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Extracellular heat shock cognate protein 70 induces cardiac functional tolerance to endotoxin: Differential effect on TNF- α and ICAM-1 levels in heart tissue

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Abstract

Endotoxin provokes cardiac dysfunction, and induction of tolerance to endotoxin has therapeutic potential. Heat shock protein 70 (HSP70) can induce endotoxin tolerance in macrophages. We recently found that heat shock cognate protein 70 (HSC70) induces pro-inflammatory cytokines via activation of TLR4 in macrophages and the myocardium. We hypothesize that HSC70 preconditioning induces cardiac tolerance to endotoxin. Pretreatment of peritoneal macrophages with HSC70 for 24 h reduced TNF- α levels following endotoxin stimulation. Preconditioning of mice with HSC70 24 h prior to endotoxin attenuated endotoxemic cardiac dysfunction. HSC70 preconditioning reduced TNF- α levels in plasma and heart tissue by 33.3% and 35.4%, respectively, and decreased ICAM-1 levels in heart tissue by 63.5% following endotoxin challenge. The effect of HSC70 on TNF- α was less robust than endotoxin preconditioning (79.7% and 75.0% reduction in TNF- α levels in plasma and heart tissue, respectively); however, HSC70 and endotoxin preconditioning had comparable effects on ICAM-1 levels in heart tissue. While HSC70 preconditioning had no effect on myocardial TLR4 protein levels, it suppressed NF- κ B activation induced by endotoxin. We conclude that HSC70 preconditioning 1) attenuates the TNF- α response to endotoxin in macrophages in vitro, 2) induces cardiac functional tolerance to endotoxin and 3) reduces NF- κ B activity, and TNF- α and ICAM-1 levels in heart tissue. Thus, the mechanism of HSC70-induced cardiac tolerance to endotoxin appears to involve down-regulation of myocardial TLR4 signaling and inflammatory responses.

Keywords

endotoxemia; Toll-like receptor 4; NF- κ B; cytokine; adhesion molecule

1. Introduction

Bacterial endotoxin induces cardiac contractile dysfunction that is associated with myocardial expression of pro-inflammatory mediators including TNF- α and ICAM-1 (1,2). TNF- α is an early cytokine in the multifactorial mechanisms of endotoxemic cardiac dysfunction, and in animal models of endotoxemia there is a time lag between peak myocardial TNF- α levels and

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contractile dysfunction (1,3). Thus, TNF- α depresses myocardial contractility by inducing other cardiac depressant factors. Among those is ICAM-1, which mediates cardiac dysfunction through leukocyte-dependent and -independent mechanisms (2,4). Ligation of ICAM-1 by endogenous agents including fibrinogen contributes to leukocyte-independent cardiac contractile depression (5). We have identified TNF- α as a link between cardiac Toll-like receptor 4 (TLR4) and myocardial ICAM-1 expression and demonstrated that endotoxin fails to depress cardiac contractility in ICAM-1 knockout mice (3). Thus, myocardial ICAM-1 appears to play a central role in endotoxemic cardiac dysfunction.

Tolerance to bacterial endotoxin induced by prior exposures to endotoxin is a well-described phenomenon. We found that a single exposure to endotoxin preserves cardiac function during subsequent endotoxemia (6). Previous studies demonstrate that extracellular heat shock protein 70 (HSP70) induces an inflammatory response, including production of pro-inflammatory cytokines, in mononuclear cells through a Toll-like receptor 4 (TLR4)-dependent mechanism (7,8). Aneja and colleagues showed that extracellular HSP70 induces tolerance to endotoxin in macrophages (9). Inducing cardiac tolerance to endotoxin by non-noxious agents has therapeutic potential; however, the role of endogenous TLR4 ligands in the induction of cardiac tolerance to endotoxin has not been determined.

