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THE ROLE OF ENDOGENOUSLY PRODUCED EXTRACELLULAR HSP72 IN MONONUCLEAR CELL REPROGRAMMING

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Abstract

Intracellular heat shock protein 72 (Hsp72) is known to serve a broad cytoprotective role. Recent data indicate that stressed cells can release Hsp72 into the extracellular compartment, although the biological function of extracellular Hsp72 remains to be fully elucidated. Because extracellular Hsp72 has been demonstrated to interact with Toll-like receptor 4, we hypothesized that endogenously produced and released Hsp72 would reprogram the mononuclear cell responses to LPS. THP-1 cells treated with LPS were used as a model for nuclear factor (NF)- κ B activation. Heat shock conditions consisted of incubation at 43°C for 1 h. Control cells were incubated at 37°C. Twenty four hours after incubation, heat shock conditioned media (HSCM) and control media (CM) were centrifuged, and the respective cells were discarded. A separate group of naive THP-1 cells were then incubated with either HSCM or CM for 18 h and then stimulated with LPS (1 μ g/mL). Heat shock significantly increased Hsp72 in HSCM compared with CM. In THP-1 cells transfected with an NF- κ B luciferase reporter plasmid, the addition of HSCM attenuated subsequent LPS-mediated luciferase activity compared with cells incubated in CM. The addition of HSCM also attenuated LPS-mediated NF- κ B-DNA binding and I κ B α degradation. Heat shock protein 72-mediated inhibition of NF- κ B activation was further corroborated by a significant decrease in TNF- α production. When HSCM and CM were subjected to Hsp72 depletion via adenosine triphosphate-agarose binding, LPS-mediated activation of NF- κ B was partially restored, suggesting that Hsp72 is partially responsible for cellular reprogramming in response to HSCM. These data demonstrate that endogenously produced and released extracellular Hsp72 has the ability to reprogram the *in vitro* response to endotoxin in cultured human mononuclear cells.

Keywords

LPS; preconditioning; heat shock proteins; endotoxin tolerance; NF- κ B; inflammation; signal transduction; monocytes

INTRODUCTION

The heat shock response is an evolutionarily conserved, primitive endogenous cellular defense mechanism characterized by the rapid intracellular expression of a class of proteins collectively known as heat shock proteins (Hsps). The structure, mode of regulation, and function of Hsps are highly conserved among different species, and Hsps have been identified in virtually all

eukaryotic and prokaryotic species examined to date (1–3). Heat shock proteins are generally thought to maintain cellular homeostasis by acting as molecular chaperones, facilitating the proper folding and assembly of nascent polypeptides, and assisting in the refolding and stabilization of damaged peptides, although until relatively recently, Hsps were considered to be exclusively intracellular proteins (1–3). However, a growing body of literature suggests that Hsps may also exist and function outside of the cell. For example, several studies suggest that cellular stress results in the increased surface expression and release of Hsps (4,5).

The release of the 70-kd Hsp72 into the extracellular environment was first reported in cultured rat embryo cells after exposure to increased temperature in the late 1980s (6). The mechanism of release seemed to be specific, in that Hsp72 release could not be reproduced by induction of cell lysis through exposure to nonionic detergents. However, the mechanism did not seem to involve classic secretory pathways either because it was not inhibited by either colchicine or monensin, both of which inhibit this pathway. Finally, Hsp72 synthesized in the presence of a lysine amino acid analog (aminoethyl cysteine) was not released from these cells, suggesting that the altered protein structure prevented interaction with an as yet unidentified, but specific, secretory mechanism (6).

The release of Hsps such as Hsp72 may serve to signal an impending danger signal to neighboring cells (7). Several properties would suggest Hsp72 to be a biologically plausible and likely candidate to serve as a host danger signal. Collectively, Hsps are the most abundant intracellular proteins, representing up to 10% of the total protein content in the cell (8). Heat shock protein 72 in particular is markedly induced in response to a diverse range of cellular insults, including increased temperature, oxidative stress, glucose deprivation, chemical exposure, I/R injury, ultraviolet radiation, and infectious agents such as LPS. Therefore, Hsp72 or additional inducible stress proteins, by virtue of their relative abundance during times of stress, are reliable markers of cell stress.

