whether HSC70 is sufficient to induce the inflammatory response and cardiac dysfunction and to evaluate the role of TLR4 in the cardiac response induced by HSC70, we compared the effect of rHSC70 on hearts from C3H/HeN (TLR4-competent) mice and C3H/

HeJ (TLR4-defective) mice. In TLR4-competent hearts, rHSC70 induced the phosphorylation of p38 MAPK but had little influence on ERK1/2 phosphorylation (Fig. 2, A and B). In addition, rHSC70 increased myocardial NF- κ B DNA-binding activity in TLR4-competent hearts (Fig. 2C). These effects were markedly reduced in TLR4-defective hearts, which indicates that extracellular HSC70 activates the p38 MAPK and NF- κ B pathways through TLR4.

We then examined the effect of rHSC70 on cytokine expression. In TLR4-competent hearts, treatment with rHSC70 increased levels of mRNA encoding TNF- α , IL-1 β , and IL-6 by 10-, 8-, and 6-fold, respectively, and peptide levels of TNF- α , IL-1 β , and IL-6 by 8-, 5-, and 6-fold, respectively (Fig. 3). In TLR4-defective hearts, both mRNA and peptide levels of these cytokines were not significantly increased after rHSC70 treatment. These results indicate that HSC70 induces myocardial cytokine expression through a TLR4-dependent mechanism.

We also assessed the effect of rHSC70 on cardiac contractility. As presented in Fig. 4, LVDP and $dP/dt_{\max}$ significantly declined in TLR4-competent hearts 30 min after rHSC70 perfusion and continued to decline throughout the washout. By contrast, TLR4-defective hearts did not exhibit functional depression during or after rHSC70 treatment. At the end of the experiment, LVDP was $72.0 \pm 6.36\%$ of baseline levels in TLR4-competent hearts and $98.3 \pm 9.54\%$ of baseline levels in TLR4-defective hearts ($P < 0.05$). Thus HSC70 depresses cardiac contractility through TLR4.

The substrate-binding domain of rHSC70 is required to induce a cardiac response

HSC70 has two functional domains: the nucleotide-binding domain (NBD) and the substrate-binding domain (SBD). ATP binding at the NBD induces the release of protein substrate from the SBD; protein substrate binding at the SBD causes the hydrolysis of ATP at the NBD. The SBD is critical for HSC70 to interact with proteins (7,20,33). We demonstrated that full-length HSC70 induces a TLR4-dependent inflammatory response and contractile depression. To determine whether the SBD is required for HSC70 to have those effects, we examined what effect an HSC70 fragment without the SBD (i.e., a recombinant NBD) had on cytokine expression and cardiac contractility, and we contrasted its effect with that of full-length rHSC70.

As shown in Fig. 5, the rHSC70 fragment, applied according to the same protocol and in the same concentration as rHSC70, did not induce cardiac functional changes in TLR4-competent hearts. Similarly, the rHSC70 fragment without the SBD failed to induce cytokine expression. Levels of TNF- α , IL-1 β , and IL-6 mRNA (expressed as a fraction of GAPDH mRNA) were 0.16 ± 0.05 , 0.35 ± 0.03 , and 0.26 ± 0.04 , respectively, which were not significantly different than those of perfusion controls (0.14 ± 0.03 , 0.34 ± 0.12 , and 0.22 ± 0.10 , respectively, $P > 0.05$). Peptide levels of TNF- α , IL-1 β , and IL-6 in hearts treated with the rHSC70 fragment were 7.08 ± 1.90 , 9.11 ± 2.51 , and 16.0 ± 2.91 pg/ml, respectively, also comparable with those of perfusion controls (4.01 ± 0.60 , 5.38 ± 0.84 , and 10.8 ± 1.68 pg/ml, respectively, $P > 0.05$). Further, cytokine mRNA and peptide levels were significantly lower in hearts treated with the rHSC70 fragment than in hearts treated with full-length rHSC70 (all $P < 0.05$). These results demonstrate that the rHSC70 fragment without the SBD is insufficient to induce either cardiac dysfunction or the inflammatory response.

