Extracellular Heat Shock Protein 70 Induces Cardiomyocyte Inflammation and Contractile Dysfunction via TLR2

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**Background:** Toll-like receptors (TLRs) are expressed on cardiomyocytes and recognize pathogen-associated molecular patterns. Whether endogenous molecules produced by tissue injury (damage associated molecular patterns, DAMPs) can induce cardiomyocyte inflammation via TLR signalling pathways and/or reduce cardiomyocyte contractility is unknown.

**Methods and Results:** Primary cardiomyocytes isolated from nuclear factor κ B (NFκB)-luciferase knock-in mice were used to assess NFκB signalling. DAMPs, HSP60, HSP70 and HMGB1, increased NFκB transcriptional activity compared to controls. HSP70 stood out compared to other DAMPs and even lipopolysaccharide (LPS). Subsequent experiments focused on HSP70. Cardiomyocytes exposed to HSP70 had a 58% decrease in contractility without a decrease in calcium flux. Exposure of cultured HL-1 cardiomyocytes to HSP70 resulted in increased expression of intercellular adhesion molecule 1 (ICAM-1), interleukin 6 (IL-6) and keratinocyte-derived chemokine (KC) compared to controls. Knock-out mice for TLR2, TLR4 and MyD88, plus background strain controls (C57BL/6) were used to assess induction of cardiomyocyte inflammation by HSP70. The cardiomyocyte expression of ICAM-1 induced by HSP70 was significantly reduced in TLR2 and MyD88 knock-out mice but not TLR4 knock-out mice; implicating the TLR2 signalling pathway. Furthermore, blocking antibodies to TLR2 were able to abrogate HSP70-induced contractile dysfunction and cell death.

**Conclusions:** Extracellular HSP70 acting via TLR2 and its obligate downstream adaptor molecule, MyD88, activate NFκB. This causes cardiomyocyte inflammation and decreased contractility. (Circ J 2011; 75: 2445–2452)

**Key Words:** Cell adhesion molecules; Contractility; DAMPs; HSP70; TLR2

Cardiomyocytes express innate immune toll-like receptors (TLRs), activation of a discrete set of these TLRs (TLR 2, 4, 5) on cardiomyocytes using exogenous pathogen-associated molecular patterns (PAMPs) such as proteoglycan for TLR2, lipopolysaccharide (LPS) for TLR4, and flagellin for TLR5, leads to an nuclear factor κ B (NFκB)-mediated cardiomyocyte inflammatory response and a concomitant decrease in contractility. Cardiomyocytes, unlike immune cells, are not routinely exposed to pathogens or PAMPs, but diverse stimuli leading to endogenous damage-associated molecular patterns (DAMPs) might be important mediators of acute cardiac dysfunction. DAMPs, like PAMPs, activate TLRs. DAMPs are rapidly upregulated by the host at the site of injury, have distinct intracellular and extracellular roles, and activate innate immune signalling. A variety of molecules have been classified as DAMPs including fibronectin, elastase, chromosomal DNA, HMGB1, and heat shock proteins. TLRs have been implicated in cardiac dysfunction following non-pathogen-related injury, such as ischemia-reperfusion, suggesting activation of TLR signalling by endogenous DAMPs. However, identification and understanding of endogenous DAMPs, which induce TLR signalling with functional consequences in cardiomyocytes, is incomplete.

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TLR-mediated cardiac dysfunction induced by PAMPs requires NFκB signalling. Accordingly, in transgenic mice with a NFκB-luciferase reporter gene expressed in cardiomyocytes, we measured responses to the DAMPs, HSP60, HSP70, and HMGB1. Among these DAMPs we found HSP70 to be the strongest inducer of NFκB activity; HSP70’s plateau effect was substantially greater than that of the “positive control” PAMP, E. coli LPS. Therefore, we tested for a direct effect of HSP70 on cardiomyocyte inflammatory and contractile function. In immune cells and endothelial cells, and endothelial cells, HSP70 is a ligand for TLRs. In the heart, TLR2 and TLR4 are most prominently involved following ischemia-reperfusion.
insults. Therefore, we focused on these TLRs in order to identify and understand the signalling pathways leading to the effect of HSP70 on cardiomyocytes.

