

Human Myocardium Releases Heat Shock Protein 27 (HSP27) after Global Ischemia: The Proinflammatory Effect of Extracellular HSP27 through Toll-like Receptor (TLR)-2 and TLR4

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The myocardial inflammatory response contributes to cardiac functional injury associated with heart surgery obligating global ischemia/reperfusion (I/R). Toll-like receptors (TLRs) play an important role in the mechanism underlying myocardial I/R injury. The aim of this study was to examine the release of small constitutive heat shock proteins (HSPs) from human and mouse myocardium after global ischemia and examine the role of extracellular small HSP in myocardial injury. HSP27 release was assessed by enzyme-linked immunosorbent assay. Anti-HSP27 was applied to evaluate the role of extracellular HSP27 in the postischemic inflammatory response and functional injury in mouse hearts. Isolated hearts and cultured coronary vascular endothelial cells were exposed to recombinant HSP27 to determine its effect on proinflammatory signaling and production of proinflammatory mediators. HSP27 levels were markedly elevated in coronary sinus blood of patients and in coronary effluent of mouse hearts after global ischemia. Neutralizing extracellular HSP27 suppressed myocardial nuclear factor (NF)- κ B activation and interleukin (IL)-6 production and improved cardiac function in mouse hearts. Perfusion of HSP27 to mouse hearts induced NF- κ B activation and IL-6 production and depressed contractility. Further, recombinant HSP27 induced NF- κ B phosphorylation and upregulated monocyte chemoattractant protein (MCP)-1 and intercellular adhesion molecule (ICAM)-1 production in both human and mouse coronary vascular endothelial cells. TLR2 knockout (KO) or TLR4 mutation abolished NF- κ B phosphorylation and reduced MCP-1 and ICAM-1 production induced by extracellular HSP27 in endothelial cells. In conclusion, these results show that the myocardium releases HSP27 after global ischemia and that extracellular HSP27 is proinflammatory and contributes to the inflammatory mechanism of myocardial functional injury. Both TLR2 and TLR4 are involved in mediating the proinflammatory effect of extracellular HSP27.

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INTRODUCTION

Global myocardial ischemia/reperfusion (I/R) associated with cardiac surgery induces the cardiac inflammatory response with overexpression of proinflammatory genes (1). Several studies have found that myocardial nuclear factor (NF)- κ B is activated in humans during cardiac sur-

gery (2) and that myocardial proinflammatory cytokine levels are increased after cardiac surgery obligating global myocardial I/R (3,4). Dysregulated inflammatory response has a significant impact on clinical outcome in patients undergoing heart surgery (5). In animal models of global myocardial I/R, myo-

cardial inflammatory response contributes to cardiac dysfunction (6,7).

Several toll-like receptors (TLRs), particularly TLR2 and TLR4, play important roles in mediating myocardial inflammatory and injurious responses to I/R (8–15). Modulation of TLR signaling has been shown to improve cardiac function after myocardial I/R (6,16,17). Thus, these innate immunoreceptors have been proposed to be potential targets in cardiac dysfunction caused by I/R (18). Moreover, TLR2 and TLR4 contribute to the mechanisms of hepatic, renal lung and cerebral I/R injury (19–22). Investigation of the mechanism by which TLRs are activated in the myocardium will be helpful for the development of novel therapeutic strategies for myocardial

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protection during cardiac surgery involving obligatory myocardial I/R. Several studies have identified high-mobility group box 1 (HMGB1) and large heat shock protein (HSP) as endogenous TLR4 activators in animal models of myocardial I/R (7,11,23,24).

The functions of intracellular HSPs are primarily transportation and stabilization of proteins. In addition, they assist in refolding denatured cellular proteins and thus confer protection against cellular stress (25). Large HSPs appear to have adverse effects when located in the extracellular space. In this regard, recombinant HSP60 and HSP70 are found to induce the production of cytokines in mononuclear cells (26). Purified large HSPs, such as HSP60, HSP70 and GP96, could signal through TLRs, particularly TLR4, to activate NF- κ B in mononuclear cells and induce dendritic cell maturation (26,27). These findings suggest that extracellular HSPs could be potential factors involved in tissue inflammatory response after they are released from cells. Interestingly, cardiac surgery with obligatory global I/R has been reported to cause the release of HSP70 (28). Our previous studies found that mouse hearts release a constitutive 70-kDa HSP (heat shock cognate protein 70, HSC70) during early reperfusion after global ischemia and that extracellular HSC70 plays a role in myocardial cytokine expression after global I/R (7). In addition, we found that recombinant HSC70 induces cytokine production in the myocardium and macrophages in a TLR4-dependent manner (29,30).

HSP27 is a member of a small HSP family and is constitutively expressed in cells. In rodents, this small HSP is 25 kDa in molecular size and is hence termed HSP25 (31). The main functions of HSP27, as a molecular chaperone, include facilitation of the refolding of partially denatured proteins into active conformations, modulation of F-actin and cell movement, and anti-apoptosis (31,32). Several studies demonstrate that HSP27 has a protective role in the cardiovascular system. In this regard, overex-

pression of HSP27 protects cardiac myocytes against ischemic injury (33). However, HSPs exert a protective effect when they are located in the cell, and they may be detrimental when located in the extracellular spaces. Cardiac microvascular endothelial cells interact with molecules in the interstitial spaces and in the circulation and play a critical role in the overall myocardial inflammatory response to injury. Currently, it remains unknown whether the heart releases small constitutive HSP during global I/R and whether extracellular small HSP plays a role in mediating myocardial inflammatory response and injury.

