Extracellular Heat Shock Protein 60, Cardiac Myocytes, and Apoptosis

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Rationale: Previously, we have found that changes in the location of intracellular heat shock protein (HSP)60 are associated with apoptosis. HSP60 has been reported to be a ligand of Toll-like receptor (TLR)-4.

Objective: We hypothesized that extracellular HSP60 (exHSP60) would mediate apoptosis via TLR4.

Methods and Results: Adult rat cardiac myocytes were treated with HSP60, either recombinant human or with HSP60 purified from the media of injured rat cardiac myocytes. ExHSP60 induced apoptosis in cardiac myocytes, as detected by increased caspase 3 activity and increased DNA fragmentation. Apoptosis could be reduced by blocking antibodies to TLR4 and by nuclear factor κB binding decoys, but not completely inhibited, even though similar treatment blocked lipopolysaccharide-induced apoptosis. Three distinct controls showed no evidence for involvement of a ligand other than exHSP60 in the mediation of apoptosis.

Conclusions: This is the first report of HSP60-induced apoptosis via the TLRs. HSP60-mediated activation of TLR4 may be a mechanism of myocyte loss in heart failure, where HSP60 has been detected in the plasma. (*Circ Res*. 2009;105:1186-1195.)

Key Words: Toll-like receptor-4 ■ apoptosis ■ heat shock protein 60 ■ cardiac myocytes ■ tumor necrosis factor ■ TLR4 ■ inflammation

Toll-like receptors (TLRs) have been recognized in the last 15 years as an important part of the immune system. The TLRs are a key component of innate immunity, a primitive immunity characterized by the rapid recognition of bacterial and other motifs as dangerous, followed by an inflammatory response that includes the production of cytokines, such as tumor necrosis factor (TNF)-α. Heat shock protein (HSP)60 is thought to be a ligand of TLR4, which has been found on the surface of cardiac myocytes.[1,2] In the immune system, activation of TLR4 is characterized by activation of nuclear factor (NF)κB followed by production of TNF-α. Limited studies have addressed the function of the TLRs in nonimmune system cells. We hypothesized that extracellular (ex)HSP60 activated TLR4 and that this would induce cardiac myocyte apoptosis.

Lipopolysaccharide (LPS) has also been identified as a ligand for TLR4. Some controversy persists as to whether observed effects with other proteins activating TLR4 do so directly, or are actually contaminated with LPS.[3] However, it is becoming clear that extracellular HSPs have an important role in cell signaling.[4] To address the issue of LPS contamination, in addition to careful controls, we examined the effect of LPS on apoptosis, and the effect of a TLR4 blocking antibody on the LPS and exHSP60 induced apoptosis.

We report here that exHSP60 binds selectively to the cardiac myocyte and induces apoptosis. Apoptosis is decreased by anti–TLR4 blocking antibodies but not by blocking antibodies to TLR-2 or CD14. These findings imply that HSP60 released during cardiac injury can have a paracrine effect on neighboring myocytes leading to cell death. This is the first report of HSP60 having a toxic effect on cardiac myocytes.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Isolated Adult Cardiac Myocytes

Isolated adult cardiac myocytes were prepared from male Sprague–Dawley rats (Harlan, Indianapolis, Ind).[5] The animal protocol was approved by the University of California, Davis Animal Research committee in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Binding Studies

Binding studies were performed using the approach of Habich et al[6] Recombinant human HSP60 (rhHSP60, StressGen, ESP-540, Low-Endotoxin) was labeled with Oregon green 488 (Molecular Probes). Cardiac myocytes were incubated with Oregon-green labeled rhHSP60 (OG-rhHSP60) in concentrations up to 0.2 μmol/L for 30

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Non-standard Abbreviations and Acronyms	
AIF	apoptosis-inducing factor
CDD	cell death assay
EU	endotoxin unit
exHSP60	extracellular heat shock protein 60
HSP	heat shock protein
IL	interleukin
LPS	lipopolysaccharide
NFκB	nuclear factor κB
rhHSP60	recombinant human heat shock protein 60
ratHSP60	heat shock protein 60 from adult rat cardiac myocytes
TLR	toll-like receptor
TNF	tumor necrosis factor

minutes at 4°C. For competition assays, cardiac myocytes were incubated with 0, 0.07 and 0.35 μmol/L rhHSP60 followed by incubation with 0.07 μmol/L OG-rhHSP60.

Purification of Released HSP60 From Adult Rat Cardiac Myocytes

In contrast to rhHSP60, the released HSP60 from adult rat cardiac myocytes is termed ratHSP60.

Apoptosis

Myocytes were treated with 1 μg/mL rhHSP60 (low endotoxin, ESP540, Assay Designs), 1 μg/mL ratHSP60, or 10 ng/mL TNF-α'20'28R&D Systems). Caspase 3 activity was measured using a kit (Promega, Madison, Wis). DNA fragmentation was measured using the cell death assay (CDD) (Roche, Alameda, Calif).

Blocking Antibodies

Following 30 minutes of preincubation with antibodies to TLR4 (20 μg/mL, HTA-125, StressGen), TLR-2 (20 μg/mL; Serotec, Raleigh, NC), and CD14 (10 μg/mL, Coulter Immunology, Hialeah, Fla), treatment with rhHSP60, TNF-α and LPS was initiated. Concentrations of the blocking antibodies were based on the literature.[7–10] For cytokine experiments neutralizing antibodies for TNF-α and interleukin (IL)-1β (both R&D Systems) were used.

Endotoxin Levels

Endotoxin levels for both the rhHSP60 (low endotoxin, ESP540, StressGen) and the ratHSP60 were measured using the Pyrogene assay (Cambrex, Baltimore, Md). Treatment with Detoxi gel (polymixin B, Pierce) reduced levels to <0.01 endotoxin unit (EU)/μg protein (<1 pg).

Statistics

Data are expressed as means±SEM. Groups were compared using an ANOVA followed by a Holm–Sidak test. Normalized data were compared by an ANOVA on Ranks followed by a Student–Neumann–Keuls test or by a Wilcoxon rank test. Binding data were analyzed using SigmaPlot pharmacokinetic functions. A $P<0.05$ was considered significant.