**Methods**

All animal studies were approved by the University of British Columbia animal ethics committee and conformed to NIH guidelines.

**Cell Line**

HL-1 cells are an immortalized cell line with adult cardiac morphological, biochemical, and electrophysiological properties, including contraction and biochemical response to cognate ligands. The cell line was kindly provided by Dr William Claycomb. Cells are grown in complete supplemented Claycomb media (JRH Biosciences, Lenexa, KS, USA). Stimulations are performed with the cells at confluence.

**Primary Murine Cardiomyocytes**

Murine ventricular myocytes were isolated from 10- to 14-week-old male mice. Strains were obtained from Jackson Laboratory (Bar Harbor, Maine) and included background strain (C57BL/6), NFκB-luciferase knock-in mice (B10.Cg-H2b-Tg(NFκB/Fos-luc)26Rinc/J), TLR2 knock-out (B6.129-Tlr2tm1Kir), TLR4 knock-out (B6.B10ScN-Tlr4tm1Klr), and MyD88 knock-out mice (courtesy of Dr William Qureshi and Dr S. Akira). Cardiomyocyte isolation and cell culture was performed as previously described.1

**Rat Primary Cardiomyocyte Isolation for Contractility Measurement**

Murine cardiomyocyte contractility is substantially less than that of rat cardiomyocytes. Therefore, to optimize the sensitivity of contractility measurements, we used rat cardiomyocytes. Isolation of rat cardiomyocytes was performed as previously described. The cells were incubated for 90 min in 95% O2, 5% CO2. The medium was then changed to fresh M199 with 5% BSA and the cardiomyocytes were incubated for 24 h to allow them to become relatively quiescent. After 24 h, cells were considered viable if they demonstrated a characteristic rod shape without membrane blebbing.

**Measurement of Cardiomyocyte NFκB Activity**

Twenty-four hours after isolation, primary cardiomyocytes from NFκB-luciferase knock-in mice were incubated with endotoxin-free HMGB1 (0.06–6 μg/ml), HSP60 (0.1–10 μg/ml, low endotoxin), HSP70 (0.1–10 μg/ml, low endotoxin) (all from StressGen, Victoria, BC, Canada), and the positive control PAMP, E. coli-derived LPS (0.1–10 μg/ml) (Invivogen, San Diego, CA, USA). After 24 h of incubation, cell lysates were collected and 60 μl of each were added to duplicate wells in a luminometry plate and read using the Dual-Luciferase Reporter Assay (Promega #1910) using a Fluostar Optima Luminometer (BMG Labtech, Durham, NC, USA). Relative light units were obtained for Firefly-Luciferase and all results were normalized to μg protein lysate.

**Calcium Flux Assay**

HL-1 cells grown to confluence on 4-well LabTek coverslip chambers (Sigma, Oakville, ON, USA) are incubated for 30 min with 2.5 μmol/L Fura-2 calcium-gated fluorescent dye (Molecular probes). Fluorescence is captured using a photomultiplier detector (Ionoptix Corp, Milton, MA, USA) and analyzed using an Ionoptix Sofdetection package that directly yields dynamic cardiomyocyte calcium flux (Ionoptix Corp). Calcium flux is calculated as the peak to baseline difference in the ratio of emissions from excitation wavelengths 340/380 nm.

**Cardiomyocyte Inflammatory Marker mRNA Expression**

Intercellular adhesion molecule 1 (ICAM-1), interleukin 6 (IL-6) and keratinocyte-derived chemokine (KC) are NFκB-regulated molecules that define a myocardial inflammatory response. ICAM-1 is most relevant to inflammatory cardiac contractile dysfunction as ligation of cardiomyocyte ICAM-1 decreases cardiac contractility via interaction with the structural cytoskeleton. Additionally, although their cardiac function is unknown, IL-6 and KC expression have shown to be significantly upregulated in myocardial inflammatory states. Thus, we assessed the cardiomyocyte inflammatory response by measuring ICAM-1, IL-6, and KC mRNA expression. HL-1 cells were incubated with HSP70 (10 μg/ml) for 6 h and harvested in 1 ml Trizol (Invitrogen, Carlsbad, CA, USA). RNA was extracted as per the manufacturer’s instructions, and DNase
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Measurement of Cardiomyocyte Fractional Shortening
Isolated rat cardiomyocytes were cultured as above for 24 h. Cells were then incubated with 10 μg/ml of HSP70 (StressGen, Victoria, BC, Canada) or a vehicle control for 6 h. Following this incubation, cardiomyocytes were stimulated at 140 V using a Grass S48 stimulator (Grass-Telefactor, Warwick, RI, USA). Images were captured using a Myocam video camera (Ionoptix Corp, Milton, MA, USA) and analyzed using a Ionoptix SoftEdge detection package (Ionoptix Corp). Fractional shortening was calculated as the difference between diastolic and systolic length, divided by the diastolic length.