The 70 kDa heat shock protein family has two major members, the inducible HSP70 and the constitutive heat shock cognate protein 70 (HSC70). Both HSP70 and HSC70 are intracellular proteins and function as molecular chaperons, playing important roles in folding and transporting newly synthesized peptides (10). In addition, expression of HSP70 is an important mechanism by which living cells adapt to stress. In a recent study (11) we found that extracellular HSC70 can induce production of the pro-inflammatory cytokines TNF- α and IL-1 β in both macrophages and the heart, an effect which requires functional TLR4. We also found that recombinant HSC70 directly interacts with TLR4 in macrophages. Thus extracellular HSC70 seems to activate TLR4 to induce pro-inflammatory cytokine expression.

We hypothesize that HSC70 preconditioning induces cardiac tolerance to endotoxin. In this study we examined: 1) whether HSC70 preconditioning of macrophages *in vitro* reduces the TNF- α response to subsequent endotoxin stimulation, 2) the effect of *in vivo* HSC70 preconditioning on cardiac function in mice during subsequent endotoxemia, and 3) the effect of HSC70 preconditioning on NF- κ B activity, and the levels of TLR4, TNF- α and ICAM-1 in the heart.

2. Materials and Methods

2.1. Animals

Male C3H/HeJ (TLR4-defective) and C3H/HeN (TLR4-competent) mice, body weight 23–28 g, were acclimated in a quarantine room for 2 weeks before use. 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). C3H/HeJ mice have a point mutation in the intracellular domain of TLR4, resulting in a complete loss of signaling function (12).

2.2. Chemicals and reagents

Recombinant bovine HSC70 (rHSC70) was purchased from Assay Designs (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 our previous study (11), we found that Polymyxin B has a minimal effect on macrophage TNF- α production induced by

recombinant HSC70. In this study, we also confirmed that the effect of HSC70 on macrophages was not influenced by Polymyxin B.

TNF- α ELISA kit was purchased from R&D Systems (Minneapolis, MN), and NF- κ B activity assay kit was purchased from Active Motif (Carlsbad, CA). Rat monoclonal antibody against mouse ICAM-1 and rabbit polyclonal against mouse TLR4 were purchased from Serotec (Oxford, UK). Endotoxin (from E. Coli. 0111:B4) and other reagents were purchased from Sigma (St. Louis, MO).

2.3. Collection, culture and treatment of peritoneal macrophages

Ten TLR4-competent and ten TLR4-defective mice were anesthetized with sodium pentobarbital (50 mg/kg iv). Peritoneal macrophages were collected by lavage. Briefly, the peritoneal cavity was flushed with 5 ml cold D-MEM/F-12 serum-free medium. Cells were harvested by centrifugation (IEC MP 4R, International Equipment Co, Needham Hts, MA) at $500 \times g$ for 10 min at 4°C. Cells were washed with serum-free medium and resuspended in full D-MEM/F-12 medium (containing 10% fetal bovine serum and 150 μ g/ml of gentamycin). Cells were plated onto 24-well plates (0.5×10^6 cells/well) and incubated at 37 °C for 2 h. Non-adherent cells were removed by 2 washes with full D-MEM/F-12 medium. More than 95% of adherent cells were macrophages, as identified by immunofluorescent staining with antiserum against murine macrophage marker protein CD68.

For endotoxin preconditioning experiments, adherent cells were incubated in medium containing endotoxin (200 ng/ml) for 24 h, then washed twice with D-MEM/F-12 medium and stimulated with endotoxin (200 ng/ml) for 18 h. For HSC70 preconditioning experiments, adherent cells were incubated in D-MEM/F-12 medium containing recombinant HSC70 (2.5, 5.0 or 10 μ g/ml) for 24 h, washed twice with D-MEM/F-12 medium and stimulated with endotoxin for 18 h. Culture media were collected and cells were harvested, washed with PBS and lysed in cold lysis buffer. Samples were stored at -70 °C for cytokine assays. Comparable numbers of total cells and viable cells (determined by Trypan blue stain) remained in the wells after different treatments.