Results

HSP60 Binding to Cardiac Myocytes Is Saturable and Specific

As shown in Figure 1A, OG-rhHSP60 bound to cardiac myocytes and this was saturable. The K_d was calculated to be 0.16 μmol/L. Pretreatment with increasing amounts of unlabeled rhHSP60 competed with 0.07 μmol/L rhHSP60, as shown in Figure 1B. Thus, binding of OG-rhHSP60 could be prevented by competition with unlabeled protein. Hence, binding of HSP60 is saturable and specific.

ExHSP60 Causes Apoptosis

ExHSP60 has been reported to be a ligand of toll-like receptor (TLR)-4. We hypothesized that if exHSP60 were a ligand of TLR4, this might lead to apoptosis. After pilot experiments to test several time points, cardiac myocytes were treated for 16 hours with both rat and rhHSP60 (1 μg/mL). This concentration of HSP60 was based on the report that 25% of British civil servants enrolled in the Whitehall study had 1.0 μg/mL or more HSP60 present in their serum.[11] As shown in Figure 1C, both rat and rhHSP60 activated caspase 3. 10 ng/mL TNF-α was used as an internal reference for comparison of apoptotic effect. Similar degrees of caspase 3 activity were seen with both rat and rhHSP60 and TNF-α. Three different treatments were used to control for the specificity of this effect. RatHSP60 was denatured by heating to 95°C for 10 minutes. Secondly, cells were treated with anti-HSP60 at a 3:1 molar ratio to ratHSP60, before adding ratHSP60. Lastly, the ratHSP60 was passed over a polymixin B column to remove any endotoxin. As shown in Figure 1C, both heat inactivation and anti-HSP60 antibody completely inhibited activation of caspase 3 by ratHSP60. In contrast, polymixin B had no effect on ratHSP60 activation of caspase 3.

At 19 hours, cells were collected and assayed for DNA fragmentation using the CDD assay (Roche). RatHSP60 had a greater effect on DNA fragmentation than either rhHSP60 or TNF-α (Figure 1D), although this was not observed with more recent preparations of rhHSP60 and TNF-α. Using the same controls, both heat inactivation and anti-HSP60 antibody blocked DNA fragmentation by ratHSP60. Polymixin B had no effect on DNA fragmentation. Examples of apoptotic cardiac myocytes are shown in Figure 1E.

The mitochondrial death pathway is an important component of apoptosis. Cardiac myocytes were permeabilized, and the cytosol was analyzed by Western blotting. As shown in Figure 1F and G, cytochrome *c* release was seen after 2 hours of treatment with rhHSP60 or TNF-α. Apoptosis-inducing factor (AIF) and endonuclease G release also occurred, providing evidence for activation of the caspase independent apoptosis pathway (Figure 1H).

TLR4, TLR-2, and CD14 Blocking Antibodies

HSP60 has been proposed to be a ligand for TLR-2 and TLR4.[1,12,13] To test the role of the TLR-2 and TLR4 receptors in HSP60-mediated apoptosis, we pretreated the cardiac myocytes with blocking antibodies. Treatment with 20 μg/mL anti–TLR4 blocking antibody completely prevented the induction of apoptosis by LPS and significantly reduced apoptosis mediated by rhHSP60 or TNF-α (Figure 2A). In contrast, TLR-2 blocking antibody had no effect (Figure 2B). CD14 complexes with TRL-4 and is critical for LPS binding. CD14 had been thought to be a monocyte/macrophage specific receptor but has been found in cardiac myocytes.[14] The CD14 blocking antibody had no effect on rhHSP60