HSC70 interacts with TLR4

Myeloid cells have a high density of TLRs on their cell surfaces. We used macrophages as a cellular model to examine the interaction of extracellular HSC70 with TLR4. First, we confirmed that HSC70 induces a TLR4-dependent inflammatory response in macrophages.

We treated peritoneal macrophages isolated from TLR4-competent and TLR4-defective mice with rHSC70 (1.0 $\mu\text{g/ml}$) and examined TNF- α levels in culture media after 4 h. Correlating with our results on hearts, rHSC70 induced TNF- α in TLR4-competent cells (488 ± 38 vs. 17 ± 2.5 pg/ml in untreated controls, $P < 0.01$) but little TNF- α in TLR4-defective cells (25 ± 2.1 pg/ml, $P < 0.01$ vs. TLR4-competent cells treated with rHSC70). Therefore the effect of rHSC70 was TLR4 dependent in macrophages. To address the issue of endotoxin contamination in our recombinant protein preparations, we used the endotoxin antagonist polymyxin B (5.0 $\mu\text{g/ml}$) and found that it had a minimal influence on rHSC70-induced TNF- α production in macrophages (413 ± 47 pg/ml in cells treated with rHSC70 plus polymyxin B vs. 488 ± 38 pg/ml in cells treated with rHSC70 alone, $P > 0.05$). These results show that the HSC70 protein per se was responsible for the observed effect on TNF- α production.

Then, we performed dual immunofluorescent staining and FRET analysis on macrophages incubated with or without rHSC70 to examine the interaction of HSC70 with TLR4. In untreated cells, HSC70 was located in the cytoplasm and no FRET signal between HSC70 and TLR4 was detected (Fig. 6). In cells incubated with rHSC70, increased cell-surface localization of HSC70 was observed and colocalization of HSC70 and TLR4 was detected. In addition, FRET signals demonstrated an interaction between HSC70 and TLR4 on the cell surface. These results indicate that extracellular HSC70 interacts with TLR4.

DISCUSSION

We found that the constitutively expressed HSC70 is released into the coronary effluent during global ischemia-reperfusion, and we detected HSC70 in the extracellular space of the myocardium after global ischemia-reperfusion. Therefore HSC70 escapes from cardiac cells during ischemia-reperfusion. Further, the HSC70 release we detected was from isolated, buffer-perfused hearts, which excludes any contribution from other organs or blood cells. Thus our results demonstrate that myocardial ischemia-reperfusion causes HSC70 to be released from the heart itself.

Several studies have demonstrated the release of HSC70 in vitro from various cell types. HSC70 is released from glia cells (30) and from K562 erythroleukemic cells treated with IFN- γ , IL-10, or hyperthermia (3). In addition, Saito et al. (23) found that HSPs including HSC70 are released from injured cells. The present study is the first to demonstrate the release of HSC70 from the heart. The HSC70 release we observed is likely due to cell injury, but active secretion of HSC70 from cells has also been reported (3,6). Further study is needed to determine what mechanism(s) and which cell type(s) are involved in myocardial HSC70 release. To our knowledge, no in vivo clinical or animal studies have examined myocardial HSC70 release or measured circulating HSC70 levels. Several studies have reported that the inducible HSP70 is released into the circulation after stressful or injurious events including myocardial infarction (9,24) and cardiac surgery with obligatory global myocardial ischemia-reperfusion (10,11,28). These studies do not, however, address whether HSC70 is also released. Since the constitutive HSC70 is much more abundant in most cells, including cardiac myocytes, than the inducible HSP70, it is possible that HSC70 release after myocardial infarction and cardiac surgery with global ischemia-reperfusion has even greater significance than HSP70 release. Further studies are needed to examine HSC70 release in those clinical situations.