2.4. In vivo preconditioning

Four groups of TLR4-competent mice, 6 mice in each group, were used to examine endotoxemic cardiac dysfunction and the effect of preconditioning using a pressure-volume catheter (Millar Instruments, Houston, TX). One group of TLR4-competent mice was injected with endotoxin (0.5 mg/kg) through a tail vein, and control mice were injected with sterile normal saline (0.2 ml) in the same manner. Left ventricular function was measured 6 h later. Two additional groups of TLR4-competent mice were pretreated with endotoxin (0.5 mg/kg, iv) or recombinant HSC70 (0.4 μ g/g body weight, iv; calculated to give a blood concentration of 5.0 μ g/ml as blood accounts for 8% of body weight in mice) and challenged with endotoxin (0.5 mg/kg, iv) 24 h after pretreatment. Left ventricular function was measured 6 h later. Hearts were collected from the four groups of mice after measurement of cardiac function, and myocardial homogenate was prepared for analysis of ICAM-1 levels.

Four groups of TLR4-competent mice, 5 in each group, were used to examine the effect of preconditioning on TNF- α levels in plasma and heart tissue, as well as NF- κ B activity in heart tissue. Two groups of mice were injected with endotoxin (0.5 mg/kg, iv) or sterile normal saline (0.2 ml, iv). Animals were sacrificed 1 h after injection, and plasma and myocardial homogenate were prepared. The other two groups of mice were injected with endotoxin (0.5 mg/kg, iv) or recombinant HSC70 (0.4 μ g/g body weight, iv), challenged with endotoxin (0.5 mg/kg, iv) 24 h later, and sacrificed 1 h afterwards. Plasma and myocardial homogenate were

prepared. TNF- α levels in plasma and myocardial homogenate, as well as NF- κ B activity in myocardial homogenate were analyzed.

Additional 6 mice were used to examine the effect of preconditioning on myocardial TLR4 levels. Three animals were treated with endotoxin (0.5 mg/kg, iv) or recombinant HSC70 (0.4 μ g/g body weight, iv). Animals were sacrificed 24 h later, and myocardial homogenate were prepared. TLR4 protein levels were analyzed by immunoblotting and compared with those of untreated mice.

2.5. TNF- α assay

Enzyme-linked immunosorbent assay (ELISA) kits were used to analyze TNF- α levels in culture medium, cell lysates, plasma and myocardial homogenates as described previously (3). Myocardial homogenate was prepared in 8 volumes of homogenate buffer [0.1 M sodium phosphate (pH 7.5) containing 0.1% Triton X100, 2 mM EGTA, 1.0 mM benzamidine and 1.0 mM phenylmethylsulfonyl fluoride] and centrifuged at $1000 \times g$ for 15 min at 4°C with an Eppendorf centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY). Supernatant was collected, and protein concentrations were analyzed before analysis of TNF- α .

Recombinant murine TNF- α was used to construct standard curves. Absorbance of standards and samples was determined spectrophotometrically at 450 nm using a microplate reader (Bio-Rad, Hercules, CA). Results were plotted against the linear portion of a standard curve.

2.6. NF- κ B activity assay

NF- κ B (p65) DNA-binding activity in the myocardial homogenate was measured using a commercial kit. This assay utilizes a 96-well plate coated with oligonucleotides containing the NF- κ B binding site. 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 (BioRadLaboratories, Hercules, CA).

2.7. Measurement of left ventricular contractility and cardiac function

Left ventricular contractility and other measures of left ventricular function were determined using a pressure-volume catheter (Millar Instruments, Houston, TX). Mice were anesthetized and anticoagulated with avertin (20 mg/ml, 0.3 ml/mouse, ip; Sigma; St. Louis, MO) and heparin (1,000 units/kg, ip; Elkins-Sinn; Cherry Hill, NJ). Animals were kept on a heating blanket and core body temperature was maintained at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. The left carotid artery was cannulated and a microcatheter (1F) inserted until a ventricular waveform was obtained. The pressure-volume loop was recorded using the MPVS-400 System with the aid of PVAN software. Six to ten pressure-volume loops during inferior vena cava occlusion were sampled and used to measure end-systolic elastance (E_{es}), which is the slope of the end-systolic pressure-volume relationship independent of changes in preload and afterload. The volume axis intercept, V_d , was considered zero volume for steady-state measurements. Pressure-volume loops recorded at steady-state were used to measure the maximum rate of change in ventricular pressure and ejection fraction.

