Extracellular HSP60 induces inflammation through activating and up-regulating TLRs in cardiomyocytes

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Aims
The molecular events leading from cardiomyocyte ischaemia to inflammatory cytokine production are not well understood. We previously found that heat shock protein 60 (HSP60) appeared in extracellular space after cardiomyocyte ischaemia. This study examined the activation and regulation of toll-like receptors (TLRs) by HSP60 in cardiomyocytes.

Methods and results
Cytokine production and TLRs regulation mediated by TLRs signalling were examined in response to exogenous HSP60 (exHSP60) and endogenous HSP60 (enHSP60) released extracellularly under ischaemia. The results showed that exHSP60 induced inflammatory cytokine production in adult rat cardiomyocytes and H9c2 cells (a standard cardiac cell line derived from embryonic cells), through a pathway dependent on TLR4, myeloid differentiation factor 88 (MyD88), p38, and nuclear factor-κB (NF-κB). Further study revealed up-regulated expression of both TLR2 and TLR4 by exHSP60, which was dependent on the activation of TLR4, MyD88, c-Jun NH2-terminal kinase (JNK), and NF-κB, but not on p38. In myocytes exposed to ischaemia, enHSP60 was released into the media, and triggered cytokine production and TLR2/4 overexpression, through the same pathways as exHSP60. In rats subjected to LAD ligation, the released enHSP60 contributed to cytokine production and TLR2/4 overexpression in the ischaemic myocardium.

Conclusion
Extracellular HSP60 induces cytokine production via TLR4-MyD88-p38/NF-κB pathway, and up-regulates TLR2/4 expression via TLR4-MyD88-JNK/NF-κB pathway. Both pathways contribute to myocardial inflammation induced by ischaemia.

Keywords
Heat shock protein • Toll-like receptor • Inflammatory cytokine • Cardiomyocyte

1. Introduction
Inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin (IL) are secreted locally in the heart or systemically in a broad range of cardiac conditions including heart failure, cardiac ischaemia—reperfusion, myocarditis, allograft rejection, and sepsis-induced dysfunction. They are secreted by virtually all resident cell types in the myocardium, including the cardiomyocytes themselves, as well as leucocytes that migrate into the heart. Through binding their cognate receptors, inflammatory cytokines have important effects on cardiac function and structure. The biological stimuli that initiate inflammatory cytokine production in myocardium include myocardial injury, haemodynamic stress, autoimmune reactions and rarely, microbial infections. Activation of inflammatory signalling may proceed through dedicated cell-surface receptors such as toll-like receptors (TLRs), in response to poorly understood endogenous ligands. TLRs are highly conserved pattern recognition receptors (PRRs) that recognize invading microbial pathogens and trigger innate
immunity. At least 10 TLRs have been identified in humans, and six in mice heart. Upon binding to specific ligands, TLRs signal through two distinct pathways, respectively, dependent on the adaptor proteins myeloid differentiation factor 88 (MyD88) and Toll/IL-1 receptor (TIR)-domain-containing adaptor protein inducing interferon (IFN-β)-mediated transcription factor (Trif). MyD88 and Trif activation leads to activation of nuclear factor-κB (NF-κB) and IFN regulatory factors (IRFs), which result in the production of inflammatory cytokines. All TLRs except TLR3 signal through MyD88-dependent pathway, TLR4, but not TLR2, partially signals through Trif-dependent pathway. Accumulative evidence demonstrates that the TLRs play critical roles in modulating inflammation and tissue damage following non-infectious insults such as myocardial ischaemia–reperfusion. A number of endogenous mediators produced by tissue damage likely serve as ligands activating TLRs in the absence of infectious microbial agents. Importantly, heat-shock proteins (HSPs) have been identified as ligands for TLRs in isolated cells. HSPs are a class of molecular chaperones that promote protein folding intracellularly. They may be released into extracellular spaces after cell trauma and interact with adjacent cells or distant cells via bloodstream delivery. We previously found that HSP60, a mitochondrial and cytosolic protein, appeared in the serum, the cardiac plasma membrane fraction, and on the surface of a subset of cardiac myocytes after permanent coronary artery occlusion in rats. Furthermore, exogenous HSP60 (exHSP60) induced apoptosis in isolated adult rat cardiomyocytes via TLR4. ExHSP60 has been reported to induce inflammatory responses through mechanisms both dependent and independent of TLR2 and TLR4 in the immune cells. Endogenous HSP60 (enHSP60) that appears in the serum after myocardial ischaemia and reperfusion has been found to play a role in activating cardiac inflammation. However, direct evidence and complete identification of the involved inflammatory signal pathway(s) activated in cardiomyocytes by extracellular HSP60 are still lacking. The present study addresses these issues.