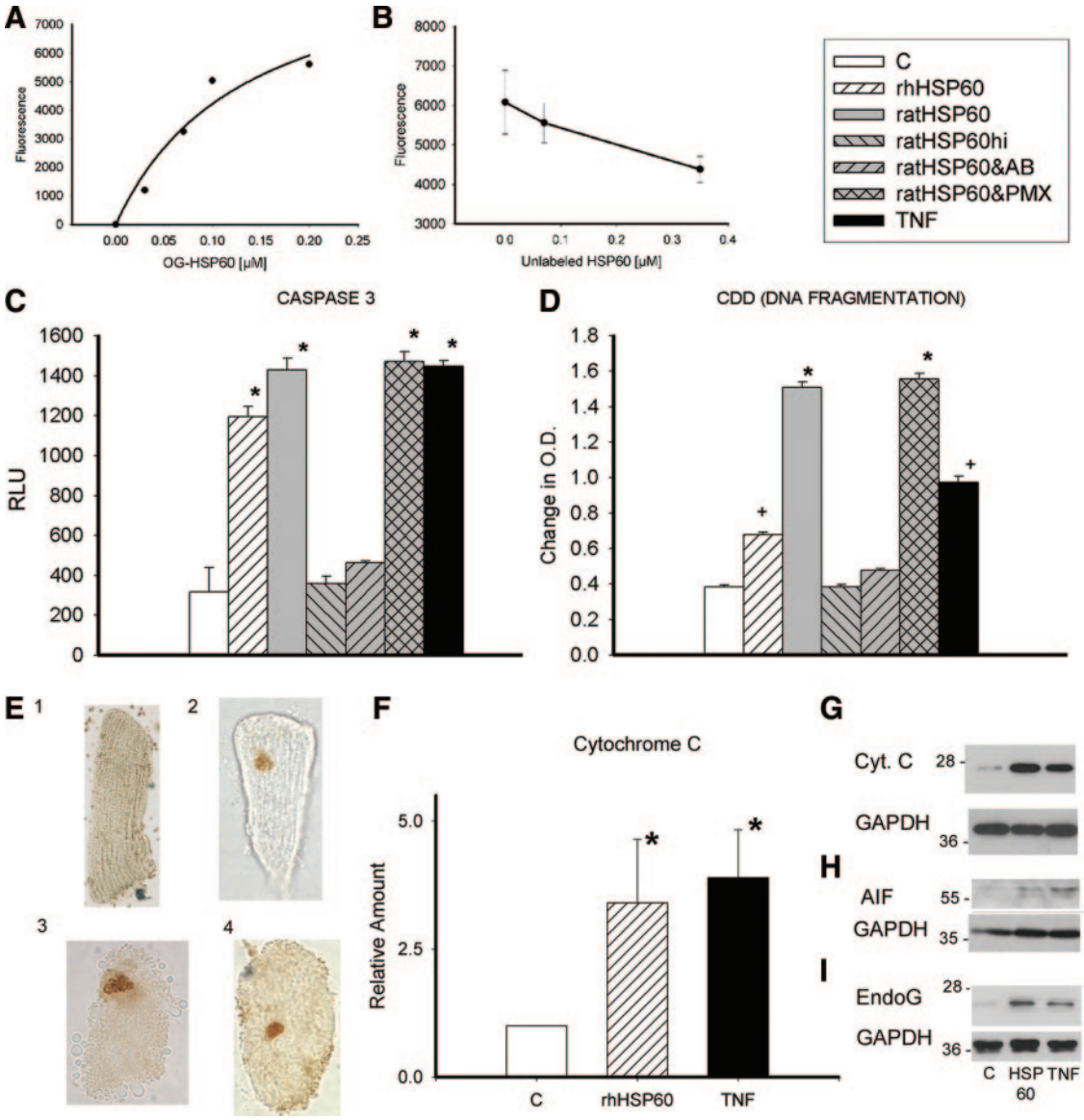

Figure 1. Extracellular HSP60 binds cardiac myocytes and causes apoptosis. A, Binding of HSP60. Cardiac myocytes were incubated with OG-rhHSP60 in increasing concentrations up to 0.2 μmol/L for 30 minutes at 4°C. B, Competition assays. Cardiac myocytes were preincubated with 0, 0.07, and 0.35 μmol/L rhHSP60, followed by incubation with 0.07 μmol/L OG-rhHSP60 4°C. Each graph summarizes 3 separate experiments. C, Caspase 3 activity, measured as change in relative light units (RLU) generated by cleavage of Z-DEVD-aminoluciferin. Cardiac myocytes were treated with 1 μg/mL low endotoxin rhHSP60 (recombinant human), 1 μg/mL ratHSP60, or 10 ng/mL TNF-α. Controls for endotoxin contamination were: heat inactivation (hi) of the protein, anti-HSP60 (AB) in a 3-fold excess, and polymixin B (PMX) treatment. The first 2 controls eliminated apoptosis, but polymixin B had no effect. This supports that apoptosis is mediated by HSP60, not a contaminant. D, DNA fragmentation by CDD assay. Groups same as in C. E, Representative images of apoptotic cardiac myocytes after TUNEL stain: no apoptosis (1); apoptotic cardiac myocytes positive for DNA fragmentation (brown-stained nuclei) (2 to 4). F, Graph summarizes cytochrome *c* release from mitochondria (n=6 per group). G, Representative Western analysis of released cytochrome *c* at 2 hours. Lower blot shows GAPDH as loading control. H, AIF release at 2 hours from the mitochondria. Lower blot shows GAPDH as loading control. *$P<0.05$ vs all others; +$P<0.05$ vs no treatment (designated as the letter C [control]) (n=6 to 12 per group). Legend for C and D is shown at top right. RLU indicates light units generated after cleavage of Z-DEVD-aminoluciferin by caspase 3.

induced apoptosis. Thus, only blocking TLR4 inhibited rhHSP60 induced apoptosis. CD14 antibody together with the TLR4 blocking antibody had no effect on the observed decrease in apoptosis with the TLR4 blocking antibody alone (Figure 2C).

Effect of Endotoxin

Endotoxin contamination has often been used as an argument against evidence showing that HSP60 or other ligands activate TLR4. The low endotoxin rhHSP60 used in our experiments is the preparation cited by critics as free of significant contamination and the gold standard for HSP60 interactions.[3] In our experiments, heat inactivation, anti-HSP60 antibody, and polymixin B treatment were used as controls. To further exclude the effects of HSP60 as being secondary to endotoxin contamination, DNA fragmentation (CDD assay) was compared after treatment with rhHSP60, TNF-α and endotoxin (Cambrex). The same protocol was used as in the previous