Similar to LPS, extracellular Hsp72 elicits nuclear factor (NF)- κ B-dependent, proinflammatory gene expression via Toll-like receptor (TLR) 4 and TLR-2 (9,10). Therefore, the release of Hsp72, whether from dying, necrotic cells or viable, but damaged, cells can activate the host innate immune response, acting as both a chaperone and a cytokine (chaperokine) (9–12). Consistent with these observations, our laboratory has recently shown that preconditioning human peripheral blood monocytes and THP-1 cells with extracellular, human recombinant Hsp72 abrogated the response to a subsequent challenge with LPS, partially through a mechanism involving inhibition of the NF- κ B pathway (13). However, these studies linking extracellular Hsps with inflammation should be interpreted cautiously because some investigators have been unable to replicate these findings using endotoxin-free, recombinant Hsps, suggesting that these effects were due to endotoxin or other bacterial contaminants in the recombinant protein (14). Accordingly, we developed an *in vitro* model whereby cultured human mononuclear cells are induced to release extracellular Hsp72 after exposure to heat shock. The conditioned media from these cells are subsequently used to precondition naive mononuclear cells before subsequent exposure to LPS. We hypothesized that endogenously produced and released extracellular Hsp72 can reprogram the cellular response to LPS in a manner analogous to that of classic endotoxin tolerance (15).

MATERIALS AND METHODS

Cell culture

The human acute monocytic leukemia cell line THP-1 was used in all experiments and was purchased from American Type Culture Collection (Manassas, Va). THP-1 were cultured in RPMI 1640 medium containing 10% fetal bovine serum, kanamycin, 2- β -mercaptoethanol, and 2% glutamine (pH 7.35).

Release of extracellular Hsp72

THP-1 cells were subjected to classic heat shock (43°C for 1 h) and allowed to recover at 37°C without changing the culture media. Media were collected at various time points after heat shock and centrifuged at 1,200 rpm for 10 min at room temperature. Extracellular Hsp72 was measured using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA; Stressgen, Vancouver, British Columbia, Canada) according to the manufacturer's protocol. In separate experiments, THP-1 cells were pretreated with inhibitors of the classical protein secretory pathways: brefeldin A (Sigma-Aldrich, St. Louis, Mo; 25 µg/mL) or monensin (Sigma-Aldrich; 8 µg/mL) for 5 h before heat shock. As an additional control, THP-1 cells were pretreated with cycloheximide (Sigma-Aldrich; 10 µg/mL) for 1 h before heat shock. Media were harvested for Hsp72 ELISA, and cytoplasmic protein was isolated for Western immunoblot of intracellular Hsp72 as previously described (13,16). As a final control, we measured the concentration of intracellular Hsp72 via ELISA. In all experiments, cell viability was determined via trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (13). As an additional control, media were harvested after heat shock for lactate dehydrogenase Cytotoxicity BioAssay (US Biological, Swampscott, Mass) that was performed according to the manufacturer's instructions.

Heat shock conditioned media experimental conditions

The first group of THP-1 cells were subjected to classic heat shock (43°C for 1 h) and allowed to recover for 24 h at 37°C without changing the culture media. After this 24-h incubation period, the cells and media were collected and centrifuged at 1,200 rpm for 10 min at room temperature. These conditions result in a consistent and reproducible release of extracellular Hsp72 in THP-1 cells. The cell pellet was subsequently discarded, and the heat shock conditioned media (HSCM) were saved and immediately added (undiluted) to a second group of naive THP-1 cells that had been previously maintained in basal growth media at 37°C. Control conditioned media (CM) were generated in a similar manner from naive THP-1 cells maintained at 37°C. The second group of naive THP-1 cells was maintained in either HSCM or CM for 18 h then stimulated with 1 µg/mL LPS (*Escherichia coli*, serotype O55:B5; Sigma-Aldrich) to stimulate activation of the NF-κB pathway. The duration of LPS stimulation was based on the experimental end point (see below). As an additional control, recombinant human Hsp72 was generated as previously described (13) and administered to naive THP-1 cells at doses consistent with the amount of Hsp72 found in control and HSCM. Recombinant Hsp72 was administered 18 h before subsequent LPS administration (1 µg/mL).

Transient transfection and luciferase assays

Nuclear factor-κB transcriptional activation in response to LPS stimulation was measured by transiently transfecting cells with an NF-κB-luciferase reporter plasmid as previously described (13). Briefly, THP-1 cells were washed twice with STBS (25 mM Tris Cl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 0.7 mM CaCl₂, and 0.5 mM MgCl₂) and pelleted. The cells were transfected using diethylaminoethyl-dextran. For each 1×10^6 THP-1 cells, 1 µg of NF-κB reporter plasmid was mixed with 10 µg diethylaminoethyl-dextran and 1 mL of STBS buffer and added to the pelleted THP-1 cells. The cells were incubated at 37°C for 20 min, washed twice with STBS, then resuspended in complete RPMI medium at a concentration of 6×10^6 THP-1 cells/9 mL media. The transfected cells were seeded for 24 h in tissue culture flasks before exposure to the experimental conditions described in the previous sentences. After 4 h of LPS stimulation in the presence of either HSCM or CM, cells were harvested for measurement of luciferase activity according to the manufacturer's instructions (Promega, Madison, Wis) using a Berthold AutoLumat LB953 luminometer. Luciferase activity is reported as fold induction over control cells and corrected for total cellular protein.