In this study, we found that HSC70-specific antibody improves cardiac functional recovery after global ischemia-reperfusion and reduces expression of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6. We also found that recombinant HSC70 depresses cardiac contractility and increases the expression of TNF- α , IL-1 β , and IL-6. Therefore,

extracellular HSC70 appears to play a role in cardiac dysfunction and in the expression of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 after global ischemia-reperfusion. It is well-known that TNF- α , IL-1 β , and IL-6 are cardiodepressant. In an earlier study (5), we linked TNF- α and IL-1 β to reduced cardiac contractility after ischemia-reperfusion. Since extracellular HSC70 plays a critical role in myocardial expression of cardiodepressant cytokines, the release of HSC70 from the myocardium may play a role in the cardiac dysfunction and inflammatory response observed in vivo after cardiac surgery with obligatory global myocardial ischemia-reperfusion.

In our previous study (5), we found that TLR4 plays a critical role in postischemic myocardial cytokine production and cardiac dysfunction. However, how myocardial TLR4 activation occurs during ischemia-reperfusion remains unknown. In this study, after finding that extracellular HSC70 contributes to the inflammatory response and myocardial functional injury after ischemia-reperfusion, we then examined whether extracellular HSC70 exerts these effects through TLR4. We found that HSC70 activates the p38 MAPK and NF- κ B pathways; induces myocardial expression of TNF- α , IL-1 β , and IL-6; and depresses cardiac contractility in TLR4-competent hearts but not in TLR4-defective hearts. HSC70 also induced a TNF- α response in macrophages from TLR4-competent mice but not from TLR4-defective mice. These results provide direct evidence that extracellular HSC70 activates NF- κ B and p38 MAPK; induces the myocardial expression of TNF- α , IL-1 β , and IL-6; and depresses cardiac contractility via a TLR4-dependent mechanism. Thus extracellular HSC70 appears to be one of the factors that activate myocardial TLR4.

In this study, we found that HSC70 induces p38MAPK and NF- κ B activation in a TLR4-dependent manner and that p38MAPK and NF- κ B activation correlates with the expression of proinflammatory cytokines. It seems that the p38MAPK and NF- κ B pathways are involved in signaling mechanisms in the HSC70-induced, TLR4-dependent inflammatory response. TLR4 signaling involves both MyD88-dependent and MyD88-independent pathways (15). In the MyD88-dependent pathway, MyD88 recruits IRAK4, which then phosphorylates IRAK1 to propagate the proinflammatory signal, leading to the phosphorylation of the IKK complex and MAPKs, including p38 MAPK (14,25). In the MyD88-independent pathway, TLR4 utilizes TRIF-related adaptor molecules to activate NF- κ B. Previous studies (2) have shown that HSP70 activates TLR4 and induces proinflammatory cytokine production in monocytes via the MyD88/IRAK/NF- κ B signaling pathway. Future studies are required to determine the relative role of the MyD88-dependent and -independent pathways in the activation of NF- κ B and p38 MAPK by HSC70.

It has been suggested that activation of TLR4 by recombinant proteins is partly due to endotoxin contamination (32). Although we used a preparation of rHSC70 with very little endotoxin contamination (<5.0 pg/ μ g protein by Limulus assay), we examined whether that amount of endotoxin in our rHSC70 preparation contributed to the inflammatory response by treating macrophages with rHSC70 in the presence of the endotoxin antagonist polymyxin B (5.0 μ g/ml). We found that the endotoxin antagonist had a minimal effect on rHSC70-induced TNF- α production. Thus endotoxin is not responsible for the proinflammatory effect of rHSC70. In addition, the recombinant HSC70 fragment, which also contains a trace amount of endotoxin, did not induce a cytokine response in the heart. These results support the conclusion that the induction of a TLR4-dependent proinflammatory response by HSC70 is due to the native properties of the protein.