2. Methods

An expanded Methods section is provided in the Supplementary material online.

2.1 Rat model of myocardial ischaemia

Myocardial ischaemia was induced by ligation of the left-anterior descending coronary artery (LAD) in anaesthetized rats, as described previously. The rats were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.), and maintained under anaesthesia by bolus injections of sodium pentobarbital (3–6 mg/kg, i.v.) as required. A neuromuscular blocking agent, succinylcholine bromide (0.6 mg/kg, i.v.), was used to allow a more stable preparation. The adequacy of anaesthesia was assessed periodically by testing blood pressure and heart-rate responses to toe pinch. After 4 h of LAD ligation, rats were sacrificed with an overdose of sodium pentobarbitone (60 mg/kg, i.v.), and blood and heart tissue were obtained for study. All animal procedures were approved by the Second Military Medical University, China, in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

2.2 Isolation of adult rat cardiomyocytes

Cardiomyocytes were isolated from adult male Sprague Dawley rats and cultured as previously described, with modifications. Briefly, hearts were digested with calcium-free Krebs–Henseleit buffer containing 1 mg/mL collagenase type 2. Dissociated cells were then filtered, collected, brought back to calcium-containing buffer, pre-plated to remove fibroblasts, and cultured in DMEM supplemented with 10% foetal bovine serum. This procedure yielded 70–80% rod-shaped cardiomyocytes. After 1 h of culture, myocytes were treated with low endotoxin HSP60 for indicated time periods.

2.3 Models of cultured rat H9c2 cardiomyocytes

The H9c2 rat-ventricular cell line was cultured as described previously. When cells were 80% confluent, they were serum-deprived for 12 h for synchronization, and then exposed to HSP60 or ischaemia. Ischaemia was induced by exposing cells to 1% O2 –94% N2 –5% CO2 in serum-free low-glucose DMEM for 12 h. The p38 inhibitor SB203580, c-Jun NH2-terminal kinase (JNK) inhibitor SP600125, NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC), or TLR2/4 neutralizing antibody (anti-TLR2/4) were added 15 min before HSP60 or ischaemia treatment. The MyD88 homodimerization inhibitory peptide (Inhi MyD88) or the control peptide (Cntl MyD88) was added 24 h before HSP60 or ischaemia treatment. siRNAs were used to specifically knock down the expression of TLR2/4, as detailed in the Supplementary material online.

2.4 Quantitative reverse transcriptase–polymerase chain reaction

Real-time RT–PCR was performed to determine the mRNA levels of TLR2, TLR4, and inflammatory cytokines, as detailed in the Supplementary material online.

2.5 Western blot and co-immunoprecipitation analysis

Western blot was used to determine the protein levels of TLR2, TLR4, MyD88, and Trif, as well as the phosphorylated and total protein levels of p38 and JNK. Co-immunoprecipitation (co-IP) was performed to examine the interaction between HSP60 and TLR2/4, and between TLR4 and MyD88/Trif.

2.6 Determination of NF-κB activation

The nuclear translocation of p65, a subunit of the NF-κB heterodimer, was used as readout for NF-κB activation. We examined p65 translocation by immunohistochemistry and by probing western blots of nuclear extracts for p65.

2.7 ELISA analysis for TNFα, IL-6, and HSP60

The TNFα, IL-6, and HSP60 levels in cell-culture supernatant and rat serum were measured using commercial ELISA kits (Enzo Life Sciences), following the manufacturer’s instructions.