This study tested the hypothesis that the myocardium releases HSP27 after global ischemia and that extracellular HSP27 mediates myocardial inflammatory response and cardiac functional injury through TLRs. The goals of this study were to determine (a) whether human and mouse myocardium releases HSP27 after global ischemia, (b) the role of extracellular HSP27 in postischemic myocardial inflammatory response and injury and (c) the effect of HSP27 on the inflammatory response and proinflammatory signaling in cardiac vascular endothelial cells.

MATERIALS AND METHODS

Patients, Surgical Procedures and Sample Collection

Fifteen patients who had elective cardiac surgery (Coronary Artery Bypass Graft Surgery, mitral or aortic valve surgery or combined coronary artery and heart valve surgery) at the University of Colorado Hospital or the Denver VA Medical Center were included in this study. All patients gave consent for blood sampling. This study was approved by the Colorado Multiple Institutional Review Board and conforms to the principles outlined in the Declaration of Helsinki.

Heparin (3.0 mg/kg body weight) was given centrally before cannulation of the aorta. Core body temperature was reduced to 32°C, and a nonpulsatile

flow of 2.4 L/min per meter² was maintained. Myocardial protection was delivered via antegrade and retrograde cold blood cardioplegia (10°C). Myocardial temperature was monitored with a septal myocardial temperature probe and maintained at 10°C.

Blood samples were taken from the coronary sinus cannula before initiating cardiopulmonary bypass (baseline) and 5 and 20 min after removal of aortic cross-clamp (reperfusion). Blood was centrifuged (704g/min for 15 min), and plasma was collected for analysis of HSP27.

Animals

Male TLR2 knockout (KO), C57BL/6 (wild-type control for TLR2 KO) and C3H/HeJ (TLR4-defective) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), and male C3H/HeN (TLR4-competent) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were maintained on a standard pellet diet. Their body weight was 22–26 g when used for the experiments. 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, 7th ed., 1996) (34).

Chemicals and Reagents

Recombinant HSP27 and HSP25 (low-endotoxin preparations) were purchased from Assay Design (Ann Arbor, MI, USA). These preparations contain endotoxin, <2.0 pg/ μ g protein, as measured by Limulus assay. The highest concentration of recombinant HSP25 used in the isolated heart experiments was 2.0 μ g/mL, and the highest concentration of recombinant HSP27 used in the cell culture experiments was 4.0 μ g/mL. Thus, endotoxin concentrations in all experiments were <8.0 pg/mL.

Monoclonal antibodies against phospho-NF- κ B p65 and total NF- κ B p65 were purchased from Cell Signaling

(Danvers, MA, USA). Polyclonal anti-human intercellular adhesion molecule (ICAM)-1 and monoclonal anti-mouse ICAM-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cytokine enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, USA). A human HSP27 ELISA kit was purchased from Assay Design. Other reagents were purchased from Sigma (St. Louis, MO, USA).

Isolated Heart Perfusion

Mice were anesthetized and anticoagulated with pentobarbital (60 mg/kg; Abbot Laboratories, North Chicago, IL, USA) and heparin (1,000 units/kg; Elkins-Sinn, Cherry Hill, NJ, USA) before heart isolation. Isolated hearts were perfused by the Langendorff technique as previously described (6,7). Perfused hearts were equilibrated for 20 min before experiments. In HSP27 perfusion experiments, hearts were perfused with recombinant HSP25 (0–2.0 µg/mL in coronary circulation) for 30 min, followed by a 70-min washout. In global myocardial I/R experiments, hearts were subjected to 20 min of normothermic global ischemia at 37°C, followed by 30–120 min of reperfusion. For anti-HSP27/25 experiments, hearts were perfused with anti-HSP27/HSP25 (1.0 µg/mL in coronary circulation) or isotype-matching nonimmune IgG (1.0 µg/mL in coronary circulation) for 10 min before ischemia and for 20 min after initiation of reperfusion.

An ultrathin latex balloon was inserted into the left ventricle, and balloon volume was adjusted to achieve left ventricular end-diastolic pressure of 8–12 mmHg during the initial equilibration. Pacing wires were fixed to the right atrium, and all hearts were paced at 450 beats/min. Left ventricular developed pressure was continuously recorded with a computerized pressure amplifier/digitizer.

Myocardial tissue was collected at the end of the experiment to assess NF-κB DNA-binding activity and cytokine levels.

Culture of Human Coronary Vascular Endothelial Cells

Human coronary vascular endothelial cells (HCVECs) were purchased from Lonza (Walkersville, MD, USA) and grown in EGM-2MV medium. Cells of passages 4 and 6 were used, and all experiments were performed by using cultures of 90% confluence.

Isolation and Culture of Mouse Coronary Vascular Endothelial Cells

Mouse coronary vascular endothelial cells (MCVECs) were isolated using a previously reported method (35). Briefly, beating hearts were immersed in ice-cold calcium-free phosphate-buffered saline (PBS) and dipped into 70% ethanol to devitalize epicardial mesothelial cells and endocardial endothelial cells. Ventricular tissue was minced into fine pieces and digested in nominally calcium-free Hanks balanced salt solution supplemented with collagenase II (1.0 mg/mL), glucose (2.0 mg/mL), taurine (2.5 mg/mL), bovine serum albumin (BSA, 0.1%) and MgCl₂ (1.4 mmol/L). Then, the tissue was digested in a solution containing 0.125% trypsin, 0.1 mmol/L EDTA (ethylenediaminetetraacetic acid) and 2.0 mg/mL glucose dissolved in Hanks balanced salt solution. Cells were separated from tissue debris and remaining myocytes by spinning at 20g for 5 min. The supernatant was centrifuged at 113g (4°C) for 8 min to collect endothelial cells. Cells from three to four hearts were pooled and resuspended in 10 mL Dulbecco modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum, penicillin (50 U/mL) and streptomycin (50 mg/mL). Cells were seeded in 24-well plates and cultured at 37°C for 2 h. Nonattached cells were removed. Experiments were performed by using cultures of 90% confluence.