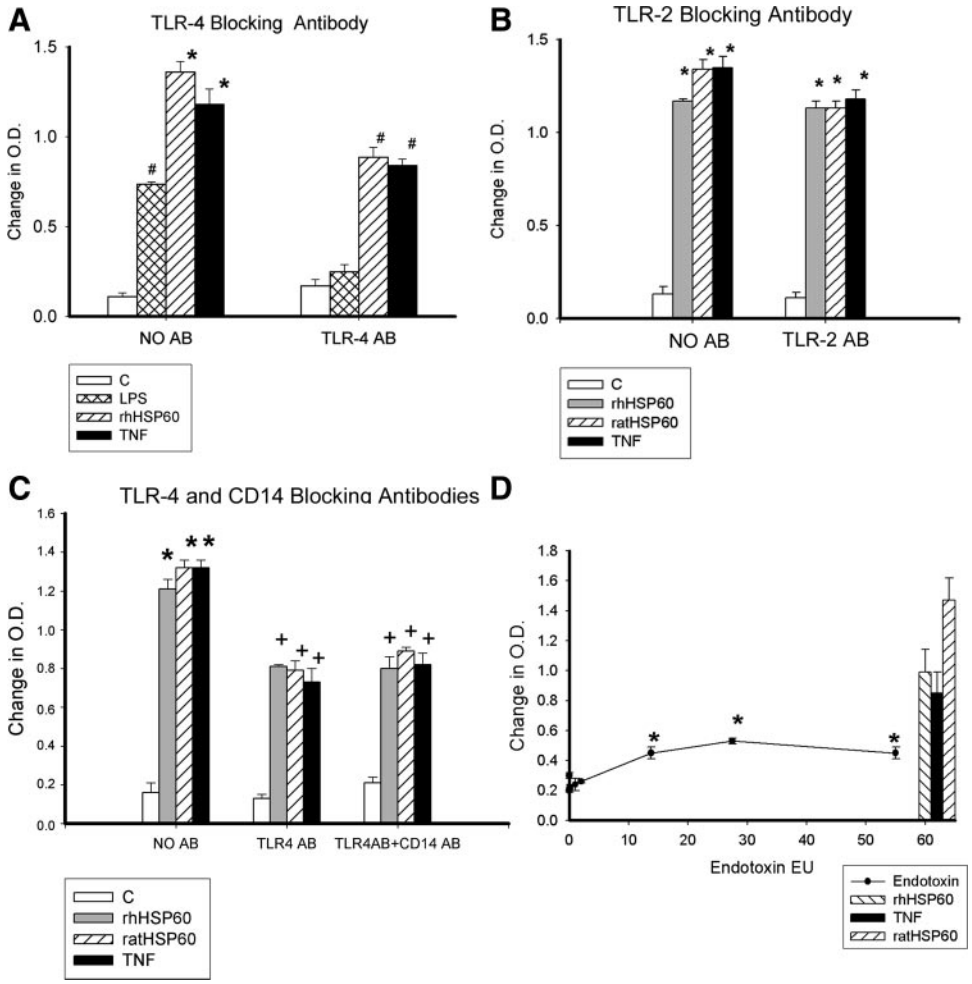

Figure 2. Blocking antibodies. A, Graph summarizes effect of TLR4 blocking antibody on HSP60-induced apoptosis (DNA fragmentation). B, Graph showing effect of TLR-2 blocking antibody on HSP60-mediated apoptosis. C, Graph summarizes effect of combination of TLR4 and CD14 blocking antibodies on HSP60-mediated apoptosis. CD14 blocking antibody alone had no effect (data not shown). D, Effect of endotoxin treatment. The effect of increasing concentrations of endotoxin on apoptosis were compared with rhHSP60 and TNF-α. DNA fragmentation was measured as an end point. As shown in the graph, only with 13.75 EU of endotoxin did significant apoptosis occur. Further increases in endotoxin concentration did not increase apoptosis. Amount of apoptosis was markedly less than that seen with rhHSP60 (first cross-hatched bar), TNF-α (black bar), and ratHSP60 (cross-hatched bar on far right). Endotoxin contamination in HSP60 preparations was $<$1 EU/mL. Results summarize 3 different experiments (n=6 to 9 per group). *$P<0.01$ vs all others; #$P<0.01$ vs no treatment (designated as the letter C [control]), LPS plus TLR4 antibody; +$P<0.05$ vs no treatment. For D, *$P<0.05$ vs control.

experiment. For these experiments, the low endotoxin rhHSP60 was treated with polymixin B–agarose before use. This reduced endotoxin levels to <0.05 EU/mL. Endotoxin from 0.1 to 2.0 EU/mL had no effect on DNA fragmentation (Figure 2D). Only when endotoxin was increased to 13.75 EU/mL was DNA fragmentation seen, and this plateaued at a level half that seen with rhHSP60 or TNF-α.

ExHSP60 Activates NFκB

TLR4 activates MYD88 and IRAK, which activate TRAF6, which leads to activation of NFκB and p38.[15,16] Therefore, we examined the effect of HSP60 on NFκB activation. As shown in Figure 3A,1 μg/mL rhHSP60 resulted in phosphorylation of IκB by 20 minutes ($P<0.05$ versus control). Likewise, treatment with rhHSP60 led to degradation of IκB, which was significantly reduced by 80 minutes ($P<0.05$ versus C, Figure 3B). NFκB was activated by 20 minutes of treatment with either rhHSP60 or ratHSP60 (Figure 3C). Activation declined after 80 minutes of treatment. To investigate the significance of NFκB activation for subsequent apoptosis, cells were pretreated with NFκB binding decoys as previously described.[17] These binding decoys reduced DNA fragmentation caused by either rhHSP60 or TNF-α (Figure 3D). Control cells were treated with a scrambled sequence.

To determine whether TLR4 binding and NFκB activation were essential for caspase 3 activation, caspase 3 activity was measured after pretreatment with TLR4 blocking antibodies and NFκB binding decoys as in the DNA fragmentation experiments (Figure 3E). TLR4 blocking antibodies completely inhibited rhHSP60 and LPS mediated activation of caspase 3, as did the NFκB binding decoys. In contrast, the TLR4 blocking antibodies and the NFκB binding decoys decreased TNF-α–mediated caspase 3 activation but did not completely block it. A scrambled decoy sequence (Scr) had no effect on caspase 3 activation by any of the treatments. The same treatments did not block release of cytochrome *c* or endonuclease G, but these assays are less sensitive (data not shown).

TLR4 Signaling and p38

TLR4 signaling is known to activate NFκB and has recently been found to activate p38.[15,16] HSP27 phosphorylation was examined as an index of p38 activation. Neither rhHSP60 nor TNF-α treatment had an effect on HSP27 phosphorylation (Figure 4A/B). Total HSP27 levels did not change with either treatment (Figure 4B). NFκB activation by rhHSP60 was thought to be mediated by TLR4. TLR4 blocking antibodies were used to test the association between TLR4 activation and NFκB activation. As shown in Figure 4C, pretreatment with TLR4 blocking antibodies prevented activation of NFκB.

rhHSP60 and TNF-α both released AIF from the mitochondria (Figure 1H), indicating that the caspase independent