HSC70 is a constitutively expressed cytoplasmic protein that functions intracellularly primarily as a molecular chaperone (36). It has two main functional domains, an ATPase or NBD and an SBD (20,33). Deletion studies (7,33) have shown that these two domains are sufficient for HSC70 to function as a chaperone in clathrin uncoating. We tested whether an

HSC70 fragment without the SBD induces cytokine production. In our isolated heart experiments, the HSC70 fragment did not induce cytokine expression nor did it influence cardiac contractility. Since the proinflammatory and cardiodepressive effects of HSC70 require the SBD, it is most likely the SBD of HSC70 that interacts with and/or activates TLR4. We applied FRET analysis to macrophages incubated with HSC70 to examine whether HSC70, when applied extracellularly, interacts with TLR4. The results showed increased cell-surface HSC70 levels and increased FRET signals between HSC70 and TLR4. Therefore, it appears that extracellular HSC70 interacts with TLR4 to activate that receptor.

In summary, this study demonstrates for the first time that 1) the myocardium releases HSC70 during global ischemia-reperfusion, 2) extracellular HSC70 contributes to the postischemic myocardial inflammatory response and to cardiac dysfunction, 3) HSC70 applied extracellularly is sufficient to induce the myocardial inflammatory response and cardiac functional changes through a TLR4-dependent mechanism, 4) the substrate-binding domain of HSC70 is required to induce these TLR4-dependent effects, and 5) extracellular HSC70 interacts with TLR4. These findings indicate that extracellular HSC70 depresses cardiac function by modulating the myocardial innate immune response and suggest that extracellular HSC70 may represent a novel target for preserving cardiac function during global ischemia-reperfusion.

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Fig. 1.

Heat shock cognate protein 70 (HSC70) released from the myocardium during ischemia-reperfusion (I/R) contributes to cardiac dysfunction and myocardial cytokine expression. Hearts isolated from C3H/HeN (wild-type) mice were subjected to global ischemia-reperfusion or perfusion only (control). **A:** coronary effluent was collected before 20-min ischemia (basal) and during the first 5 min (R5) and the last 5 min (R60) of reperfusion. Although immunoblotting did not detect HSC70 in the coronary effluent before ischemia, HSC70 was present in both samples collected during reperfusion. Two HSC70-immunoreactive bands with lower molecular size were also detected in the R5 sample. Myocardial tissue sections were immunofluorescently stained to localize HSC70 after

ischemia-reperfusion (20-min ischemia/5-min reperfusion). *Top*: HSC70 (red) and nuclei (blue); *bottom*: cell surfaces (green). In control hearts, HSC70 is intracellular and associated with myocyte striations. After ischemia-reperfusion, the striation pattern is lost, and HSC70 is localized in the extracellular space (arrows). *B*: hearts were subjected to global ischemia-reperfusion (20-min ischemia/60-min reperfusion) with or without treatment with polyclonal anti-HSC70 (HSC Ab, 0.2 μ g/ml) or control IgG (IgG, 0.2 μ g/ml) for 10 min before ischemia and for 30 min after initiation of reperfusion. Control hearts were perfused with perfusion buffer without being subjected to ischemia. Anti-HSC70 improved the recovery of left ventricular developed pressure (LVDP) and dP/dt max while control IgG had no effect. *C*: anti-HSC70 reduced myocardial levels of cytokine (TNF- α , IL-1 β , and IL-6) mRNA and peptides after ischemia-reperfusion while control IgG had no effect. Data are means $\pm$ SE. rHSC, recombinant HSC70; M, myocyte ($n = 6$ in each group). * $P < 0.05$ vs. I/R; ** $P < 0.05$ vs. control.

Fig. 2.