Immunoblotting

Cells were lysed with M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA). Cell lysate was mixed with an equal vol-

ume of 2× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) supplemented with 5% (vol/vol) β-mercaptoethanol. Crude proteins were separated by 4–20% polyacrylamide gel and transferred onto nitrocellulose membrane (Bio-Rad). The membrane was incubated in PBS containing 5% nonfat dry milk, and then incubated overnight with a primary antibody diluted with PBS containing 0.1% Tween 20 and 5% nonfat dry milk. After thorough washes, the membrane was treated with peroxidase-labeled secondary antibody (1:5,000 dilution with PBS containing 0.1% Tween 20 and 5% dry milk) for 60 min. Protein bands were developed by using the enhanced chemiluminescence (ECL) substrate, and densitometric analysis was performed by using ImageJ software.

ELISA

HSP27 in plasma, and cytokines in myocardial homogenate and in culture media, were analyzed using ELISA kits. Recombinant proteins were used to construct standard curves. Absorbance of standards and samples was determined spectrophotometrically at 450 nm by using a microplate reader (Bio-Rad). Results were plotted against the standard curve. Plasma HSC70, and HSC70 and HSP25 in coronary effluent, were analyzed by custom ELISA as previously reported (29).

Statistical Analysis

Data are expressed as mean ± standard error (SE). Comparisons between groups were performed by using StatView software (Abacus Concepts, Calabasas, CA, USA) with one-way analysis of variance with the *post hoc* Fisher test. A difference was considered significant at $P \leq 0.05$. Significant differences were confirmed with the Mann-Whitney nonparametric test.

RESULTS

Human and Mouse Myocardium Releases HSP27 after Global Ischemia

We examined HSP release from the myocardium. In patients, levels of HSP27

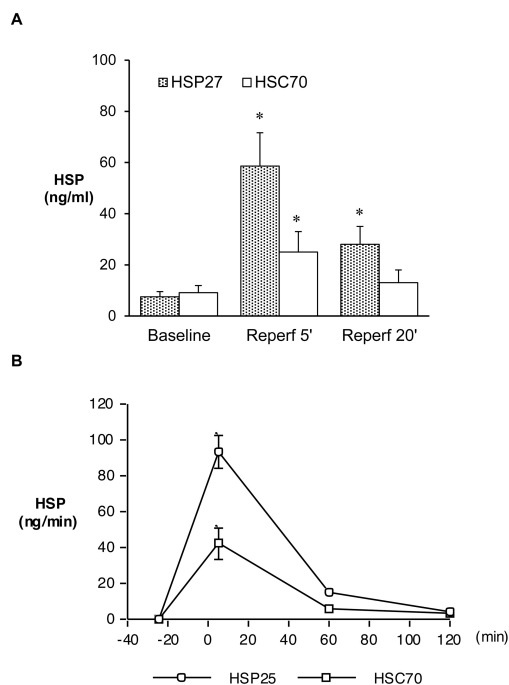


Figure 1. Human and mouse hearts release HSP27/HSP25. (A) Coronary sinus blood is collected from patients before aortic clamping (baseline) and after de-clamping (reperfusion). Heat shock protein 27 (HSP27) and heat shock cognate protein 70 (HSC70) are analyzed by ELISA. Higher levels of HSP27 in coronary sinus blood are evident during reperfusion. Data are presented as mean \pm SE. $n = 15$; $*P < 0.05$ versus baseline. (B) Hearts from mice are subjected to global ischemia and reperfusion (I/R; 120 min/R 120 min). Coronary effluent is collected before ischemia and at 0–5, 55–60 or 115–120 min of reperfusion, and HSP25 and HSC70 are analyzed. Hearts release higher levels of HSP25 in early reperfusion. Data are mean \pm SE. $n = 8$; $*P < 0.05$ versus baseline.

and HSC70 increased in coronary sinus blood after de-clamping. HSP27 levels are higher during the first 5 min after aortic cross-clamp release (Figure 1). Similarly, mouse hearts release HSP25 (rodent form of HSP27) and HSC70 into coronary effluent immediately after ischemia, and significantly greater HSP25 release than HSC70 release is found (Figure 1). These data demonstrate that I/R causes myocardial release of constitutive HSPs and that the heart releases more HSP27/HSP25.

Extracellular HSP27 Mediates Myocardial Inflammatory Response and Cardiac Dysfunction

To determine the role of extracellular HSP27 in the inflammatory response to I/R in mouse hearts, we assessed the effect of polyclonal antibodies against HSP27/HSP25 on myocardial NF- κ B ac-

tivity and cytokine production. As shown in Figure 2, anti-HSP27/HSP25 reduced myocardial NF- κ B activity and the production of interleukin (IL)-6 after I/R. Similarly, anti-HSP27/HSP25 improved cardiac functional recovery from I/R (Figure 2). These results support the hypothesis that extracellular HSP27 plays a role in mediating the inflammatory response to I/R and contributes to the mechanism of myocardial injury caused by global I/R.