Effect of Blocking TLR Binding
Isolated rat cardiomyocytes were cultured in a Matrigel (BD Biosciences)-coated 96-well plate for 24 h in M199 media+5% BSA. Cells were incubated overnight with 25 μg/ml of blocking antibodies to TLR2, TLR4 or isotype control antibodies (eBioscience, San Deigo, CA, USA). Cells were then incubated with HSP70 (10 μg/ml) for 4 h followed by measurement of fractional shortening, as described above. Cell viability after treatment was determined by counting the number of rod-shaped cells per high power field (HPF, 100× magnification).

Statistical Analysis
All values are expressed as mean±SD. For each experimental condition and timepoint, 3 independent replicate analyses were performed, unless otherwise noted. An ANOVA and a post-hoc Bonferroni correction was used to identify significant differences between the groups. The analyses were performed using SigmaPlot (San Jose, CA, USA) and statistical significance was set at P<0.05.

Results
DAMPS, and HSP70 in Particular, Induce NFκB Signalling
TLR-mediated cardiac dysfunction requires NFκB signalling.1 Therefore we used primary cardiomyocytes isolated from a mouse strain containing an NFκB-Luciferase reporter gene to compare the effect of the DAMPs, HSP60, HSP70 and HMGB1, to a potent positive control PAMP (LPS) on NFκB transcrip-
Figure 3. HSP70 stimulation of the HL-1 cardiomyocyte cell line is able to significantly increase the expression of several inflammatory markers compared to controls (CL). ICAM-1 expression (1.6-fold±0.2, P=0.006), KC expression (6.9-fold±0.3, P<0.001) and IL-6 expression (2.3±0.3, P=0.003) were all significantly increased compared to controls. *P<0.05 vs. control. ICAM-1, intercellular adhesion molecule 1; IL-6, interleukin-6; KC, keratinocyte-derived chemokine.

Figure 4. HSP70 induces ICAM-1 expression in cardiomyocytes through TLR2 and MyD88. Primary cardiomyocytes derived from background strain mice (C57BL/6), TLR2 knockout, TLR4 knockout and MyD88 knockout mice were incubated for 6h with 10μg/ml HSP70. ICAM-1 is highly induced in wildtype and TLR4 knockout mice, while mice deficient in TLR2 or MyD88 do not upregulate ICAM-1 in response to HSP70. ICAM-1, a cell adhesion molecule; TLR, toll-like receptors.
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HSP70 Reduces Fractional Shortening of Isolated Rat Cardiomyocytes Without Altering Calcium Flux

Following 6 h of exposure to 10 μg/ml HSP70, contractility of isolated rat cardiomyocytes was significantly decreased compared to saline-control treated cells (P<0.001). HSP70-treated cardiomyocytes shortened only 58±2.4% of control-stimulated cells (Figures 2A,B). Generally, inhibition of cardiomyocyte contractility relates either to a reduction in calcium flux or to an uncoupling of excitation-contraction. Using real-time Fura-2 based fluorescence as a surrogate of calcium flux in spontaneously beating HL-1 cardiomyocytes exposed to HSP70, we found no significant decline in calcium flux due to HSP70 vs. controls (mean flux 2.3±0.3 in each case) (Figure 2C).

HSP70 Induces a Cardiomyocyte Inflammatory Response

HL-1 cultured cardiomyocytes were stimulated with HSP70 (10 μg/ml) for 6 h. A qRT-PCR analysis of mRNA expression revealed an increase in several inflammatory markers. The cell adhesion molecule, ICAM-1, had increased expression by 1.6±0.2-fold that was significantly elevated compared to the control (P=0.03). KC expression was increased by 6.9±0.3-fold compared to the control (P=0.001). IL-6 expression was increased by 2.3±0.3-fold compared to the control (P=0.003) (Figure 3).