2.8. Immunoblotting of ICAM-1 and TLR4

ICAM-1 and TLR4 levels in myocardial homogenate were analyzed by immunoblotting as previously described (3). Myocardial homogenate was mixed with an equal volume of sample buffer [100 mM Tris-HCl (pH 6.8), 2% SDS, 0.02% bromophenol blue and 10% glycerol]. Crude protein (20 μ g) was fractioned on 4–20% acrylamide gradient gels and transferred onto nitrocellulose membranes. Membranes were incubated in PBS containing 5% non-fat dry milk for 45 min to block non-specific binding. Then, membranes were probed with a rat monoclonal antibody against mouse ICAM-1 or a rabbit polyclonal antibody against mouse TLR4 (1:1000

dilution with PBS containing 0.05% Tween 20 and 5% dry milk) for 60 min. Subsequently, membranes were incubated with peroxidase-labeled goat anti-rat IgG or goat anti-rabbit IgG (1:5000 dilution with PBS containing 0.05% Tween 20 and 5% dry milk) for 45 min. Protein bands were developed using enhanced chemiluminescence (ECL) technique and membranes exposed on Kodak X-Omat film. Densitometry was performed using a computerized densitometer (Molecular Dynamics, Sunnyvale, CA).

2.9. Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean (SE). Analysis of variance (ANOVA) was performed, and differences were considered significant when $P < 0.05$, as verified by Fisher post-hoc test.

3. Results

3.1. HSC70 preconditioning attenuates macrophage TNF- α response

We stimulated macrophages with HSC70 or endotoxin and measured levels of released and cell-associated TNF- α . Because cell-associated levels of this cytokine were very low, we will present only the results on released TNF- α . Stimulation with recombinant HSC70 (2.5–10 $\mu\text{g}/\text{ml}$) for 24 h increased TNF- α levels in the medium of TLR4-competent cells in a dose-dependent fashion (Figure 1). HSC70 had no effect on TNF- α levels in TLR4-defective cells (Figure 1). However, HSC70 is much less potent in the induction of TNF- α . HSC70 in a concentration of 10 $\mu\text{g}/\text{ml}$ increased TNF- α level to 1.11 ± 0.13 ng/ml in TLR4-competent cells while endotoxin in a much lower concentration (0.2 $\mu\text{g}/\text{ml}$) induced a robust TNF- α response (3.12 ± 0.17 ng/ml, Figure 2).

We preconditioned macrophages with HSC70 or endotoxin and measured TNF- α levels in the medium following subsequent exposure to endotoxin. HSC70 preconditioning reduced TNF- α levels in a dose-dependent manner (Figure 2). Among the concentrations we tested, 10 $\mu\text{g}/\text{ml}$ of HSC70 was the most effective, reducing TNF- α levels comparable to those in endotoxin-preconditioned cells.

3.2. HSC70 preconditioning preserves cardiac function during subsequent endotoxemia

We next examined whether in vivo preconditioning with HSC70 induces cardiac functional tolerance to endotoxin. We have previously used the Langendorff method to demonstrate that low-dose endotoxin (0.5 mg/kg) causes depression of cardiac intrinsic contractility (1,2). In the present study, we characterized endotoxemic cardiac dysfunction in intact animals using a microcatheter. Six hours after endotoxin injection, mice exhibited decreased left ventricular contractility compared to saline-treated controls (Table 1). LVDP was 78.6 ± 3.8 mmHg in untreated controls and decreased to 56.1 ± 1.9 mmHg 6 h after endotoxin ($P < 0.05$ vs. untreated controls). Hearts preconditioned with endotoxin 24 h earlier were resistant to the myocardial depressive effects of subsequent endotoxin exposure (LVDP 74.6 ± 3.7 mmHg after 6 h endotoxin exposure, $P < 0.05$ vs. no preconditioning). Similarly, HSC70 preconditioning attenuated the myocardial dysfunction induced by endotoxin (72.6 ± 2.8 mmHg, $P < 0.05$ vs. no preconditioning). The preload-independent Emax significantly improved in mice preconditioned with endotoxin or HSC70 (Figure 3). Thus HSC70 preconditioning induces cardiac functional tolerance to endotoxin.