Electromobility shift assays

Nuclear factor- κ B-DNA binding in response to LPS stimulation was measured by electromobility gel shift assay (EMSA) as previously described (13). After 1 h of LPS stimulation in the presence or absence of either HSCM or CM, cells were harvested for isolation of nuclear proteins. All nuclear protein extraction procedures were performed on ice with ice-cold reagents. Cells were washed twice with phosphate-buffered saline (PBS) and harvested by scraping. Cells were pelleted in 1 mL of PBS at 6,000 rpm for 5 min. The pellet was washed twice with PBS and resuspended in lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*-ethane sulfonic acid, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.2% vol/vol Nonidet P-40, 1 mM dithiothreitol [DTT], and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]). The suspension was incubated on ice for 5 min and centrifuged at 4°C and 6,000 rpm. The supernatant was discarded, and one cell pellet volume of extraction buffer (20 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, pH 7.9, 420 mM NaCl, 0.1 M EDTA, 1.5 mM MgCl₂, 25% vol/vol glycerol, 1 mM DTT, and 0.5 mM PMSF) was added to the nuclear pellet and incubated on ice for 15 min. Nuclear proteins were isolated by centrifugation at 14,000 rpm at 4°C for 15 min. Protein concentrations of the resultant supernatants were determined using the Bradford assay. Nuclear proteins were stored at -70°C until used for EMSA.

Electromobility gel shift assays were performed as previously described (13). A double-stranded oligonucleotide probe (5'-GTGGAATTCCTCTGA-3') corresponding to the NF- κ B consensus sequence was labeled with γ -[³²P] adenosine triphosphate (ATP) using T4 polynucleotide kinase (GIBCO BRL, Gaithersburg, Md) and purified in Bio-Spin chromatography columns (BioRad, Hercules, Calif.). For each sample, 10 μ g of nuclear proteins was preincubated with EMSA buffer (12 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, pH 7.9, 4 mM Tris-HCL, pH 7.9, 25 mM KCL, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 50 ng/mL poly [d (I-C)], 12% glycerol vol/vol, and 0.2 mM PMSF) on ice for 10 min before addition of the radiolabeled oligonucleotide probe for an additional 10 min. Supershift analyses were conducted using anti-p50 and anti-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif), which were added 5 min before the radiolabeled probe was added. Protein-nucleic acid complexes were resolved using a nondenaturing polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide/bisacrylamide) and run in 0.5 $\times$ 45 mM Tris-HCL, 45 mM boric acid, 1 mM EDTA for 1 h at constant current (30 mA). Gels were transferred to Whatman 3M paper, dried under a vacuum at 80°C for 1 h, and exposed to photographic film at -70°C with an intensifying screen.

Western blot analysis

I κ B α degradation in response to LPS stimulation was measured by Western blot analysis. After 15, 30, and 60 min of LPS stimulation in the presence or absence of either HSCM or CM, cells were harvested for isolation of cytosolic proteins as previously described. Whole cell lysates containing 50 μ g of protein were boiled in equal volumes of loading buffer (125 mM Tris-HCL, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2- β -mercaptoethanol). Proteins were separated electrophoretically on 8% to 16% Tris-glycine gradient gels (Novex, San Diego, Calif) and subsequently transferred to nitrocellulose membranes (Novex) using the Novex Xcell mini-gel system. For immunoblotting, membranes were blocked with 5% nonfat dried milk in Tris-buffered saline (TBS) for 1 h. Primary antibody against I κ B α (Santa Cruz Biotechnology) was applied at 1:250 dilutions for 1 h. After washing twice with TBS containing 0.05% Tween 20, secondary antibody (peroxidase-conjugated goat antirabbit immunoglobulin G; Stressgen, Victoria, British Columbia, Canada) was applied at 1:10,000 dilution for 1 h. Blots were washed in TBS containing 0.05% Tween 20 twice for 10 min, incubated in commercial enhanced chemiluminescence reagents (Amersham, Buckinghamshire, UK), and exposed to photographic film.

Reverse-Transcriptase–Polymerase Chain Reaction

THP-1 cells were preconditioned with either CM or HSCM as described above. RNA was extracted with TRIzol and reverse-transcriptase–polymerase chain reaction (RT-PCR) as previously described (17). Toll-like receptor 4 primers used were sense 5'-CAGGGCTTTTCTGAGTCGTC-3' and antisense 5'-TGAGCAGTCGTGCTGGTATC-3' (PCR product, 224 bp). Polymerase chain reaction amplification for all primers was performed for 40 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 45 s, followed by one cycle of 72°C for 10 min. Product was run on a 1.5% agarose gel containing 10 µg ethidium bromide.