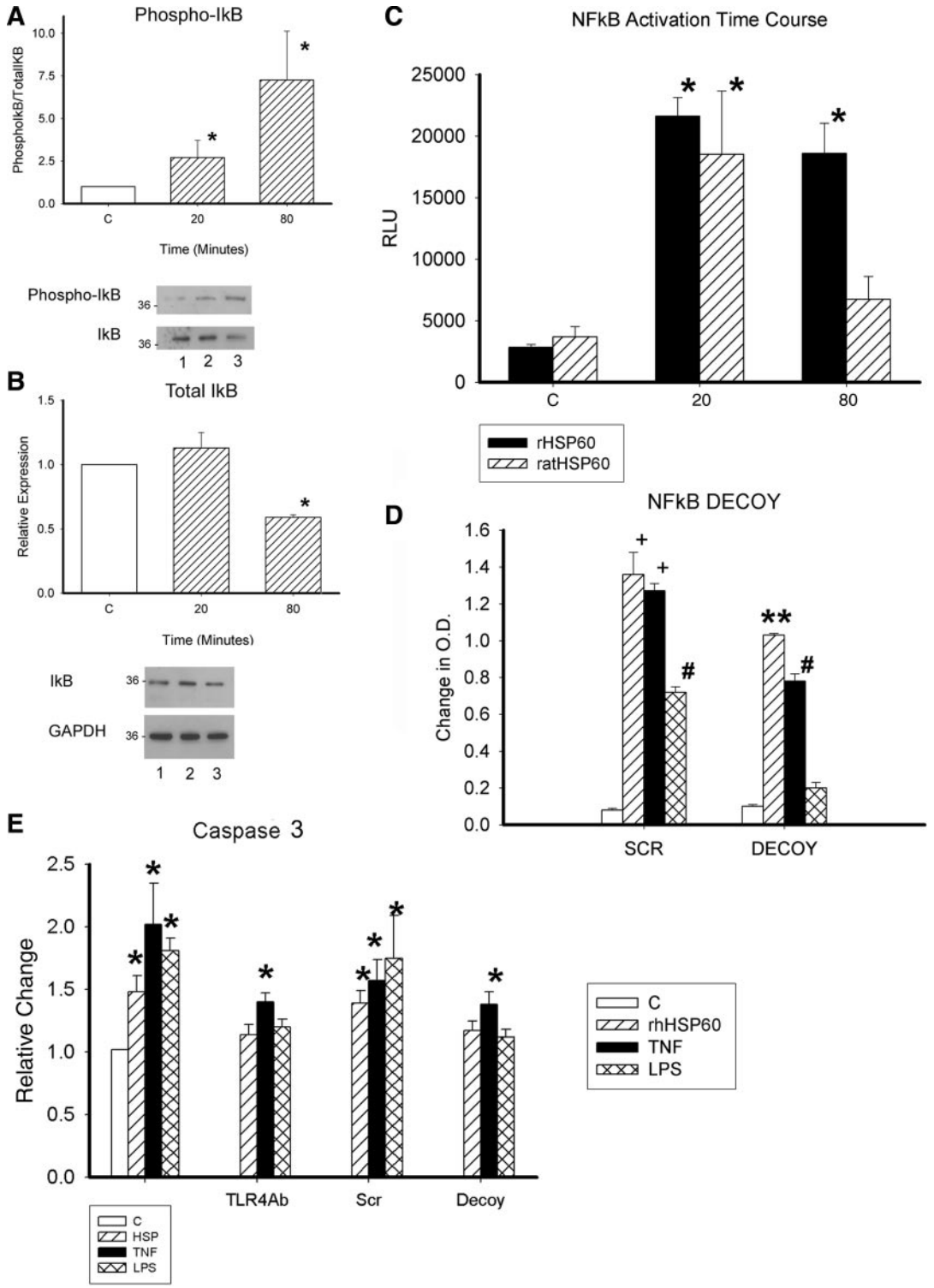

Figure 3. NFκB activation by HSP60. A, IκB-α phosphorylation. Graph summarizes results of 5 experiments. Results normalized to total IκB-α. Phosphorylation increased at 20 and 80 minutes. Representative Western analysis is shown (bottom). The letter C designates no treatment (control). B, IκB degradation. Graph summarizes results of 4 separate experiments. Total IκB was normalized to GAPDH as a loading control. IκB was significantly decreased at 80 minutes. Representative Western analysis is shown (bottom). C, Graph showing NFκB activation within 20 minutes after adding either rhHSP60 or ratHSP60. Activation declined over the ensuing hour. Reaction is chemiluminescent, and data are relative light units (RLU). D, Graph summarizing effect of NFκB binding decoy, which inhibited LPS-mediated apoptosis and partially inhibited HSP60 and TNF-α–mediated apoptosis. SCR indicates scrambled sequence decoy; C, control (no treatment). E, Upstream inhibitors and caspase 3 activity. Graph summarizes the effect of TLR4 blocking antibodies and the NFκB decoy on caspase 3 activity; bars on the far left are in the absence of antibody or decoy. TLR4 blocking antibody and NFκB decoy both block HSP60 and LPS-mediated caspase 3 activation. TNF-α–induced caspase 3 activity was reduced but not abolished by these treatments. *$P<0.05$ vs control (no treatment, designated as the letter C); +$P<0.05$ vs all; **$P<0.05$ vs control (C), C decoy, LPS decoy, LPS, TNF-α decoy; #$P<0.05$ vs C, C decoy, LPS decoy (n=4 to 11 per group).

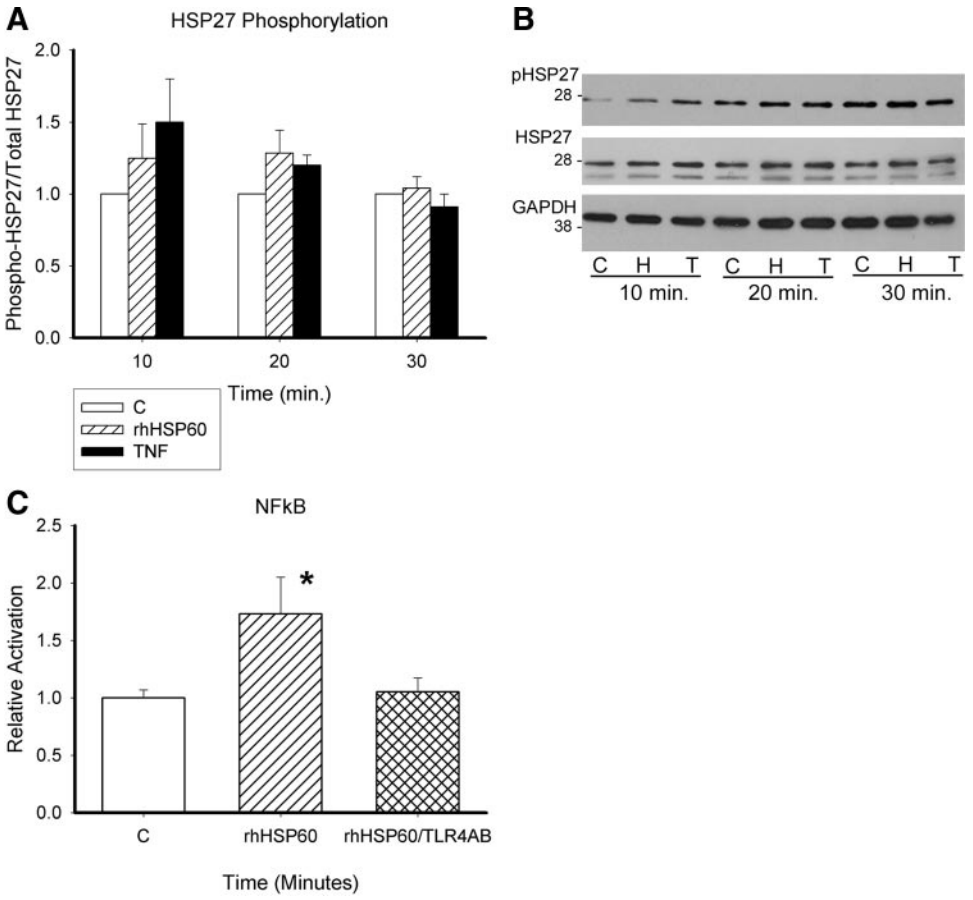

Figure 4. TLR4 signaling and p38 activation. A, p38. HSP27 phosphorylation is an index of p38 activation. Neither HSP60 nor TNF-α treatment resulted in HSP27 phosphorylation. Control (no treatment) is designated as the letter C. B, Representative Western analysis for phospho-HSP27, total HSP27, and GAPDH (same blot). Although there is variation in the amount of phospho-HSP27, this when averaged over groups was not significant. The letter C indicates control; H, HSP60; T, TNF-α C, TLR4 blocking antibodies and NFκB. Antibodies were given 30 minutes before treatment with HSP60. NFκB activity was measured at 20 minutes. TLR4 blocking antibodies inhibited NFκB. Different rhHSP60 lots likely account for variation in amount of activation. Activation was normalized to control. *$P<0.05$ vs C and HSP60/TLR4AB.