HSP70 Acts via TLR2 to Induce a Cardiomyocyte Inflammatory Response

Both TLR2 and TLR4 signalling have been implicated in the TLR response to HSP70 in immune cells. Therefore, we measured the cardiomyocyte inflammatory molecule known to functionally reduce contractility, ICAM-1, in response to HSP70 in cardiomyocytes isolated from mice deficient in TLR2, TLR4 or the downstream adaptor molecule MyD88. As illustrated in Figure 4, HSP70 increased ICAM-1 mRNA expression in cardiomyocytes from the background murine strain by 2.3±0.2-fold (P<0.001). Cardiomyocytes derived from TLR4-deficient mice similarly increased ICAM-1 mRNA expression following exposure to HSP70 by 2.0±0.5-fold (P=NS vs. wildtype cardiomyocytes). In contrast, HSP70 exposure in TLR2 knock-out and MyD88 cardiomyocytes did not increase ICAM-1 mRNA expression (P>0.001 compared to the wildtype response). These results implicate TLR2 and its downstream adaptor molecule, MyD88, in the HSP70-induced cardiomyocyte inflammatory response.

HSP70-Induced Reduction in Fractional Shortening Is Also Dependent Upon TLR2

To confirm that, like the inflammatory response, the cardiomyocyte functional response is similarly dependent on the TLR2 signalling pathway, we repeated measurements of fractional shortening following overnight incubation with blocking antibodies to TLR2, TLR4 or the isotype control. Following the overnight incubation, cardiomyocyte fractional shortening in controls was less than that observed in controls in Figure 2, which is likely related to interrupted incubation with blocking or isotype control antibodies (control fractional shortening 12±0.8%). Nevertheless, incubation with 10 μg/ml HSP70 resulted in a similar 25±2.6% decrease in fractional shortening from the control value in isotype control antibody incubated cells (Figure 5). The TLR4-blocking antibody had no effect on the HSP70-induced reduction in fractional shortening. Importantly, pre-treatment with the TLR2 blocking antibody reversed this effect while TLR4 blocking antibody, as well as isotype control antibody, had no effect. TLR, toll-like receptors.
Heat shock proteins and other DAMPs are induced by oxidative stress and other acute injury and, intracellularly, serve to protect the cell against these insults. Overexpressed intracellular HSP70 has shown to reduce infarct size and preserve cardiac contractility in several animal myocardial infarction models. However, DAMPs are also released extracellularly and appear to have very different effects; many of these effects mediated through innate immune receptors. For instance, HMGB1 is implicated in the exacerbation of acute cardiac dysfunction in a model of septic shock. PAMPS induce cardiac NFκB transcriptional activity and cause a TLR-induced NFκB-dependent decline in contractility, with LPS being the most potent PAMP in the heart. Accordingly, we surveyed 3 major DAMPs (HSP60, HSP70 and HMGB1) and found that all 3 induced NFκB transcriptional activity in cardiomyocytes. HSP70 in particular exhibited a very strong induction of cardiomyocyte NFκB transcriptional activity, over 3-fold greater than LPS.