Flow cytometry

THP-1 cells were preconditioned with either CM or HSCM as described above and were washed and incubated with isotype control or TLR-4 antibodies (eBioscience, San Diego, Calif) for 1 h on ice. Cells were washed to remove unbound antibody and fixed with 1% paraformaldehyde (EM Sciences, Hatfield, Pa). Cellular surface expression of TLR4 was then analyzed via FACSCalibur using CellQuest Pro software.

Enzyme-linked immunosorbent assay

The presence of Hsp72 in HSCM and CM was measured using a commercially available sandwich ELISA (Stressgen, Vancouver). TNF- α production in response to LPS stimulation was also measured by a commercially available sandwich ELISA (R and D Biosystems, Minneapolis, Minn). All procedures were performed as recommended by the manufacturers.

Depletion of Hsp72 from HSCM and CM using ATP-agarose beads

First, we depleted Hsp72 from HSCM and CM using ATP-agarose beads (Sigma-Aldrich; Hsp72 is known to readily bind ATP). The ATP agarose powder was reconstituted with 0.92 mL of endotoxin-free reagent water per milliliter of lyophilized agarose. Heat shock conditioning media and CM were collected as described above and combined with ATP agarose at a concentration of 50 µL/mL media. The media were then allowed to sit at room temperature and rock for 1 h. As an additional control, HSCM and CM that were not treated with agarose were separated from the cells and rocked for 1 h as well. All treated media were then filtered underneath a sterile hood and then combined with transfected cells and subjected to the experimental conditions as described above.

Depletion of Hsp72 from HSCM and CM via immunoprecipitation

We next depleted Hsp72 from HSCM and CM using a blocking antibody to Hsp72. Heat shock conditioning media and CM were collected as described above and combined with anti-Hsp72 (Stressgen, Vancouver; 4.5 µg/mL), as well as protein A/G agarose beads (Santa Cruz Biotechnology) and allowed to sit at room temperature for 2 h. As a final control, HSCM media were treated with an isotype control antibody, protein A/G agarose beads, before LPS treatment. The HSCM and CM were centrifuged at 4,000 rpm $\times$ 5 min at 4°C and washed. All treated media were then filtered underneath a sterile hood and then combined with transfected cells and subjected to the experimental conditions as described above.

Statistical analysis

Continuous quantitative data were evaluated using one-way ANOVA and Student-Newman-Keuls test (SigmaStat version 3.10; Systat Software, Point Richmond, Calif). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Hsp72 is released into the extracellular compartment after heat shock of THP-1 cells

To induce the release of Hsp72 into the extracellular compartment (i.e., the cell culture media), THP-1 cells were exposed to heat shock conditions (43°C for 1 h) and allowed to recover at 37°C for up to 24 h. The media from these cells, HSCM, was collected for measurement of extracellular Hsp72. Control media was obtained in a similar fashion but from cells not exposed to the heat shock conditions and maintained at 37°C. As shown in Figure 1, heat shock resulted in an early and sustained release of extracellular Hsp72. Heat shock conditioned media obtained 24 h after heat shock contained an approximately 2-fold increase in Hsp72 when compared with CM (18.5 ± 5.9 vs. 8.5 ± 5.1 ng/mL; $P < 0.05$). In subsequent experiments, THP-1 cells were pretreated with inhibitors of the classical protein secretory pathways, brefeldin A or monensin for 5 h before heat shock. Neither inhibitor had a significant effect on Hsp72 release. Finally, THP-1 cells were pretreated with cycloheximide (10 µg/mL) for 1 h before heat shock. Cycloheximide treatment inhibited synthesis of intracellular Hsp72, as determined by both ELISA and Western immunoblot. However, cycloheximide treatment did not completely eliminate intracellular Hsp72 induction, suggesting that there may be a prestored basal level of intracellular Hsp72. Cycloheximide treatment did not affect extracellular Hsp72 release. As a final control, we measured extracellular Hsp72 in cell culture media at 37°C and 43°C. No Hsp72 was detected in the cell culture media (data not shown). Cell viability and lactate dehydrogenase cytotoxicity assays showed no significant differences in cell survival after these heat shock conditions (data not shown). Collectively, these data confirm that heat shock significantly increases Hsp72 levels in the media of cultured THP-1 cells and confirm previous studies suggesting a nonclassical mechanism of Hsp72 release (6,18–22).