To further determine the impact of extracellular HSP25 on myocardial cytokine production and cardiac function, we perfused recombinant HSP25 to isolated mouse hearts. Data in Figure 3 show that HSP25 induces myocardial NF- κ B activation and IL-6 production in a dose-dependent fashion. Recombinant HSP25 also depresses cardiac function. Thus, extracellular HSP25 is sufficient to

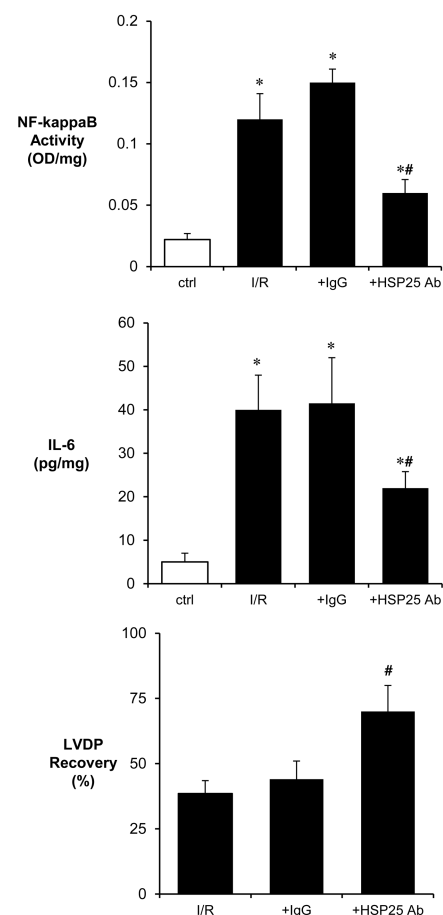


Figure 2. Extracellular HSP25 mediates postischemic inflammatory response and functional injury in mouse hearts. Mouse hearts are subjected to global ischemia and reperfusion (I/R; 120 min/R 30 or 120 min). Hearts are treated with an antibody against HSP27/HSP25 (HSP25 antibody (Ab), 1.0 μ g/mL) or control IgG (1.0 μ g/mL) for 10 min before ischemia and for 20 min after initiation of reperfusion. HSP25 antibody reduces myocardial NF- κ B activity and IL-6 levels and improves cardiac functional recovery. Data are mean \pm SE. $n = 8$; $*P < 0.05$ versus perfusion control (ctrl); $^{\#}P < 0.05$ versus I/R and I/R + IgG. OD, optical density.

induce myocardial inflammatory response and cardiac functional injury.

Because IL-6 secretion is sensitive to a very low level of endotoxin (36), we determined whether the trace amount of endotoxin in the recombinant HSP25 preparation has a proinflammatory effect on mouse hearts. We denatured the re-

combinant protein by heating the stock solution at 80°C for 30 min before use and examined whether the denatured HSP25 preparation has any effect on IL-6 secretion. The results in Figure 3 show that heat-denatured recombinant HSP25 preparation failed to induce IL-6 production in mouse hearts.

Extracellular HSP27 Induces Inflammatory Responses in HCVECs

To examine whether extracellular HSP27 induces the inflammatory response in HCVECs, we treated cells with recombinant human HSP27 and analyzed cellular ICAM-1 levels. Figure 4A shows that cells treated with HSP27, in a concentration of either 2.0 or 4.0 µg/mL, had higher ICAM-1 levels compared with untreated cells. Normalized ICAM-1 band density increased 2.6- and 3.9-fold ($P < 0.01$), respectively, after treatment with 2.0 and 4.0 µg/mL HSP27. Thus, extracellular HSP27 is potent to induce ICAM-1 in HCVECs.

We analyzed the levels of monocyte chemoattractant protein-1 (MCP-1), IL-6, IL-8 and tumor necrosis factors (TNF)-α in the culture medium after treatment of HCVECs with HSP27 for 24 h. Whereas HSP27 did not affect TNF-α levels, it increased the levels of MCP-1, IL-6 and IL-8 in a dose-dependent manner (Figure 4B). To exclude the possibility that the trace amount of endotoxin in the recombinant HSP27 preparation contributes to the inflammatory response, we performed experiments by using denatured HSP27 preparation. Heat-denatured recombinant HSP27 preparation failed to induce IL-6 secretion in HCVECs (Figure 4B). Thus, extracellular HSP27 per se is proinflammatory to HCVECs and has a differential effect on the release of proinflammatory cytokines.

Both TLR2 and TLR4 Mediate the Proinflammatory Effect of Extracellular HSP27 in MCVECs

We examined MCP-1 and ICAM-1 responses of WT, TLR2 KO and TLR4-defective MCVECs to extracellular HSP27. Compared to WT cells, either