HSC70 induces the activation of myocardial p38 MAPK and NF- κ B through Toll-like receptor 4 (TLR4). Hearts isolated from C3H/HeN (TLR4-competent) and C3H/HeJ (TLR4-defective) mice were perfused with recombinant HSC70 (0.5 μ g/ml) for 30 min followed by 60 min washout. Control hearts were perfused without exposure to HSC70. **A** and **B**: representative blot and mean densitometry data (means $\pm$ SE) of 2 separate experiments show that HSC70 induced phosphorylation of p38 MAPK and ERK1/2 in TLR4-competent hearts. In TLR4-defective hearts, ERK 1/2 phosphorylation was minimally changed, but the effect of HSC70 on p38 MAPK phosphorylation was abrogated. **C**: HSC70 increased NF- κ B DNA-binding activity in TLR4-competent hearts, an effect that was greatly attenuated in

TLR4-defective hearts. NF- κ B DNA-binding data are means $\pm$ SE ($n = 6$ in each group). * $P < 0.05$ vs. HeN HSC. p-p38, phosphorylated-p38; p-ERK1/2, phosphorylated-ERK1/2.

Fig. 3.

HSC70 induces expression of cardiodepressant cytokines through TLR4. Hearts isolated from C3H/HeN (TLR4-competent) and C3H/HeJ (TLR4-defective) mice were perfused with recombinant HSC70 (0.5 μ g/ml) for 30 min followed by 60-min washout. Control hearts were perfused with perfusion buffer without HSC70. **A:** TLR4-competent hearts expressed cytokine (TNF- α , IL-1 β , and IL-6) mRNA in response to HSC70 treatment; TLR4-defective hearts did not. **B:** cytokine (TNF- α , IL-1 β , and IL-6) peptide levels increased in TLR4-competent hearts treated with HSC70 but not in TLR4-defective hearts. Data are means $\pm$ SE ($n = 6$ in each group). * $P < 0.05$ vs. HeN HSC; ** $P < 0.05$ vs. HeN control.

Fig. 4.

HSC70 induces cardiac contractile depression through TLR4. Hearts isolated from C3H/HeN (TLR4-competent) and C3H/HeJ (TLR4-defective) mice were perfused with recombinant HSC70 (0.5 $\mu\text{g/ml}$) for 30 min followed by 60-min washout. Perfusion with HSC70 depressed LVDP (A) and $\text{dP/dt}_{\text{max}}$ (B) in TLR4-competent hearts but not in TLR4-defective hearts. Data are expressed as means $\pm$ SE ($n = 6$ in each group). * $P < 0.05$ vs. HeJ.

Fig. 5.

The effect of HSC70 on cardiac contractility is abrogated in the absence of the substrate-binding domain. Hearts isolated from C3H/HeN (TLR4-competent) mice were perfused with either recombinant full-length HSC70 (0.5 $\mu\text{g/ml}$) or recombinant HSC70 fragment (without the substrate-binding domain, 0.5 $\mu\text{g/ml}$) for 30 min followed by 60-min washout. *A* and *B*: without the substrate-binding domain, the ability of HSC70 to depress cardiac contractility was lost. Data are means $\pm$ SE ($n = 6$ in each group). * $P < 0.05$ vs. full-length HSC70.

FIG. 6.

HSC70 interacts with TLR4 in macrophages. Macrophages from C3H/HeN (TLR4-competent) mice were incubated with recombinant HSC70 (0.5 $\mu\text{g/ml}$) for 15 min or left untreated (control). Double immunofluorescent staining labeled HSC70 red and TLR4 green (*left*). Nuclei were counterstained blue. In untreated cells (*top*), HSC70 was located in the cytoplasm and no fluorescence resonance energy transfer (FRET) signal between HSC70 and TLR4 was detected after photo-bleaching. In cells incubated with HSC70 (*bottom*), increased cell-surface localization of HSC70 and colocalization of HSC70 and TLR4 (yellow areas, arrows) were observed. In addition, FRET signals (*bottom right*, arrows) were detected in the 2 photobleached cells at *bottom right* but not in the nonphotobleached (control) cell at *top left*.