3.3. Preconditioning with HSC70 and endotoxin has differential effects on TNF- α levels

In a previous study we found that plasma and myocardial TNF- α levels peak 1 h after administration of endotoxin (1). In naïve mice challenged with endotoxin, TNF- α levels in plasma and heart tissue were 2100 ± 183 pg/ml and 48 ± 6.3 pg/mg (both $P < 0.05$ vs. untreated

controls), respectively, 1h after endotoxin challenge (Figure 4). Endotoxin preconditioning markedly reduced TNF- α levels in plasma and heart tissue (427 ± 50 pg/ml and 12 ± 1.8 pg/mg, respectively; 79.7% and 75.0% reduction; both $P < 0.05$ vs. endotoxin alone) resulting from subsequent endotoxin challenge. HSC70 preconditioning also reduced TNF- α levels in plasma and heart tissue after endotoxin challenge (1401 ± 150 pg/ml and 31 ± 5.2 pg/mg; respectively; 33.3% and 35.4% reduction; both $P < 0.05$ vs. endotoxin alone and vs. endotoxin preconditioning); however, the reduction in TNF- α levels was less pronounced compared to endotoxin preconditioning..

3.4. Preconditioning with HSC70 and endotoxin reduces ICAM-1 levels in heart tissue

Our previous studies show that endotoxin increases myocardial ICAM-1 levels (2,3). Naive hearts challenged with endotoxin exhibited high levels of ICAM-1 after 6 h endotoxin challenge (Figure 5). Both endotoxin preconditioning and HSC70 preconditioning significantly reduced ICAM-1 levels in heart tissue after endotoxin challenge (76.4% and 63.5% reductions, respectively, $P < 0.05$), with comparable reduction in ICAM-1 levels in the two groups.

3.5. Preconditioning with HSC70 and endotoxin attenuates myocardial NF- κ B DNA-binding activity without an effect on myocardial TLR4 level

Our previous study found myocardial NF- κ B DNA-binding activity peaks at 1 h after injection of endotoxin (13). We analyzed myocardial NF- κ B p65 DNA-binding activity at 1 h after endotoxin challenge. Endotoxin increased myocardial NF- κ B DNA-binding activity in hearts (Figure 6A). Preconditioning with endotoxin or HSC70 significantly reduced NF- κ B DNA-binding activity following subsequent endotoxin exposure (Figure 6A). To examine whether the attenuated pro-inflammatory signaling in preconditioned hearts is due to an alteration in TLR4 levels, we examined TLR4 levels in myocardial tissue homogenate. As shown in Figure 6B, myocardial TLR4 levels were unchanged after preconditioning with either endotoxin or HSC70.

4. Discussion

4.1. HSC70 has proinflammatory and endotoxin tolerance-inducing effects in macrophages

In this study, we found that recombinant HSC70 induced a dose-dependent increase in TNF- α level in TLR4-competent but not in TLR4-defective macrophages. Apparently, recombinant HSC70 activates TLR4 to elaborate an inflammatory response in macrophages. In a previous study (11), we addressed whether this effect of recombinant HSC70 is due to endotoxin contamination. We found that the effect of HSC70 on macrophage TNF- α response is not influenced by treatment with the endotoxin antagonist Polymyxin B. We also found that recombinant HSC70, but not a recombinant HSC70 fragment, induces myocardial expression of proinflammatory cytokines in a TLR4-dependent manner. Further, we demonstrated that recombinant HSC70 interacts with cell-surface TLR4 in macrophages. Together, these observations indicate that the HSC70 protein itself induces the TNF- α response in macrophages through TLR4.