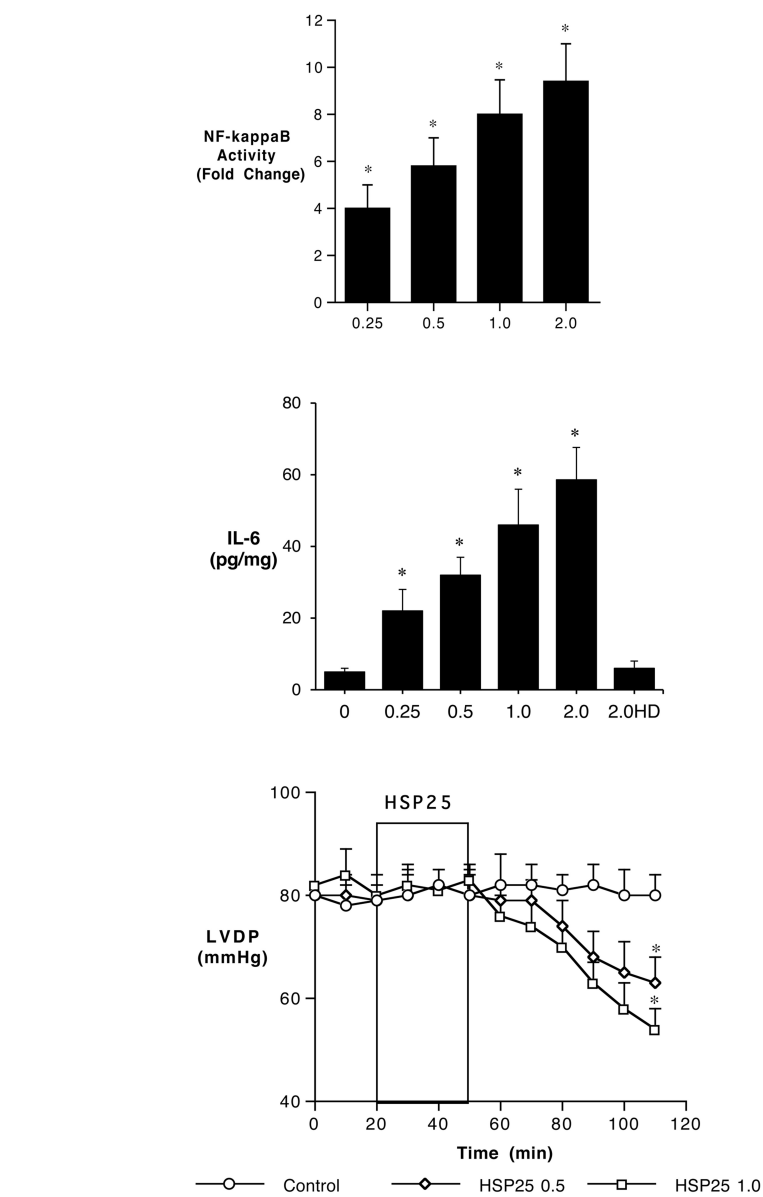


Figure 3. Recombinant HSP25 induces myocardial NF-κB activation and IL-6 production and depresses cardiac function. Hearts from WT mice are perfused with recombinant heat HSP25 (0.25–2.0 µg/mL) for 30 min followed by a 70-min washout. Recombinant HSP25 increases myocardial NF-κB activity (expressed as fold-change above buffer perfusion control) and IL-6 levels and depresses cardiac contractility in a dose-dependent manner. Heat-denatured HSP25 at 2.0 µg/mL (2.0HD) fails to induce IL-6 production. Data are mean ± SE. n = 6; * $P < 0.05$ versus buffer perfusion control.

TLR2 KO cells or TLR4-defective cells exhibited markedly attenuated MCP-1 and ICAM-1 responses to extracellular HSP27 (Figure 5). TLR2 KO or TLR4 mutation abolished the increase in MCP-1 levels at 4 h and markedly attenuated the increase at 24 h (Figure 5). ICAM-1 levels in-

creased by 3.3- and 6.4-fold, respectively, in C57BL/6 (WT control for TLR2 KO) cells and C3H/HeN (WT control for TLR4 mutant) cells after stimulation with 4.0 µg/mL HSP27 for 24 h. However, the same treatment resulted in much smaller increases (1.8- and 2.2-fold, respectively)