HSCM treatment does not result in NF-κB activation

We next determined whether HSCM treatment in and of itself resulted in increased NF-κB activation in THP-1 cells transiently transfected with a 3× NF-κB promoter luciferase reporter plasmid. Naive THP-1 cells were treated with HSCM (containing endogenously produced and released Hsp72 as shown above) for 1, 3, 6, 12, and 18 h. As shown in Figure 2, HSCM did not increase NF-κB activation compared with baseline at any time point, although there was a nonsignificant trend for NF-κB activation at 12 h. As an additional control, naive THP-1 cells were treated with human recombinant Hsp72. We used a concentration that has been previously shown by our group to reprogram the subsequent cellular response to LPS in THP-1 cells (13). Again, Hsp72 at a concentration of 0.03 µg/mL did not significantly increase NF-κB activation compared with baseline at any time point.

HSCM reprograms the response to LPS in THP-1 cells

In these experiments, we determined if HSCM (containing endogenously produced and released Hsp72 as shown in the previous paragraphs) reprograms the subsequent cellular response to LPS. We addressed this question by measuring activation of NF-κB in THP-1 cells transiently transfected with a 3× NF-κB promoter luciferase reporter plasmid and treated with LPS in the presence of either CM or HSCM. Treatment with CM and LPS resulted in an approximately 35-fold induction of NF-κB activity over baseline (Fig. 3). In comparison, treatment with the combination of HSCM and LPS significantly attenuated NF-κB activity compared with cells treated with the combination of CM and LPS. As an additional control, we subjected fresh media alone (i.e., not containing THP-1 cells) to heat shock and then used these media for LPS exposure in transfected, naive THP-1 cells. Fresh media subjected to heat shock, in the absence of THP-1 cells, had no effect on subsequent cellular responses to LPS (data not shown). Total (measured by RT-PCR) and cell surface (measured by flow cytometry) TLR-4 expression was not altered by treatment with HSCM (Fig. 4). In addition, we measured the level of the anti-inflammatory cytokine IL-10 in the HSCM before and after LPS

administration by ELISA. There were no significant differences in IL-10 concentration between experimental groups (data not shown). Collectively, these data indicate that heat shock leads to the release of a factor(s) into culture media that reprograms the *in vitro* mononuclear cell response to LPS. Given the preliminary studies suggesting that heat shock produces an early and sustained release of Hsp72, as well as the fact that human recombinant Hsp72 induces cellular reprogramming in THP-1 cells (13), extracellular Hsp72 in the HSCM seems to be a likely candidate for these effects.

HSCM inhibits LPS-mediated I κ B α degradation and NF- κ B activation in THP-1 cells

To further validate our findings regarding HSCM and cellular reprogramming, we next examined the effect of HSCM on LPS-mediated activation of two key upstream signal transduction events in the I κ B α /NF- κ B pathway: I κ B α degradation and NF- κ B–DNA binding. To evaluate degradation of I κ B α , THP-1 cells were combined with either HSCM or CM, treated with LPS for varying lengths of times (0, 15, 30, or 60 min), and harvested for Western blot analysis. As shown in Figure 5, the combination of CM and LPS led to a more rapid degradation of I κ B α compared with the combination of HSCM and LPS. Activation of the NF- κ B pathway occurs after phosphorylation I κ B α by the I κ B α kinase (IKK), which is then polyubiquitinated and degraded by the 26 S proteasome system (23). There were no significant differences in activation of IKK (measured via kinase assay) or phospho-I κ B α (measured by Western immunoblot) between THP-1 cells preconditioned with HSCM or CM (data not shown).

To evaluate NF- κ B–DNA binding, THP-1 cells were combined with either HSCM or CM, treated with LPS for 1 h, and then harvested for EMSA. As shown in Figure 6, the combination of CM and LPS led to a relatively greater degree of NF- κ B–DNA binding when compared with the combination HSCM and LPS. Collectively, these data demonstrate that HSCM reprograms the cellular response to LPS, and that part of the reprogramming process involves attenuation of I κ B α degradation and subsequent attenuation of NF- κ B–DNA binding.

HSCM attenuates LPS-mediated TNF- α production

To determine if the observed effects on the I κ B α /NF- κ B pathway leads to a downstream, functional effect on I κ B α /NF- κ B–dependent proinflammatory gene expression, we measured the production of TNF- α after exposure to the experimental conditions via ELISA. Treatment with CM and LPS and CM resulted in a significant increase in TNF- α production (Fig. 7). In comparison, treatment with the combination of HSCM and LPS significantly attenuated TNF- α production compared with cells treated with the combination of CM and LPS. These data demonstrate that the reprogramming effects of HSCM include a functional effect on an I κ B α /NF- κ B–dependent gene target.