Intracellularly, HSP70 is a chaperone protein involved with protein folding, modulating cell cycle progression, repressing gene expression and having anti-apoptotic functions. Increased intracellular levels of HSP70 reduce damage caused by ischemic injury and deletion of its inducible isoform induces cardiac hypertrophy and impaired contractility. These beneficial effects are thought to be through its intra-cellular roles. However, HSP70 is also released extracellularly via necrotic cell death and other reports have shown HSP70 to be actively released by cells under stress. Extracellularly, HSP70 stimulates pro-inflammatory cytokine production, augments chemokine synthesis, and increases expression of co-stimulatory molecules in a variety of non-cardiomyocyte cell lines. Serum HSP70 levels are measureable and are associated with an adverse prognosis of several diseases including renal disease, hypertension, atherosclerosis, aging, and sickle cell disease. High HSP70 serum levels are linked with a variety of cardiovascular diseases. Dybdahl et al found elevated serum levels of HSP70 in acute myocardial infarction patients. Additionally, HSP70 serum levels negatively correlated with left ventricular ejection fraction. Similarly, Satoh et al found a positive correlation between HSP70 levels and cardiac release of troponin and creatine kinase, as well as increased infarct size in acute myocardial infarction patients. One report using global ischemia-reperfusion in an isolated heart model suggested that heat shock cognate protein 70 (90% homologous to HSP70) is acutely induced by ischemia in cardiomyocytes and is secreted and rapidly detected in coronary effluent. Inhibitory antibody to heat shock cognate protein 70 was able to attenuate ischemia-induced cardiac dysfunction. Our results add to these observations by demonstrating that extracellular HSP70 directly decreased cardiomyocyte contractility and increased cell death; an effect that depended on the signaling pathway defined by TLR2, MyD88, and NFκB.

Although HSP70 has shown to co-localize with multiple receptors, among TLRs, TLR2 and TLR4 are the most likely functional candidates. Increased expression of ICAM-1 in response to inflammatory stimuli has been shown to reduce cardiac contractility. Therefore, we used ICAM-1 as an important measure of inflammation to assess whether mice deficient in TLR2 or TLR4 could mount a response to HSP70. TLR4 knockout cardiomyocytes increased ICAM-1 expression following exposure to HSP70 to the same extent as wild-type cardiomyocytes, suggesting that HSP70 does not appreciably signal via TLR4. In contrast, we found that cardiomyocytes derived from TLR2 knockout mice had no significant increase in ICAM-1 expression in response to HSP70. TLR2 signals almost exclusively via MyD88 whereas TLR4 has alternative pathways available (TRIF). MyD88 knockout cardiomyocytes did not significantly increase ICAM-1 levels in response to HSP70. In accord with these results, blocking antibodies to TLR2 prevented the HSP70-induced decrease in cardiomyocyte contractility. These results indicate that extracellular HSP70 signals via TLR2 and its downstream adaptor, MyD88,

Discussion

The major new findings of this study are that extracellular DAMPs (HSP70 in particular) induce NFκB transcriptional activity in cardiomyocytes at levels exceeding that of the “positive control”, PAMP (LPS). HSP70 signals through TLR2 and its adaptor molecule, MyD88, and induces NFκB expression, which leads to a cardiomyocyte inflammatory response as measured by ICAM-1, IL-6 and KC mRNA expression. Furthermore, this is associated with a reduction in cardiomyocyte contractility and an increase in cell death.

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Figure 6. HSP70 induces cell death in cardiomyocytes through TLR2. Rat primary cardiomyocytes were isolated and incubated overnight with a TLR2 blocking antibody or saline (SL). A 4-h stimulation with 10 μg/ml HSP70 led to significant cell death, while cells treated with a TLR2 blocking antibody had no significant increase in cell death. *P=0.005 vs. control. TLR, toll-like receptors.

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to induce NFκB signalling, which leads to a cardiomyocyte inflammatory response and ultimately decreased cardiomyocyte contractility. We went on to confirm that HSP70 signaling via TLR2 not only induces inflammation in cardiomyocytes but results in cell death. The results of this study are similar to those reported by Kim et al who showed that HSP60 is able to induce apoptosis, activate NFκB and in turn induce an inflammatory response. Unlike HSP70’s effects we describe in this manuscript, HSP60 acted uniquely via TLR4, although it did not act through known TLR4 binding sites.

A major limitation to this study is our use of isolated cardiomyocytes rather than a whole animal model. This limits our ability to infer whether the mechanisms we discuss above occur in human health and disease. If chosen to be done in vivo, we would have been unable to distinguish whether HSP70’s cardiac effects were due to direct signalling within the heart compared with interaction with the endothelium, immune cells or other cells.

In conclusion, HSP70 acts via TLR2 and MyD88 to activate NFκB. This induces expression of pro-inflammatory molecules such as ICAM-1, IL-6 and KC and decreases cardiomyocyte contractility and results in cell death. A reduction in contractility in response to DAMPs might represent an adaptive response to preserve viable myocardium in the face of injury, while intense inflammatory activity might adversely result in impaired cardiac function.

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References