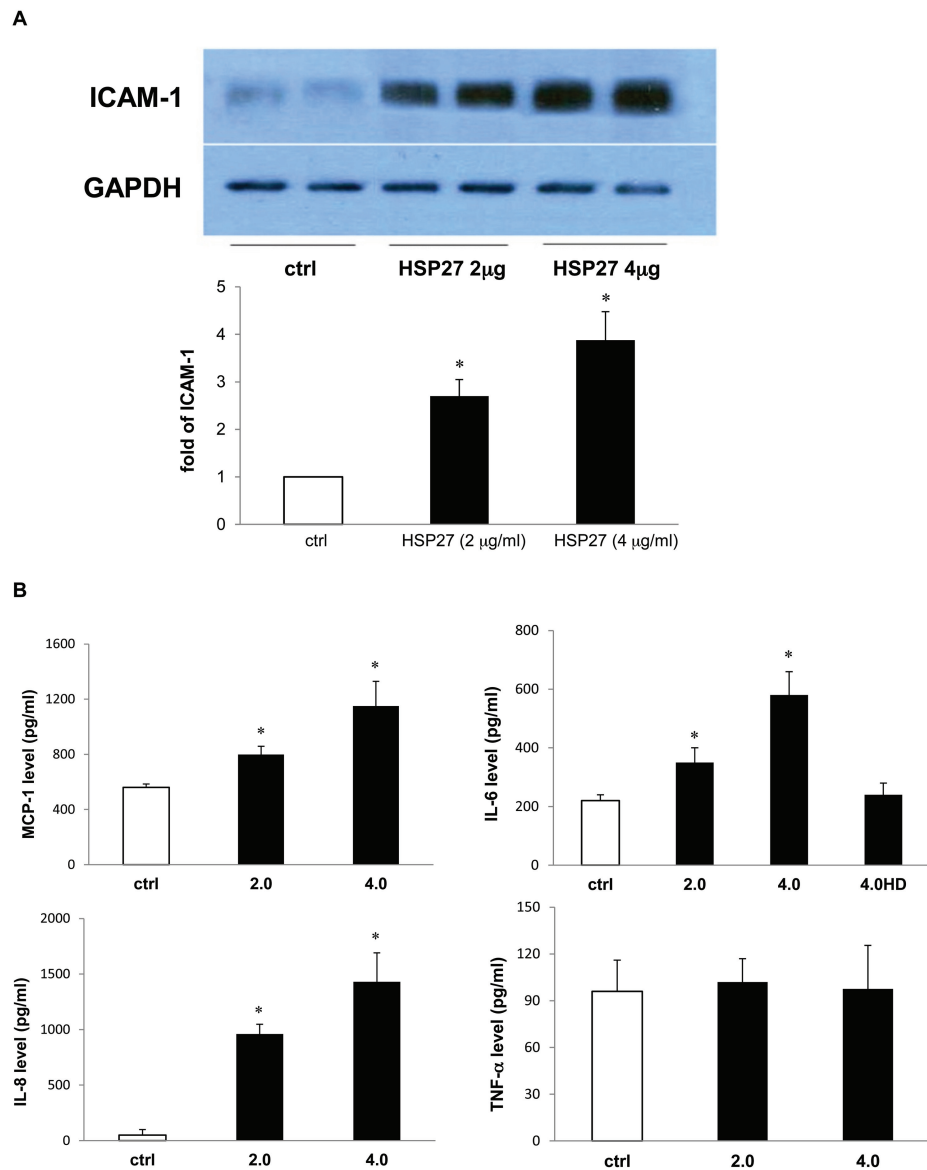


Figure 4. Extracellular HSP27 induces the expression of ICAM-1, MCP-1, IL-6 and IL-8 in HCVECs. HCVECs are treated with recombinant HSP27 (0, 2.0 or 4.0 $\mu\text{g}/\text{mL}$). The culture medium collected at 24 h is analyzed for cytokines by ELISA. ICAM-1 levels in cell lysate are analyzed by immunoblotting. (A) A representative blot and normalized band density data show that ICAM-1 levels increase in a dose-dependent manner. (B) Extracellular HSP27 induces the secretion of MCP-1, IL-6 and IL-8, but not TNF- α . Heat-denatured HSP27 at 4.0 $\mu\text{g}/\text{mL}$ (4.0HD) fails to induce IL-6 secretion. Data are mean \pm SE. $n = 5$; * $P < 0.05$ versus untreated control (ctrl).

in TLR2 KO cells and TLR4-defective cells (Figure 5). These results show that both TLR2 and TLR4 play an important role in mediating the inflammatory responses of cardiac vascular endothelial cells to extracellular HSP27.

TLR2 and TLR4 Play a Major Role in NF- κB Activation Induced by Extracellular HSP27

The NF- κB pathway is involved in the inflammatory responses in coronary vascular endothelial cells (37). We examined

the effect of extracellular HSP27 on phosphorylation of NF- κB p65 and determined whether its effect is reduced by TLR2 KO or TLR4 mutation.

Treatment of WT MCVECs with 4.0 $\mu\text{g}/\text{mL}$ HSP27 induced rapid phosphorylation of NF- κB p65 in WT cells (Figure 6). However, extracellular HSP27 failed to induce NF- κB p65 phosphorylation in either TLR2 KO cells or TLR4-defective cells. It appears that activation of NF- κB by extracellular HSP27 requires a coordination of these two innate immune receptors.

DISCUSSION

The results of this study demonstrate (a) human and mouse myocardium releases HSP27 after global ischemia, (b) extracellular HSP27 is involved in the mechanism of postischemic myocardial inflammatory responses and cardiac functional injury, (c) extracellular HSP27 induces the production of proinflammatory mediators in mouse hearts and in human and mouse coronary vascular endothelial cells, (d) both TLR2 and TLR4 are involved in mediating the proinflammatory effect of extracellular HSP27 and (e) NF- κB appears to play a major role in the inflammatory responses to extracellular HSP27.

It has been reported that cardiac surgery with obligatory global I/R induces the release of HSP70, a 70-kDa inducible form of HSP (28). Several studies found that recombinant HSP70 induces the production of cytokines in mononuclear cells (26). In addition, HSPs of 60- and 70-kDa have been found to induce myocardial production of proinflammatory mediators (11,24). Particularly, we observed that the myocardium releases a constitutive form of HSP70 (that is, HSC70) and that HSC70 is potent and induces myocardial inflammatory response (7,29). In this study, we observed that the levels a smaller constitutive HSP, namely HSP27 (termed HSP25 in rodents), in coronary sinus blood are markedly elevated in patients after cardiac surgery with cardiopulmonary bypass. In addition, we found that HSP25 is released into the coronary effluent during