HSCM-mediated reprogramming is partially dependent on Hsp72

In these experiments, we determined if Hsp72 plays a role in the ability of HSCM to reprogram cellular responses to LPS. Given the intrinsic ability of Hsp72 to bind ATP (24), we incubated HSCM and CM with ATP-agarose to deplete the media of Hsp72. Incubation with ATP-agarose efficiently removed Hsp72 from HSCM as measured by Western blot analysis of the eluted ATP-agarose precipitates (data not shown). In addition, ATP-agarose decreased the concentration of Hsp72 measured in HSCM by 2-fold (25.8 ng/mL HSCM vs. 13.1 ng/mL HSCM + ATP-agarose; $P < 0.05$), as measured by ELISA. Pretreatment of HSCM with ATP-agarose restored subsequent cellular responses to LPS to nearly the same level of that seen in cells treated with LPS and CM (Fig. 8). In separate experiments, we depleted Hsp72 from HSCM and CM using immunoprecipitation via a blocking antibody to Hsp72. Again, immunoprecipitation decreased the concentration of Hsp72 measured in HSCM by 2-fold (19.6 ng/mL HSCM vs. 9.3 ng/mL HSCM + Hsp72 antibody; $P < 0.05$), as measured by ELISA. Similar to the ATP-agarose results above, immunoprecipitation of Hsp72 from HSCM restored

subsequent cellular responses to LPS to nearly the same level of that seen in cells treated with LPS and CM (Fig. 9). Finally, as an additional control, naive THP-1 cells were treated with recombinant human Hsp72 at an equivalent dose to that found in CM (8 ng/mL) and HSCM (18.5 ng/mL) 18 h before subsequent treatment with LPS. Treatment with 18.5 ng/mL significantly attenuated LPS-mediated induction of NF- κ B activity, whereas there was no significant difference after treatment with 8 ng/mL Hsp72 (data not shown). Collectively, these data indicate that Hsp72 accounts, in part, for the reprogramming effect of HSCM.

DISCUSSION

Extracellular Hsp72 has been found in the blood, cerebrospinal fluid, and bronchoalveolar lavage fluid of critically ill children and adults with a variety of inflammatory disease states. For example, increased extracellular Hsp72 levels after cardiopulmonary bypass have been detected in both adults (25,26) and children (D. S. Wheeler, unpublished data, 2007). Increased extracellular Hsp72 levels correlate with poor outcome in critically ill patients with liver disease (27), coronary artery disease (28–31), preeclampsia (32), sickle cell disease vaso-occlusive crisis (33), diabetic ketoacidosis (34), and septic shock (5). Extracellular Hsp72 levels in the cerebrospinal fluid of children with traumatic brain injury also seem to correlate with poor outcome (35). Finally, extracellular Hsp72 has been found in the pulmonary edema fluid of adults with acute lung injury (36) and the urine of children after renal transplantation (37). Given the signaling properties recently ascribed to Hsp72, these data suggest that the release of Hsp72 can potentiate an already active host immune response, thereby leading to poor outcome. Alternatively, extracellular Hsp72 can serve as a yet undefined cytoprotective function at lower levels as a normal response to infection or stress, and once a certain critical threshold is attained, it can potentiate the dysregulated inflammatory response that subsequently results in multiple organ failure. Recent experimental data (38) and the finding that extracellular Hsp72 levels greater than 15 ng/mL correlated with improved outcome after multiple trauma in adults (39) support this concept. These questions remain an active area of investigation in many laboratories, including our own.

The source of extracellular Hsp72 seems to be either non-specific release from necrotic cells, active release from viable cells, or a combination of both mechanisms, depending on the biological context (40). Consistent with previous studies (6,18–22), release of extracellular Hsp72 does not seem to be dependent on the classic protein secretory pathway because two different inhibitors of this pathway, monensin and brefeldin A, had no effect on extracellular Hsp72 release. Similarly, release of extracellular Hsp72 was early and sustained up to 24 h after heat shock and did not require new protein synthesis. Collectively, these studies suggest a nonclassical mechanism of extracellular Hsp72 release. Notably, recent studies point to the ABC family of transporter proteins as one potential mechanism (21).

Although the issue of how Hsp72 enters the extracellular compartment is certainly important, elucidating the biological function(s) of extracellular Hsp72 is equally important and not fundamentally dependent on the mechanism of release. Here, we have attempted to begin elucidating a potential biological function of extracellular Hsp72 using an *in vitro* system that models the extracellular release of endogenously produced Hsp72 and the subsequent effect of extracellular Hsp72 on “bystander” cells that have not been directly subjected to heat shock. As the primary experimental end point to address this question, we used LPS-mediated activation of the NF- κ B pathway. In addition, there is current controversy surrounding the true biological effects of recombinant forms of extracellular Hsp72 and the potential confounding effects of endotoxin contamination (14,41). The current model system circumvents this latter issue by potentially making use of endogenously produced extracellular Hsp72.