apoptosis pathway was activated. To further investigate the role of this pathway in HSP60 induced apoptosis, experiments were repeated in the presence of the caspase inhibitor DEVD-cho (10 μmol/L). Pretreatment with DEVD-cho completely blocked activation of caspase 3 by either rhHSP60 or TNF-α (Figure 5A). In contrast, inhibition of caspase 3 failed to block DNA fragmentation (Figure 5B). Even 5 times the concentration of DEVD-cho needed to inhibit caspase 3 had no effect on DNA fragmentation induced by ratHSP60 (Figure 5B).

Cytokines

Activation of TLR4 leads to production of cytokines. Therefore, we examined TNF-α, IL-1β and IL-6 mRNA by real time PCR after 3 hours of treatment. IL-1β and TNF-α mRNA more than doubled after treatment with rhHSP60 and almost doubled with TNF-α treatment ($P<0.05$, Figure 5D/E). IL-6 mRNA did not change (Figure 5C).

NFκB Activation and Apoptosis

The dichotomous nature of NFκB is well known, with NFκB activation leading either to cell protection or inflammation/apoptosis. The factors differentiating between these 2 opposite effects are poorly understood. It has recently been reported that the p50/p65 dimer is associated with greater cell injury after hypoxia and increased expression of the proapoptotic proteins bim and noxa and decreased Bcl-Xl.[18,19] We investigated expression of bim and Bcl-Xl (no access to suitable noxa antibody). rhHSP60 treatment did not alter expression of these proteins at 19 hours (Figure 6A).

ExHSP60 treatment led to increased IL-1β and TNF-α mRNA. To determine the role of these cytokines in apoptosis, myocytes were treated with neutralizing antibodies for IL-1β and TNF-α. TNF-α antibodies inhibited exHSP60-induced apoptosis, as measured by DNA fragmentation (Figure 6B). IL-1β antibodies alone did not block apoptosis, but a combination of IL-1β and TNF-α antibodies did. Thus, DNA fragmentation after treatment with exHSP60 occurred via the production of TNF-α as a downstream event after TLR4 activation.

Recombinant Versus Endogenous HSP60

We postulated that ratHSP60 released after injury to the myocyte (hypoxia/reoxygenation) would have posttranslational modification that would result in a greater injury to cultured myocytes than with rhHSP60. Purification of the protein is shown in Online Figure I. Others have reported that HSP60 is glycosylated and can lead to cell death, but we were unable to demonstrate this using purified ratHSP60 from which all albumin had been removed (data not shown).[20]

Both HMGB1 and LPS bind TLR4. To determine whether these molecules and rhHSP60 bound to the same site on TLR4, competitive binding experiments were done. Neither compound interfered with rhHSP60 binding, as shown in Online Figure II. This is consistent with the finding that CD14, necessary for LPS binding, was not needed for rhHSP60 activation of TLR4.