Pretreatment of macrophages with HSC70 significantly reduced TNF- α levels after subsequent endotoxin exposure. Similarly, Aneja and colleagues (9) reported that HSP70, which is highly homologous to HSC70, induced endotoxin tolerance in a human macrophage cell line. Since either HSP70 or HSC70 can activate TLR4, it is likely that a shared sequence or domain of these two proteins pre-activates TLR4 to induce endotoxin tolerance. It should be noted that HSC70 preconditioning had a less robust effect on TNF- α levels than endotoxin pretreatment. Similarly, HSC70 is much less potent in the induction of TNF- α . HSC70 in a concentration of 10 μ g/ml increased TNF- α level to 1.11 ± 0.13 ng/ml in TLR4-competent cells while endotoxin

in a much lower concentration (0.2 µg/ml) induced a robust TNF- α response (3.12±0.17 ng/ml, Figure 2). It appears that pre-activation of TLR4 to a certain level is required to induce a tolerant state. A much higher concentration of HSC70 is needed to induce cellular tolerance to endotoxin due to its lower potency.

4.2. HSC70 induces cardiac functional tolerance to endotoxin

In vivo, endotoxin tolerance develops after repeated sublethal doses of endotoxin and is characterized by an attenuated response to subsequent endotoxin challenge. Endotoxin-tolerant animals have reduced pro-inflammatory cytokine production (14,15), pyrogenesis (16,17) and mortality (18,19) during endotoxemia. We previously reported that endotoxin tolerance in the heart can also develop following a single exposure to endotoxin (6,20).

Our results on macrophages show that HSC70 provokes an inflammatory response that is dependent on TLR4, and that preconditioning with HSC70 induces cellular tolerance to endotoxin manifested by a reduction in TNF- α levels following endotoxin challenge. In a previous study we determined that endotoxin preconditioning confers cardiac resistance to endotoxemic myocardial depression and that this protective effect is associated with increased expression of myocardial HSP70 (20). In addition, we have shown that liposomal delivery of HSP72 during isolated heart perfusion attenuates endotoxin-induced myocardial contractile dysfunction. (21) The findings of the current study support and expand upon these previous observations, in that, preconditioning intact animals with HSC70 also induced cardiac functional tolerance to endotoxemia, and this was associated with the reduction of TNF- α and ICAM-1 levels in heart tissue. Thus, HSC70 preconditioning and endotoxin preconditioning induce equivalent cardiac functional tolerance to subsequent endotoxemia and appear to do so by similar mechanisms.

4.3. HSC70 preconditioning has a differential effect on TNF- α and ICAM-1

Although endotoxin tolerance is a well-described phenomenon, the mechanisms underlying endotoxin tolerance are still incompletely understood. Several hypotheses have been formulated, including: 1) down-regulation of TLR4, 2) reduced production of pro-inflammatory factors, and 3) up-regulation of endogenous protective mechanisms. We earlier found that cardiac functional resistance to endotoxemia is associated with up-regulated expression of multiple cardiac proteins including antioxidant enzymes (6), I κ B- α (13) and HSP70 (20).

TNF- α is well known to mediate myocardial contractile dysfunction during endotoxemia; however, there is a temporal discordance between peak TNF- α levels and the development of contractile dysfunction suggesting that its role may be the induction of other downstream cardiac depressant factors (1,3). Among those is ICAM-1, which mediates cardiac dysfunction through leukocyte-dependent and -independent mechanisms (2,4). Particularly, ligation of ICAM-1 by endogenous agents including fibrinogen significantly depresses cardiac contractile depression (5). Our previous study found TNF- α links TLR4 activation to myocardial ICAM-1 expression and demonstrated that ICAM-1 plays a central role in endotoxemic cardiac dysfunction (2,3). In this study we investigated the effects of HSC70 and endotoxin preconditioning on TNF- α and ICAM-1 levels. We found that HSC70 and endotoxin preconditioning had different effects on TNF- α levels in vivo. Endotoxin preconditioning markedly reduced TNF- α levels in plasma and heart tissue during subsequent endotoxin challenge, whereas the effect of HSC70 preconditioning on TNF- α levels was moderate. By contrast, our in vitro experiments demonstrated that HSC70 preconditioning in macrophages led to a much greater reduction in TNF- α levels. Thus, the in vivo effect of HSC70 preconditioning on TNF- α levels differs from the in vitro effect. It is likely that macrophages

are more sensitive to HSC70 preconditioning than other TNF- α -producing cells in the myocardium.