2.8 Statistics and data analysis

All the data were expressed as the means ± SD. Data were analysed by a one-way ANOVA followed by the Fisher’s least significant difference (LSD) test to determine the differences between groups. SAS 9.0 statistical software (SAS Institute, Inc., USA) was used for data analysis. A P < 0.05 was considered statistically significant.

3. Results

3.1 ExHSP60 induces cytokine production in cardiomyocytes through TLR4–MyD88 pathway

In primary adult-rat cardiomyocytes, as well as H9c2 cells, exHSP60 significantly increased the TNFα and IL-6 mRNA levels as well as
the release of these inflammatory cytokines. Time-course studies showed the greatest increase at 6 h (Supplementary material online, Figure S1). Therefore exHSP60 was incubated for 6 h in the following experiments.

A dose–response was observed for exHSP60 in both adult and H9c2 cardiomyocytes. 5 μg/mL exHSP60 increased the mRNA levels and the release of TNFα and IL-6 more than 1 μg/mL exHSP60. The TLR4 agonist, lipopolysaccharide (LPS, 1 μg/mL), was used as a positive control. LPS significantly induced cytokine production in both adult cardiomyocytes and H9c2 cells. To exclude the possibility that endotoxin contaminated in the recombinant exHSP60 may induce inflammation, treatment with heat-inactivated HSP60 (1 μg/mL, heated at 100°C for 5 min) was done. This failed to induce cytokine production, supporting that the observed pro-inflammatory effects are a specific effect of HSP60. (Supplementary material online, Figure S1)

A series of experiments was done to investigate the signalling pathway(s) activated by exHSP60. First, we examined the interaction of exHSP60 with TLR2/4 by co-IP experiments. When the lysates of H9c2 cells were precipitated with antibodies against HSP60, TLR4 but not TLR2 was co-precipitated. In control, untreated cells, only a trace amount of TLR4 was co-precipitated with HSP60. In contrast, in exHSP60-treated cells, a significant amount of TLR4 was co-precipitated (Figure 1A and B). Secondly, we investigated the adaptor proteins, MyD88 and Trif, which TLR4 signals through. We co-precipitated cell lysates with anti-TLR4 antibodies, and observed that MyD88, but not Trif was co-precipitated, with a greater amount in exHSP60-treated cells (Figure 1C). Thirdly, we examined the activities of p38, JNK, and NF-κB, the effector molecules downstream of the TLR4-MyD88 pathway. These studies showed that p38 and JNK were activated by exHSP60, as shown by the increased phosphorylation of p38 and JNK (Figure 1D and E). NF-κB was also activated by exHSP60, as shown by p65 translocation from the cytoplasm to the nucleus (Figure 1F and G). Fourthly, we examined the effects of inhibiting upstream signalling molecules on exHSP60-induced p38, JNK, and NF-κB activation, as well as cytokine production. The results (Figure 1H–K) showed that TLR4 siRNA (100 nM), anti-TLR4 (5 μg/mL), and Inhi MyD88 (100 μM) prevented the activation of p38, JNK, and NF-κB, and significantly inhibited TNFα and IL-6 mRNA expressions in exHSP60-treated myocytes. SB203580 (20 μM) and PDTC (100 μM) also inhibited TNFα and IL-6 expressions. However, the negative control siRNA (NC siRNA, 100 nM), the control antibody (IgG, 5 μg/mL), Cntl MyD88 (100 μM), and SP600125 (20 μM) did not affect the pro-inflammatory effects of exHSP60. Surprisingly, TLR2 siRNA (100 nM) significantly inhibited exHSP60-induced cytokine production, but anti-TLR2 antibody (5 μg/mL) did not show any effects (Figure 1J and K).

Furthermore, we investigated whether exHSP60 itself increased TLR2/4 expression in the H9c2 cells. The results showed that the mRNA and protein levels of TLR2 and TLR4 were both increased by exHSP60, and reached a maximum at 6 h, remaining elevated for 24 h (Figure 2A–C). To determine the signal pathway that mediated the increase in TLR2/4 expression by exHSP60, TLR2/4 siRNA (100 nM), Inhi MyD88 (100 μM), SB203580 (20 μM), SP600125 (20 μM), and PDTC (100 μM) were used to interfere with the signal transduction. We observed that TLR2 siRNA specifically down-regulated both TLR2 and TLR4 mRNA in exHSP60-treated cells. SP600125 and PDTC abolished the induction of TLR2/4 expression by exHSP60, whereas SB203580, at the same doses that inhibited cytokine production, had no effect on TLR2/4 expression. Thus, induction of TLR2/4 expression by exHSP60 is dependent on TLR4, MyD88, JNK, and NF-κB, but not TLR2 or p38 (Figure 2D and E).