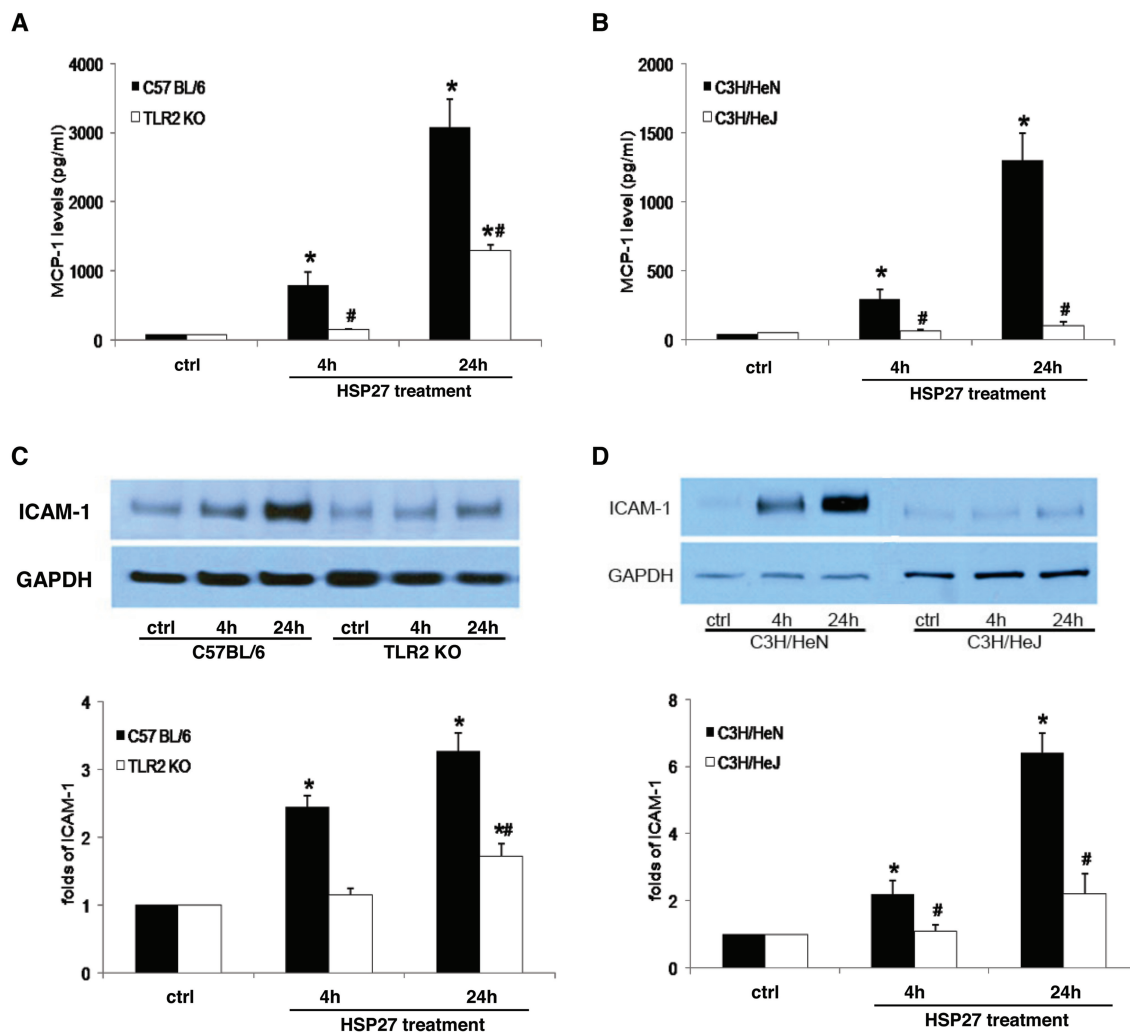


Figure 5. Induction of the inflammatory response by extracellular HSP27 requires both TLR2 and TLR4. MCVECs from TLR2 KO, TLR4-defective (C3H/HeJ) and WT mice are treated with 4.0 μ g/mL HSP27. (A, B) Either TLR2 KO or TLR4 mutation reduces MCP-1 secretion induced by extracellular HSP27. (C, D) ICAM-1 expression induced by extracellular HSP27 is reduced by TLR2 KO and TLR4 mutation. Results are presented as mean \pm SE. n = 5; *P < 0.05 versus untreated control (ctrl); #P < 0.05 versus WT (C57 BL/6 or C3H/HeN).

global I/R in mouse hearts. In contrast, no HSP70 release is observed (not shown). The HSP27 release from isolated, buffer-perfused hearts, which exclude any contribution from other organs or blood cells, indicates that myocardial I/R causes the release of HSP27 by cardiac cells. Therefore, HSP27 escapes from cardiac cells during I/R. It has been reported that stress induces active release of HSPs by cells (38). It remains unclear from the present study whether myocardial release of HSP27 is due to active secretion or passive leak. However, global I/R is stressful to the heart and could cause myocardial

injury. It is likely that both mechanisms contribute to HSP27 release from the heart during I/R.

Our results show that the heart releases constitutive HSPs in response to I/R and that extracellular HSP27 could be a factor involved in the induction of myocardial inflammatory response after global I/R. We found that HSP27/25-specific antibody improves cardiac functional recovery after global I/R and reduces production of proinflammatory cytokine IL-6 in mouse hearts. In testing whether extracellular HSP27 is sufficient to induce myocardial inflammatory re-

sponse and injury, we found that recombinant HSP25 depresses cardiac contractility and increases the expression of IL-6. Therefore, extracellular HSP27 is sufficient to induce myocardial inflammatory response and to depress cardiac contractility, and this small HSP, when being released into the extracellular spaces, plays a role in mediating postischemic myocardial inflammatory response and cardiac dysfunction. Further, the results indicate that myocardial inflammatory response induced by HSP25 mediates cardiac dysfunction, since the cardiac contractility starts to decline