Interestingly, HSC70 preconditioning and endotoxin preconditioning resulted in a comparable reduction in ICAM-1 levels in heart tissue after a subsequent endotoxin challenge. To determine whether preconditioning reduces ICAM-1 levels by altering myocardial TLR4 levels and signaling, we examined the effect of preconditioning myocardial TLR4 protein levels after 24 h and NF- κ B DNA-binding activity following subsequent endotoxin challenge. We found that preconditioning attenuates myocardial NF- κ B activation in response to endotoxin without an effect on myocardial TLR4 protein levels. Thus, preconditioning suppresses myocardial TLR4 signaling. It appears that attenuated NF- κ B activation and reduced TNF- α levels are responsible, at least in part, for lower ICAM-1 levels. However, the effect of HSC70 preconditioning on ICAM-1 levels can be only partly explained by suppression of TNF- α production. An effect downstream of TNF- α may also be involved in the marked reduction in ICAM-1 levels in HSC70-preconditioned mice. It should be noted that a TNF- α -independent mechanism for myocardial ICAM-1 expression during endotoxemia does exist. (22–25). Further studies are required to elucidate the mechanism by which HSC70 preconditioning modulates myocardial ICAM-1 expression.

5. Conclusion

This study demonstrates that HSC70 preconditioning induces cardiac functional tolerance to endotoxin in mice that is equivalent to that seen with endotoxin tolerance. HSC70 preconditioning has a significant impact on the TNF- α response to endotoxin in macrophages *in vitro*. However, its *in vivo* effect on cardiac function is associated with only a moderate reduction of TNF- α levels but a significant down-regulation of ICAM-1 levels in heart tissue. In addition, HSC70 preconditioning attenuates endotoxin-induced myocardial NF- κ B activation. Thus, the mechanism by which HSC70 preserves cardiac function involves the suppression of TLR4 signaling, and production of TNF- α and ICAM-1. Since ICAM-1 has a central role in endotoxemic myocardial dysfunction, the cardiac tolerance may be primarily due to down-regulation of ICAM-1 levels in heart tissue during subsequent endotoxemia.

Acknowledgments

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Figure 1. HSC70 induces the TNF- α response in macrophages through TLR4

Peritoneal macrophages isolated from C3H/HeN (TLR4-competent) and C3H/HeJ (TLR4-defective) mice were treated with recombinant HSC70 (2.5–10 μ g/ml) for 24 h. HSC70 dose-dependently increased TNF- α levels in TLR4-competent cells but had no effect on TLR4-defective cells. Data are expressed as mean $\pm$ SEM. $n = 5$ in each group; * $P < 0.05$ vs. baseline.

Figure 2. HSC70 preconditioning suppresses endotoxin-induced TNF- α response in macrophages Peritoneal macrophages isolated from C3H/HeN (TLR4-competent) mice were preconditioned with endotoxin (LPS, 200 ng/ml) or recombinant HSC70 (2.5–10 μ g/ml) for 24 h. Cells were then challenged with endotoxin (200 ng/ml) for 18 h. Endotoxin stimulation induced TNF- α response in control cells. Preconditioning with endotoxin or HSC70 reduced TNF- α levels after endotoxin challenge, with HSC70 preconditioning less potent than endotoxin preconditioning. Data are expressed as mean $\pm$ SEM. $n = 5$ in each group; * $P < 0.05$ vs. control.