In the current model, we show that preconditioning naive cells with HSCM 18 h before subsequent LPS stimulation significantly attenuates activation of NF- κ B-dependent proinflammatory gene expression. The kinetics of this effect (i.e., 18 h of preconditioning with HSCM) is similar to that reported for “classic” endotoxin tolerance (15,42). These data are further supported by the demonstration that preconditioning with HSCM attenuates LPS-mediated degradation of I κ B α and LPS-mediated NF- κ B–DNA binding. These inhibitory effects on the NF- κ B pathway seem to have functional significance, as demonstrated by inhibition of LPS-mediated TNF- α expression in cells preconditioned with HSCM.

We also attempted to provide direct evidence that Hsp72 is responsible for the modulating effects of HSCM on LPS-dependent signaling. We approached these experiments by depleting Hsp72 from HSCM. We took advantage of the intrinsic ability of Hsp72 to bind ATP (24) and used an ATP-agarose-based strategy and centrifugation to deplete Hsp72 from HSCM. In these experiments, the addition of ATP-agarose to HSCM, followed by centrifugation, efficiently removed Hsp72 from HSCM. Furthermore, the addition of Hsp72-depleted HSCM partially abrogated the inhibitory effects of HSCM on LPS-mediated signaling. These data were further corroborated by experiments in which Hsp72 was depleted in the HSCM by immunoprecipitation. These data indicate that the modulating effects of HSCM on LPS signaling are, in part, dependent on the presence of Hsp72. It is certainly possible that HSCM contains other proteins/factors that are capable of modulating LPS signaling (e.g., other ATP-binding proteins), and this is a current area of investigation in our laboratory. The specificity of this assertion is further supported by the observation that subjecting fresh media (i.e., without cells) to heat shock had no effect on LPS-dependent signaling. In combination, these data support the concept that stressed cells can release Hsp72 (and possibly other factors) that are capable of reprogramming the ability of bystander mononuclear cells to respond to LPS.

The mechanism by which extracellular Hsp72 alters the subsequent response to LPS is not currently well understood. Although the basic concept has been known for decades, the fundamental mechanisms occurring at the molecular level that lead to cellular reprogramming remain to be fully elucidated (15,43). In the current study, HSCM preconditioning altered the temporal kinetics of I κ B α degradation, an important step for activation of NF- κ B. Recently, Hsp72 was shown to inhibit I κ B α degradation through direct inhibition of the 26 S proteasome (44). The fact that HSCM preconditioning altered the temporal kinetics of I κ B α degradation yet had no effect on IKK kinase activity or phosphorylation of I κ B α certainly seems to be consistent with such a mechanism. It is therefore tempting to speculate that inhibition of proteasome activity can play a role in the phenomenon of cellular reprogramming, although this will require further study.

The clinical significance of our current observations remains to be elucidated. We have demonstrated that stressed cells can release Hsp72 into the extracellular compartment, and that the released Hsp72 can reprogram the *in vitro* response to endotoxin in naive cells. Whether extracellular Hsp72 (1) augments the host immune response, leading to further cellular injury; (2) abrogates the host immune response to induce a state of immunoparalysis in which the host is unable to mount an adequate response to bacterial challenge; or (3) serves as a “danger signal” to augment the host immune response to more efficiently respond to bacterial challenge are questions that are currently being investigated in our laboratory.

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FIG. 1. Enzyme-linked immunosorbent assay for Hsp72 demonstrating that heat shock increases Hsp72 levels in the media of cultured THP-1 cells

A, Cells were subjected to heat shock as described in the text, and media were harvested up to 24 h after heat shock and assayed for Hsp72 concentration. $*P < 0.05$ compared with control. The 24-h time point was selected for all subsequent experiments. B, THP-1 cells were subjected to heat shock as described in the text, and media were assayed for Hsp72 concentration at 24 h after heat shock (HSCM). Control media (CM) were obtained from THP-1 cells incubated at 37°C for 24 h. Data represent the mean $\pm$ SEM of 5 individual experiments. $*P < 0.05$ vs. CM. C, THP-1 cells were pretreated with vehicle (PBS), monensin (8 μ g/mL), or brefeldin A (25 μ g/mL) at 37°C for 5 h before subsequent heat shock. Neither monensin nor brefeldin A inhibited release of extracellular Hsp72. D, THP-1 cells were pretreated with cycloheximide (10 μ g/mL) for 1 h before heat shock. Intracellular Hsp72 expression was determined via Western immunoblot at 0, 2, 4, 6, 12, and 24 h after heat shock. Lanes 1, 3, 5, 7, 9, and 11 represent THP-1 cells pretreated with cycloheximide, whereas lanes 2, 4, 6, 8, 10, and 12 represent THP-1 cells not treated with cycloheximide. E, THP-1 cells were pretreated with cycloheximide (10 μ g/mL) for 1 h before heat shock. Extracellular Hsp72 was measured at 0, 2, 4, 6, 12, and 24 h after heat shock via ELISA. F, THP-1 cells were pretreated with cycloheximide (10 μ g/mL) for 1 h before heat shock. Intracellular Hsp72 was measured at 0, 2, 4, 6, 12, and 24 h after heat shock via ELISA. $*P < 0.05$ compared with control.