In sum, the above results showed that exHSP60 stimulated cytokine production via TLR4-MyD88-p38/NF-κB pathway, and enhanced TLR2/4 expression via TLR4-MyD88-JNK/NF-κB pathway in H9c2 myocytes.

3.2 EnHSP60 in the extracellular space contributes to cytokine production induced by ischaemia in cultured cardiomyocytes through the TLR4-MyD88 pathway

EnHSP60 is normally located in the mitochondria and cytoplasm. HSP60 was barely detectable by ELISA in the media of normal H9c2 myocytes. However, 3.7 ± 0.7 ng/mL of HSP60 was detected in the media after 12 h of ischaemia (Figure 3A). To examine whether the extracellular enHSP60 contributed to cytokine production induced by ischaemia, anti-HSP60 antibodies (10 μg/mL) were used to neutralize enHSP60. This significantly inhibited cytokine induction by ischaemia (Figure 3F and G).

The association between enHSP60 and TLR2/4, and between TLR4 and MyD88/Trif was investigated further with co-IP experiments. In accordance with the results for exHSP60, a large amount of TLR4 co-precipitated with enHSP60 from ischaemic myocytes. However, no significant binding was seen between TLR2 and enHSP60 (Figure 3B). Additionally, TLR4 co-precipitated with MyD88, but not Trif. The amount of MyD88 bound with TLR4 was much greater in ischaemic myocytes than control cells (Figure 3C). In accordance with the ability of exHSP60 to activate p38 and JNK, enHSP60 contributed to the activation of p38 and JNK in ischaemic myocytes. The activity of p38 and JNK was increased by 13.2- and 3.7-fold, respectively, under ischaemia, which was significantly reduced to 5.3- and 1.6-fold of control by anti-HSP60 antibodies (10 μg/mL). Anti-TLR4 antibodies (5 μg/mL) and Inhi MyD88 (100 μM) also inhibited p38 and JNK activation (Figure 3D and E).

Signal inhibitors were also used to investigate the role of key proteins in cytokine production. Anti-HSP60 (10 μg/mL), anti-TLR4 (5 μg/mL), TLR4 siRNA (100 nM), Inhi MyD88 (100 μM), SB203580 (20 μM), and PDTC (100 μM) all significantly inhibited both the increase in TNFα and IL-6 production and release, while SP600125 (20 μM) had no effect (Figure 3F and G).

Ischaemia increased TLR2 and TLR4 mRNA by 3.3- and 2.0-fold, respectively, and correspondingly increased their protein levels by 4.2- and 3.1-fold. Anti-HSP60 (10 μg/mL), anti-TLR4 (5 μg/mL), TLR4 siRNA (100 nM), Inhi MyD88 (100 μM), SP600125 (20 μM), and PDTC (100 μM) significantly inhibited TLR2 and TLR4 overexpression, but SB203580 (20 μM) had no effect. (Figure 4).