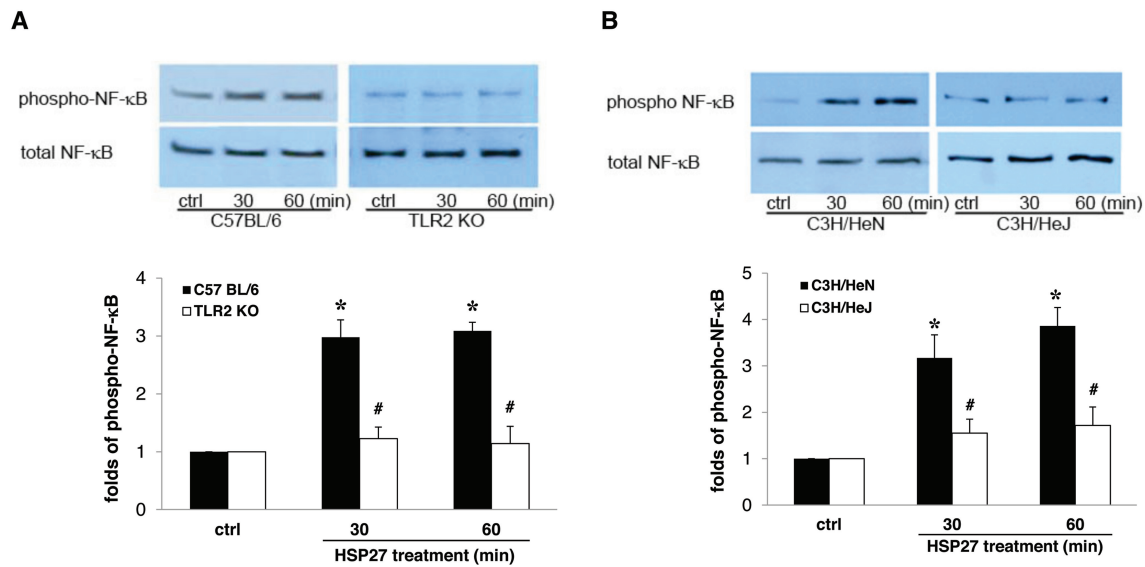


Figure 6. TLR2 KO and TLR4 mutation abrogates extracellular HSP27-induced NF-κB phosphorylation. MCVECs from TLR2 KO, TLR4-defective (C3H/HeJ) and WT mice were treated with 4.0 μg/mL HSP27 for 30 or 60 min. (A, B) Either TLR2 KO or TLR4 mutation abrogated NF-κB phosphorylation induced by extracellular HSP27. Normalized band density is presented as mean ± SE. n = 5; *P < 0.05 versus untreated control (ctrl); #P < 0.05 versus WT (C57 BL/6 or C3H/HeN).

after washing out HSP25, and the decline becomes significant 60 min later. This time course correlates with myocardial production of proinflammatory mediators. In support of this notion, we observed in a previous study that myocardial inflammatory response has a mechanistic role in cardiac dysfunction caused by I/R (6).

In HCVECs, HSP27 induces the expression of adhesion molecule (ICAM-1), cytokine (IL-6) and chemokine (MCP-1 and IL-8) in a dose-dependent manner. The findings support the notion that HSP27 released from cardiac cells contributes to the mechanisms of myocardial inflammatory responses to I/R. Like most HSPs, HSP27 is a highly conserved molecular chaperone and plays an important role in stabilization and refolding of cellular proteins (39). Intracellular HSP27 appears to have a role in modulating the cellular inflammatory response. In this regard, HSP27 is found to be required for IL-1 and TNF-induced proinflammatory response, including expression of cyclooxygenase-2, IL-6 and IL-8 in fibroblasts (40). In skin keratinocytes, HSP27 is found to be associ-

ated with the IκB kinase complex after TNF-α stimulation and silencing HSP27 enhances NF-κB activation and IL-8 release (41). In addition, HSP27 has been found to have an antiinflammatory function in monocytes through upregulation of IL-10 expression (42). It appears that HSP27 has distinct functions in regulating the inflammatory response, depending on cell type and intracellular or extracellular location.

It is reported that recombinant HSP27 induces cytokine production in bone marrow-derived dendritic cell and that its effect is attenuated in cells from TLR4 mutant mice (43). This study suggests that TLR4 is one of the signaling pathways involved in mediating the proinflammatory effect of extracellular HSP27. We found that extracellular HSP27 induces inflammatory responses in MCVECs, and the proinflammatory effects of extracellular HSP27 are significantly reduced in TLR2 KO or TLR4-defective MCVECs. These findings indicate that extracellular HSP27 may activate both TLR2 and TLR4 to induce inflammatory responses in coronary vascular endothelial cells. Thus, extracellular

HSP27 appears to be an endogenous activator for TLR2 and TLR4.

A number of endogenous proteins function as danger-associated molecular patterns (DAMPs) to induce the inflammatory responses through interaction with TLR2 and/or TLR4 when they are located in the extracellular spaces. The list of DAMPs includes HMGB1 (44), HSP70 (26), HSP60 (24) and cold-inducible RNA-binding protein (45). The results of the present study demonstrate that the myocardium releases HSP27 during I/R and that extracellular HSP27 induces the inflammatory response in cardiac cells through TLR2 and TLR4. Thus, extracellular HSP27 functions as a DAMP. TLR2 and TLR4 have been proposed to be potential targets for myocardial protection during I/R (18). Our findings indicate that prevention of the release of HSP27 may suppress TLR2/4-mediated myocardial inflammatory responses and protect the myocardium against I/R injury.

The main consequence of TLR2 and TLR4 signaling is the activation of NF-κB that promotes the expression of cytokines, chemokines and adhesion mole-

cules (37,46). We found that extracellular HSP27 induces rapid NF- κ B activation in mouse hearts and WT MCVECs. However, extracellular HSP27 failed to induce NF- κ B p65 phosphorylation in either TLR2 KO cells or TLR4-defective cells. Therefore, activation of NF- κ B by extracellular HSP27 appears to require a coordination of TLR2 and TLR4. Importantly, abrogation of NF- κ B activation by TLR2 KO or TLR4 mutation correlates with markedly reduced levels of MCP-1 and ICAM-1, indicating that the TLR2/4-dependent NF- κ B activation plays an important role in the inflammatory responses to extracellular HSP27.

CONCLUSION

This study demonstrates that human myocardium releases HSP27 after global I/R associated with cardiac surgery and that extracellular HSP27 contributes to the mechanism underlying postischemic myocardial inflammatory response and cardiac functional injury. Further, extracellular HSP27 induces the inflammatory responses in human and mouse coronary vascular endothelial cells through a TLR2/4-dependent mechanism, and the NF- κ B pathway activated by a coordination of TLR2 and TLR4 appears to have a major role in the inflammatory responses to extracellular HSP27. This study provides novel insights into the mechanism of myocardial inflammatory responses to global I/R and offers a potential therapeutic target for suppression of myocardial inflammatory responses in patients undergoing cardiac surgery with obligatory global ischemia.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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