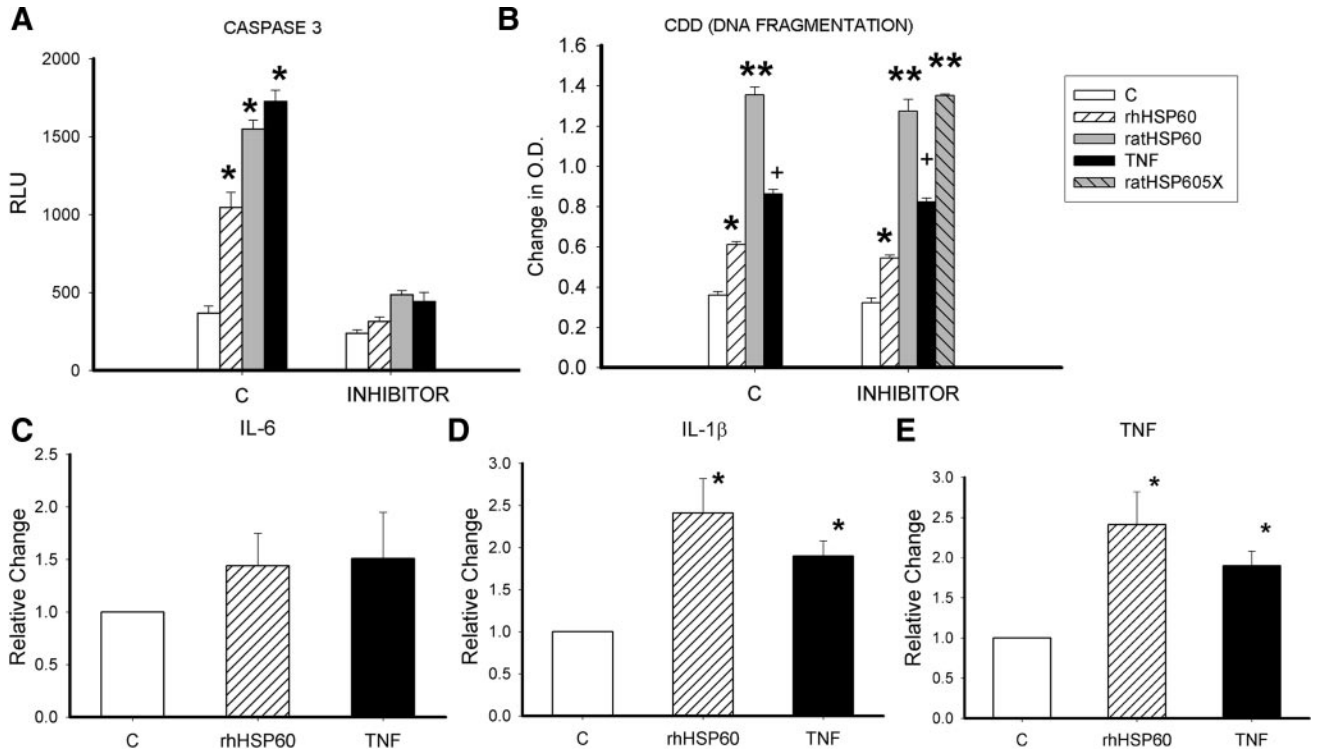

Figure 5. A, Effect of caspase 3 inhibitor, DEVD-cho, on caspase 3 activation by HSP60 and TNF-α. Relative light units (RLU), generated by cleavage of a DEVD compound, are shown. B, Effect of caspase 3 inhibitor, DEVD-cho, on DNA fragmentation by CDD assay. Results summarize 3 different experiments (n=9 to 12 per group, except for 5× inhibitor, where n=3). Figure legend inset applies to graphs A and B. RatHSP605X, 5-fold greater DEVD-cho to demonstrate there is no effect on DNA fragmentation. C through E, Real-time PCR normalized to GAPDH. C, IL-6. D, IL-1β. E, TNF-α. *P<0.05 vs C (control); **P<0.05 vs all; +P<0.05 vs rhHSP60 and C (n=4 to 7 per group).

Discussion

These experiments demonstrate that exHSP60 selectively binds cardiac myocytes. Binding is both saturable and competitive. Binding of exHSP60 is followed by rapid activation of NFκB, followed by cytochrome *c* and AIF release from the mitochondria, caspase 3 activation, and DNA cleavage. Thus, exHSP60 causes apoptosis in cardiac myocytes. The involved signaling pathways are summarized in Figure 7. Treatment with rhHSP60 increased expression of Il-1β and TNF-α. Activation of TLR4 is associated with increased expression and release of cytokines. A lingering controversy over the effects of HSP60 has been that any effect is really that of a

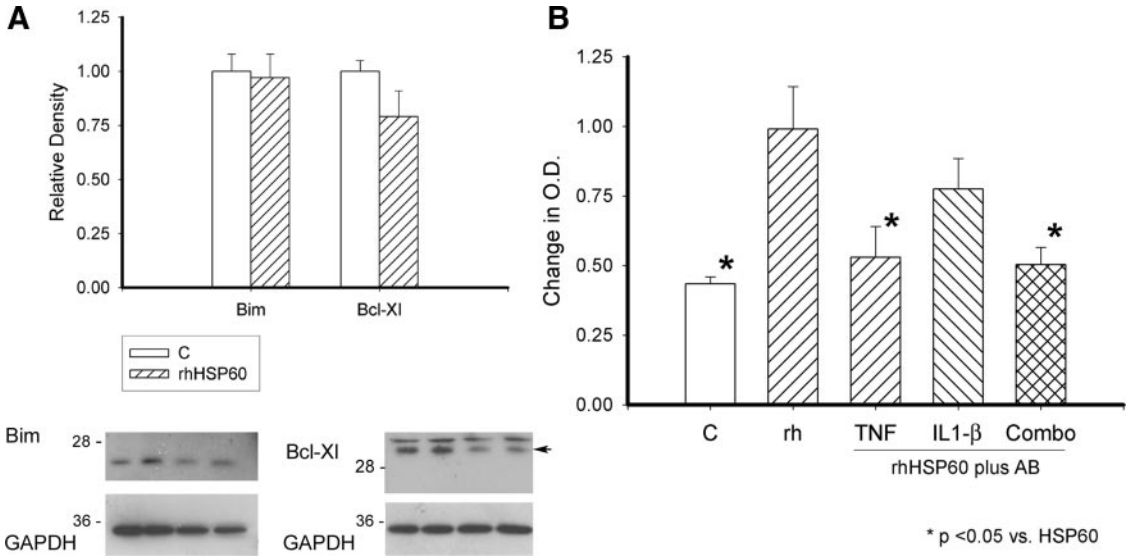

Figure 6. A, Expression of Bim and Bcl-Xl after treatment with rhHSP60. Normalized to GAPDH and control. Representative Western analyses are shown (bottom). B, Effect of cytokine-neutralizing antibodies on apoptosis (DNA fragmentation). TNF and IL-1β indicates each antibody alone; Combo, combined TNF and IL-1β antibodies. *P<0.05 vs rh (rhHSP60) (n=7 to 10 per group).

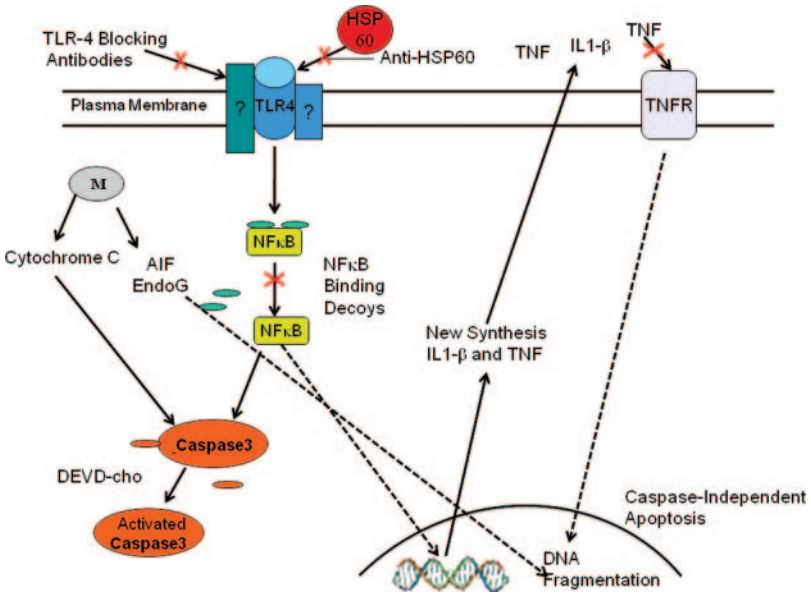

Figure 7. Diagram summarizing involved signaling pathways and effect of inhibitors. Inhibition of TLR4 or NFκB completely blocked caspase 3 activation but did not completely inhibit DNA fragmentation. This could be secondary to differences in binding HSP60 to TLR4 (compared to LPS), to involvement of a multiprotein complex including TLR4 as a receptor for HSP60, or to the involvement of a second receptor, as discussed in the text. M indicates mitochondrion. Solid lines indicate signaling from mitochondrion, receptors, and nucleus; dashed lines, signaling to the nucleus.

small amount of endotoxin. Here, it is shown that the levels of endotoxin associated with HSP60 following purification are trivial; furthermore, a far greater amount of endotoxin was needed to cause apoptosis, and the amount of apoptosis was significantly less than that seen with rhHSP60 or TNF-α.