The above results showed that ischaemia-induced cytokine production in H9c2 cells was dependent on enHSP60-TLR4-MyD88-p38/NF-κB. In contrast, enhanced TLR2 and TLR4 expressions were dependent on enHSP60-TLR4-MyD88-JNK/NF-κB. These signalling cascades match those activated by exHSP60.
**Figure 1** ExHSP60 induced inflammatory cytokine production in H9c2 cardiomyocytes via TLR4-MyD88-p38/NF-κB pathway. Low endotoxin recombinant HSP60 was administered at 1 μg/mL for 6 h unless otherwise indicated. (A and B) The association between HSP60 and TLR4 and (C) between TLR4 and MyD88 in HSP60-treated cells shown by cell-lysate immunoprecipitation (IP) followed by SDS–PAGE and immunoblotting (IB), using indicated antibodies. ‘control’ vs. ‘HSP60’ above each lane refers whether or not the cells were treated with HSP60 in the media. IP with isotype IgG (IP: IgG) was performed as a control to exclude the non-specific binding of antibodies to cellular proteins. The media was aspirated and cells were washed twice with PBS before harvesting for IP experiments. (D) Representative western blot images and (E) quantification of phosphorylated (p-) and total p38 and JNK. (F) Immunofluorescence staining and (G) western blotting for p65, showing its translocation from cytosol to nucleus in cells treated with HSP60. The cytosolic and nuclear fractions of cell lysates were immunoblotted for p65, as well as the nuclear marker histone and the cytoplasmic marker GAPDH (G). (H) Representative western blot images and (I) quantification of p-p38/p38, p-JNK/JNK, and nuclear p65/histone. (J) TNFα and (K) IL-6 mRNA levels in control and HSP60-treated cells pre-treated with 100 nM negative control (NC)/TLR2/TLR4 siRNA, 5 μg/mL control (IgG)/anti-TLR2/anti-TLR4 antibody, 100 μM MyD88 homodimerization control/inhibitory peptide (Cntl/Inhi MyD88), 20 μM p38 inhibitor SB203580, 20 μM JNK inhibitor SP600125, or 100 μM NF-κB inhibitor PDTC. All the mRNA levels were determined using real-time RT–PCR, normalized to GAPDH, and expressed as the fold change of the control cells receiving no treatment. Data are means ± SD from three independent experiments. (aP < 0.05, (bP < 0.01 vs. control receiving no treatment; (cP < 0.05, (dP < 0.01 vs. ‘control + HSP60’; (eP < 0.05, (fP < 0.01 vs. the same treatment minus HSP60).
Figure 2  ExHSP60 enhanced TLR2 and TLR4 expression in H9c2 cardiomyocytes via TLR4-MyD88-JNK/NF-κB pathway. Low endotoxin recombinant HSP60 was administered at 1 μg/mL for 6 h unless otherwise indicated. (A) Time-course for changes in TLR2 and TLR4 mRNA levels. (B) Representative western blot images and (C) quantification of the indicated proteins. (D) TLR2 and (E) TLR4 mRNA levels in control and HSP60-treated cells pre-treated with 100 nM NC/TLR2/TLR4 siRNA, 100 μM Cntl/Inhi MyD88 peptide, 20 μM SB203580, 20 μM SP600125, or 100 μM PDTC. Data are means ± SD from three independent experiments. (aP < 0.05, bP < 0.01 vs. control receiving no treatment; aP < 0.05, bP < 0.01 vs. ‘control + HSP60’;  aP < 0.05, bP < 0.01 vs. the same treatment minus HSP60).
3.3 EnHSP60 in circulation contributed to cytokine production induced by myocardial ischaemia in intact rats

In anaesthetized rats, HSP60 was detectable by ELISA in the serum after 4 h of LAD ligation (8.3 ± 1.5 ng/mL), but undetectable after sham surgery (Figure 5A). Co-IP experiments detected significant interaction between HSP60 and TLR4, but not HSP60 and TLR2, in ischaemic myocardium (Figure 5B). Significant binding between TLR4 and MyD88 was detected in both sham and ischaemic myocardium, with more binding in the latter. However, no binding was observed between TLR4 and Trif in either sham or ischaemic myocardium (Figure 5C).
In ischaemic myocardium, the activity of p38 was increased by 53.3-fold (Figure 5D and E), and the mRNA level of TNFα and IL-6 was increased by 3.7- and 3.3-fold, respectively (Figure 5F). The serum content of TNFα and IL-6 was increased from 16.9 to 81.8 pg/mL, and from 34.0 to 164.7 pg/mL, respectively (Figure 5G). By using anti-HSP60 antibodies (20 μg/kg, intravenously injected 15 min before LAD ligation) to neutralize serum HSP60 that appeared after LAD ligation, we observed reduced activation of p38 and decreased generation of TNFα and IL-6, confirming that extracellular HSP60 (extracellular) in circulation contributed to p38 activation and cytokine production in the setting of myocardial ischaemia (Figure 5D–G).