Figure 3. HSC70 preconditioning induces cardiac tolerance to endotoxin

C3H/HeN (TLR4-competent) mice were preconditioned with endotoxin (0.5 mg/kg, iv) or recombinant HSC70 (0.4 μg/g body weight, iv) for 24 h, then challenged with endotoxin (0.5 mg/kg, iv) for 6 h. Without preconditioning, endotoxin challenge resulted in cardiac dysfunction (loop shift and reduction of Emax). Preconditioning with endotoxin or HSC70 significantly improved cardiac function. Data are expressed as mean ± SEM. n = 6 in each group; * $P < 0.05$ vs. untreated control; # $P < 0.05$ vs. LPS treatment alone.

A **B**

Figure 4. HSC70 preconditioning moderately reduces TNF- α levels during endotoxemia
 C3H/HeN (TLR4-competent) mice were preconditioned with endotoxin (LPS, 0.5 mg/kg, iv) or recombinant HSC70 (0.4 μ g/g body weight, iv) for 24 h, then challenged with endotoxin (0.5 mg/kg, iv) for 1 h. Without preconditioning, endotoxin challenge resulted in increased TNF- α levels in plasma (A) and heart tissue (B). Preconditioning with endotoxin significantly reduced TNF- α levels in both plasma and myocardium after endotoxin challenge, but HSC70 preconditioning had less effect. Data are expressed as mean $\pm$ SEM. n = 6 in each group; * P <0.05 vs. endotoxin challenge without preconditioning, # P <0.05 vs. endotoxin preconditioning.

Figure 5. HSC70 preconditioning reduces ICAM-1 levels in heart tissue during endotoxemia
 C3H/HeN (TLR4-competent) mice were preconditioned with endotoxin (LPS, 0.5 mg/kg, iv) or recombinant HSC70 (0.4 μ g/g body weight, iv) for 24 h, then challenged with endotoxin (0.5 mg/kg, iv) for 6 h. Without preconditioning, endotoxin stimulation increased ICAM-1 levels in heart tissue. Preconditioning with endotoxin or HSC70 reduced ICAM-1 levels after endotoxin challenge, and the effects on the two groups were comparable. Data are expressed as mean $\pm$ SEM. $n = 5$ in each group; * $P < 0.05$ vs. endotoxin challenge without preconditioning.

A **B**

Figure 6. HSC70 preconditioning attenuates myocardial NF-κB activation

A. C3H/HeN (TLR4-competent) mice were preconditioned with endotoxin (LPS, 0.5 mg/kg, iv) or recombinant HSC70 (0.4 μg/g body weight, iv) for 24 h, then challenged with endotoxin (0.5 mg/kg, iv) for 1 h. Preconditioning with endotoxin or HSC70 reduced myocardial NF-κB DNA-binding activity after endotoxin challenge. Data are expressed as mean ± SEM. n = 5 in each group; * $P < 0.05$ vs. endotoxin challenge without preconditioning. **B.** A representative immunoblot show that treatment with endotoxin or HSC70 for 24 h had no effect on myocardial TLR4 level.

Table 1

Cardiac functional parameters

	Control	Endotoxin challenge	Endotoxin preconditioning +endotoxin challenge	HSC70 preconditioning +endotoxin challenge
HR (BPM)	481±19.5	550±23.5	512±38.2	531±29.2
Developed pressure (mmHg)	78.6±3.8	56.1±1.9 [*]	74.6±3.7 [†]	72.6±2.8 [†]
Stroke volume (μl)	21.5±2.2	13.7±1.8 [*]	17.9±1.4 ^{*†}	17.2±1.1 ^{*†}
Ejection fraction (%)	50.0±5.0	35.2±4.6 [*]	43.0±4.4 ^{*†}	41.0±3.2 ^{*†}
Cardiac output (ml/min)	10.4±1.2	7.51±1.2 [*]	9.22±1.4 [†]	9.31±0.8 [†]
dP/dt max (mmHg/sec)	6222±575	3206±209 [*]	6554±562 [†]	6220±389 [†]
dP/dt min (mmHg/sec)	-6095±379	3538±350 [*]	-6378±459 [†]	-5888±681 [†]

Mean ± SEM; n = 6 in each group;

^{*} P<0.05 vs. control;[†] P<0.05 vs. endotoxin challenge