A number of studies have established that HSP60 is a ligand for TLR4 in the immune system.[10,21] Cells outside the immune system have not been well studied. TLR4 has been shown to be present in myocytes, and recent studies have suggested a role for TLR4 activation in cardiac hypertrophy and the response to sepsis.[22–24] A mutant, nonfunctional TLR4 receptor is associated with smaller infarct size, but this reduced infarct size does not translate into preservation of function.[25,26]

As our understanding of the TLRs increases, it has become apparent that these receptors have pleiotropic effects, with both protective and destructive events triggered by their activation. In the immune system, TLR-2 and TLR4 have been implicated in signaling leading to apoptosis. TLR4, as well as TLR-2, has been described in macrophages as causing apoptosis, as well as the induction of the innate immune response.[27] In microglial cells, TLR4–mediated activation induced cell death, which is a mechanism by which activated immune cells are eliminated.[27] In the immune system, this would have an advantage, because self-destruction of an activated macrophage turns off the inflammatory response, preventing indefinite propagation.

Pathway to Apoptosis

In the present study, blocking antibodies were used to determine whether TLR4–mediated apoptosis secondary to exHSP60. As expected, TLR4 antibodies blocked LPS-mediated apoptosis. TLR4 antibodies also consistently reduced DNA fragmentation from either HSP60 or TNF-α treatment, but did not completely abolish it. Caspase 3 activation by either HSP60 or LPS was completely inhibited by the TLR4 blocking antibodies, but TNF-α–mediated activation of caspase 3 was not completely inhibited. TLR4 blocking antibodies completely inhibited activation of NFκB. Blocking antibody to TLR-2 had no effect on HSP60-mediated apoptosis, although HSP60 has been reported to be a ligand of TLR-2.[12] The competitive binding studies indicate that HSP60 binds to a different site on the TLR4 receptor than LPS or HMGB1; thus, the TLR4 blocking antibody may not completely inhibit activation by HSP60. Distinct binding sites for HSP60 and LPS is further supported by the fact that LPS requires CD14 for binding to TLR4, whereas this is not the case for HSP60. Alternatively, TLR4 may be part of a multiprotein receptor complex or more than 1 receptor may be involved. Certainly, the TLRs have been increasingly found to form complex receptors, rather than confining themselves to homodimerization.[28,29] Differences in inhibition of end points may also reflect differences in the intensity of downstream signaling pathways' activation.

Similarly, there are several possible reasons for inability to block the release of cytochrome *c* and endonuclease G. Cytochrome *c* and endonuclease G release may be mediated by TNF-α synthesized in response to TLR4 activation. HSP60 may be acting through a different receptor and thus is not blocked, or the TLR4 blocking antibodies, which were developed to block LPS, may not completely inhibit HSP60 binding/activation of TLR4, because HSP60 clearly binds at a different site than LPS.

Location and Function

HSP60 has been found in the plasma of healthy individuals with 25% of them having levels similar or higher to the concentrations used in the present study.[11] In addition, we have found HSP60 in the plasma of rats, although levels were much lower than those reported in British civil servants, but the intracardiac levels were likely much higher.[30] Paracrine signaling by HSP60 released by injured cells could extend injury. Posttranslational modification of HSP60 may be a key factor in the toxicity of exHSP60. We theorized that the ratHSP60 compared to rhHSP60 might have undergone *O*-glycosylation; however, we were unable to demonstrate

O-glycosylation of the purified HSP60. Glycosylated intracellular HSP60 has been reported to be associated with increased apoptosis in diabetes.[20] The differences in HSP60-mediated apoptosis between the rat and recombinant human (rh) preparations were less apparent with later lots of the recombinant protein. Nonetheless, it is likely that posttranslational modification does influence the toxicity of extracellular HSP60, and further work will be needed to define the posttranslational modifications of the protein, and their effect on function.

Mechanism of HSP60 Release From Cardiac Myocytes

Recently, we have reported that HSP60 is released from cardiac myocytes via exosomes, a pathway not associated with glycosylation.[31] It is not known in what form HSP60 is released in other settings. Analysis of banked plasma samples, such as those of the Whitehall study, involves the processing of samples subjected to repeat freeze thaw cycles, which can disrupt exosomes. The stability of exosomes within the plasma is unknown, and although HSP60 is released in exosomes, it may not remain associated with them. Ongoing work in our laboratory is addressing the fate, function, and stability of exosomes.

HSP60 has been implicated in atherosclerosis.[32] TLR4 has been demonstrated to have a role in atherosclerosis and in outward arterial remodeling in the atherosclerotic ApoE3 mouse.[33,34] The innate immune system and the TLRs have been studied extensively in immune cells; however, work addressing the function of the TLRs outside the immune system is nascent.[35]

In the present study, we report that extracellular HSP60 induces apoptosis in cardiac myocytes. Apoptosis, as assessed by DNA fragmentation, can be reduced by blocking antibodies to TLR4 and by NFκB decoys, but not completely inhibited, even though similar treatment blocked LPS-induced apoptosis. Three distinct controls for endotoxin showed no evidence for involvement of a molecule other than HSP60 in the mediation of apoptosis. Further work will be needed to identify the complex to which HSP60 is binding and to further define the pathway leading from HSP60 binding to apoptosis.

The present findings have implications for cardiac myocyte loss in heart failure. Previously, we have shown that HSP60 levels are doubled in the failing heart. In ongoing work, we have found that TNF-α drives the increase in HSP60 in the heart through activation of NFκB (Y Wang, L Chen, N Hagiwara, AA Knowlton, manuscript submitted for publication). HSP60 in heart failure cardiac myocytes localizes to the plasma membrane and the cell surface.[30] HSP60 can also be found in the plasma in heart failure along with TNF-α[30] ExHSP60, as we show in the present study, activates TLR4, leading to cytokine production and myocyte apoptosis. Release of TNF leads both to further myocyte apoptosis and increased HSP60. Thus there is potentially a vicious cycle of TNF-α and HSP60 expression leading to greater levels of HSP60, which may then be released from the cell, followed by apoptosis mediated by TLR4 activation by HSP60 and subsequent TNF-α release.

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Disclosures

None.

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