Similar to our findings in the H9c2 cells exposed to ischaemia, increased TLR2 and TLR4 expressions were observed in the ischaemic myocardium. As with the H9c2 cells, anti-HSP60 antibodies significantly decreased the increase in TLR2 and TLR4 (Figure 6).

4. Discussion

The present study provides direct evidence that extracellular HSP60 activates TLR4 and MyD88, but not TLR2 or Trif, in cardiomyocytes. We demonstrate for the first time that extracellular HSP60, either exogenous or endogenous (released by ischaemic myocardium), induces...
Figure 5  Endogenous HSP60 in circulation contributed to cytokine production induced by myocardial ischaemia in intact rats. Anti-HSP60 antibodies or control IgG were intravenously injected at 20 μg/kg 15 min before 4 h of LAD ligation. (A) Serum content of HSP60. (B) The association between HSP60 and TLR4 and (C) between TLR4 and MyD88 in ischaemic cardiac tissue shown by tissue homogenate immunoprecipitation (IP) followed by SDS–PAGE and immunoblotting (IB). (D) Representative western blot images and (E) quantification of phosphorylated (p-) and total p38 in myocardial ischaemic area. (F) TNFα and IL-6 mRNA levels in myocardial ischaemic area. (G) Serum content of TNFα and IL-6. Data are means ± SD. (n = 6/group. *P < 0.05, **P < 0.01 vs. sham; aP < 0.05, bP < 0.01 vs. LAD ligation).
TLR2 and TLR4 expression. During myocardial ischaemia, HSP60 released from cardiomyocytes stimulates inflammatory cytokine production via TLR4-MyD88-p38/NF-κB pathway, and up-regulates TLR2 and TLR4 expression via TLR4-MyD88-JNK/NF-κB pathway. Inflammatory cytokines serve as effectors of innate immunity and function as an early warning system to discriminate self and potential pathogens. Accumulated evidence has demonstrated that they are synthesized not only by immune cells but also by cardiomyocytes in response to ischaemia or mechanical stretch. The importance of inflammatory cytokines to stressed cardiomyocytes is well accepted; however, the mechanisms that trigger cytokine production are not well understood.

Emerging data have identified the TLRs as critical mediators of cytokine production. TLRs are a family of PRRs expressed by immune cells that recognize pathogen-associated molecular patterns (PAMPs). In mammals, TLRs expressed on antigen presenting cells...
and macrophages serves as key PRRs with central roles in induction of innate immune responses, which are characterized by cytokine production, as well as the subsequent development of adaptive immune responses. TLRs consist of an extracellular leucine-rich repeat region that is involved in microbial recognition, and an intracellular TIR domain that is necessary for signaling. Recognition of PAMPs by TLRs stimulates the recruitment of TIR-domain-containing adapters, especially MyD88 and Trif. MyD88 is recruited by all TLRs with the exception of TLR3. Recruitment of MyD88 activates the IL-1 receptor-associated kinases (IRAKs), which interact with TNF receptor-associated factor (TRAF) 6 and in turn activate transforming growth factor-β-activated protein kinase 1 (TAK1), a mitogen-activated protein kinase (MAPK) kinase (MAPKK). Ultimately, the MAPKs (ERK, JNK, p38) and the transcription factor NF-κB are activated, leading to the expression of inflammatory cytokine genes. Trif is recruited by TLR3 and TLR4. Recruitment of Trif leads to the activation of NF-κB, MAPKs, and the IFN-inducing transcription factor IRF3. However, in the current study, TLR4 activated only the MyD88 pathway, but not the Trif pathway. Both the MyD88- and Trif-dependent pathways lead to a common final outcome, cytokine production, which contribute to innate immune responses.

In addition to immune cells, TLRs have been found in various cell types including cardiomyocytes. Six types of TLRs (TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9) have been reported in murine cardiomyocytes. Using the method of real-time PCR, we detected the expression of nine types of TLR (TLR1-9) in cardiomyocytes isolated from adult rats, as well as the H9c2 cell line (data not shown). The present study showed that MyD88 but not Trif was co-precipitated with TLR4 in rat cardiomyocytes, despite the abundant expression of Trif (Figure 2B), suggesting that TLR4 recruits MyD88 but not Trif in response to HSP60 binding. In parallel with the increased recruitment of MyD88 to TLR4, we observed activation of p38, JNK, and NF-κB, as well as enhanced expression of TNFα and IL-6. These results were in accordance with the effects of TLR4-MyD88 signalling in immune cells as described earlier. Additionally, we observed that the increase in TNFα and IL-6 was abolished by the inhibition of either p38 or NF-κB, but not by JNK inhibition. This is in agreement with the pro-inflammatory role of p38 and NF-κB and anti-inflammatory role of JNK described for cardiomyocytes. In contrast, but in contrast to the pro-inflammatory role of JNK described for immune cells.

Studies with TLR deficient mice have demonstrated crucial roles of TLR2 and TLR4 in ischaemia–reperfusion injury-mediated inflammatory responses in the heart. However, there are two questions here. First, how do TLR2 and TLR4 become activated by ischaemia–reperfusion? Secondly, whether TLR2 and TLR4 on cardiomyocytes or on infiltrated immune cells, or both, contribute to cardiac inflammation. Previous studies performed mainly in immune cells identified a number of endogenous molecules that induce cytokine production via TLR2 and TLR4, such as HSP60, HSP70, and hyaluronan. Among these molecules, HSP60 was found by us to be abnormally translocated to cardiomyocyte plasma membrane and the extracellular space after myocardial infarction, at least in part via exosomes. This suggests the possibility that HSP60 may activate TLR2 and TLR4 located on the cell surface of cardiomyocytes, as well as immune cells in circulation or infiltrated into the myocardium, and this is supported by the findings of the current study.

In adult-rat cardiomyocytes, extracellular HSP60 had pro-apoptotic effects dependent on TLR4 but not TLR2. In neonatal-rat cardiomyocytes, recombinant HSP60, at the same concentration used in the current study, was found to activate IRAK-1, a kinase critical for the innate immune signalling including that of TLRs, supporting the inflammatory actions of extracellular HSP60 on cardiomyocytes. In accordance with the above reports, the present study provides direct evidence that ex-HSP60 released from ischaemic cardiomyocytes contributed to cytokine induction via the TLR4-MyD88-p38/NF-κB pathway and induces inflammation in cardiomyocytes. We also demonstrated that exHSP60 released from ischaemic cardiomyocytes potentially a significant source of inflammatory cytokines in addition to the recruited inflammatory cells expressing TLR4, which may act as a potent trigger mechanism for cardiac inflammation in these diseases.

The present study to our knowledge is the first to report that HSP60 induced TLR2 and TLR4 expression in cardiomyocytes, through TLR4-MyD88-JNK/NF-κB pathway. The overexpression of TLR2 may partially contribute to HSP60-induced inflammation, as evidenced by the inhibition of TLR2 siRNA on cytokine generation. Previously, the TLR4 ligand LPS was reported to enhance TLR4 expression in neonatal-rat-ventricular myocytes. TLRs overexpression in response to TLR ligands including HSP60 and LPS may amplify the TLR-mediated signalling and cellular effects.

Extracellular HSP60 has been found to cause cardiac myocyte apoptosis and may be a factor in the progression of heart failure. Here we provide new insights into the underlying mechanisms by which HSP60 causes cardiac myocyte inflammation. The current work shows an unexpected interaction between HSP60 and TLR2/4. HSP60 did not co-IP with TLR2, but knockdown of TLR2 expression with siRNA blocked the increase in inflammatory cytokine expression mediated by HSP60 and TLR4. Similarly, HSP60 increased the expression of both TLR2 and TLR4, which was dependent on MyD88, JNK, and NF-κB. HSP60–TLR4-mediated cytokine production depended on the activation of MyD88, p38, and NF-κB. TLR4 signals through MyD88 and Trif, but in the current study only MyD88 is involved in signalling. Thus the current study provides several novel findings with regards to the complex interaction of extracellular HSP60 and innate immunity.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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