FIG. 2. Luciferase assay demonstrating that HSCM does not increase NF-κB activation

THP-1 cells were transiently transfected with an NF-κB–luciferase reporter plasmid, incubated with either HSCM or recombinant Hsp72 (0.03 μg/mL) for 1, 3, 6, 12, and 18 h, then harvested for luciferase assay. Data represent the mean ± SEM of 3 individual experiments, with each condition performed in duplicate within each experiment.

FIG. 3. Luciferase assay demonstrating that HSCM attenuates LPS (1 μ g/mL)-mediated activation of NF- κ B

THP-1 cells were transiently transfected with an NF- κ B-luciferase reporter plasmid, incubated with either CM or HSCM $\pm$ LPS for 4 h, then harvested for luciferase assay. Data represent the mean $\pm$ SEM of 5 individual experiments, with each condition performed in duplicate within each experiment. * $P < 0.05$ vs. HSCM + LPS.

FIG. 4. Reverse transcriptase-polymerase chain reaction and flow cytometry demonstrating that HSCM does not inhibit expression of TLR-4 in THP-1 cells

THP-1 cells were preconditioned with HSCM (labeled "HS") or CM (labeled "C") for 18 h. A, RNA was isolated for RT-PCR, and actin or TLR-4 expression was measured. B, Cells (1×10^6) were treated with isotype control or TLR-4 antibody (eBiosciences). The red line represents isotype control antibody. The purple-filled area represents cells treated with fresh media and stained for TLR-4. The green tracing represents cells treated with conditioned media and stained for TLR-4. This was run in triplicate.

FIG. 5. Representative Western blot demonstrating that HSCM attenuates LPS-mediated degradation of IkBα

THP-1 cells were incubated with either CM or HSCM, treated with LPS (1 μ g/mL) for the indicated times, then harvested for Western blot analysis. Gel is representative of three individual experiments with similar results.

FIG. 6. Representative EMSA demonstrating that HSCM attenuates LPS-mediated NF-κB–DNA binding

THP-1 cells were incubated with either CM or HSCM, treated with LPS (1 $\mu\text{g/mL}$) for 1 h, then harvested for EMSA. As shown, incubation with CM for 18 h before LPS treatment significantly increased NF-κB binding (lane 3) compared with cells treated with CM (lane 1) or HSCM (lane 2) alone. Conversely, incubation with HSCM for 18 h significantly attenuated LPS-mediated NF-κB binding (lane 4). Lanes 5 and 8 represent cold-competitor controls for CM + LPS and HSCM + LPS, respectively. Lanes 6 and 9 represent p65 supershifts for CM + LPS and HSCM + LPS, respectively. Lanes 7 and 10 represent p50 supershifts for CM + LPS and HSCM + LPS, respectively. Gel is representative of three individual experiments with similar results.

FIG. 7. Enzyme-linked immunosorbent assay for TNF- α demonstrating that HSCM attenuates LPS (1 μ g/mL)-mediated TNF- α production

THP-1 cells were incubated with either CM or HSCM $\pm$ LPS for 24 h, then the cell culture media were assayed for TNF- α concentration. Data represent the mean $\pm$ SEM of five individual experiments, with each condition performed in triplicate within each experiment. * $P < 0.05$ vs. HSCM + LPS.

FIG. 8. Luciferase assay demonstrating that treating HSCM with ATP-agarose (depletion of Hsp72 from HSCM) reverses the ability of HSCM to attenuate LPS (1 μ g/mL)-mediated activation of NF- κ B

THP-1 cells were transiently transfected with an NF- κ B-luciferase reporter plasmid, incubated with either CM or HSCM $\pm$ LPS for 4 h, then harvested for luciferase assay. As indicated, some cells underwent treatment with CM or HSCM that had been previously treated with ATP-agarose. Data represent the mean $\pm$ SEM of five individual experiments, with each condition performed in duplicate within each experiment. * P < 0.05 vs. CM + LPS.

FIG. 9. Luciferase assay demonstrating that treating HSCM with anti-Hsp72 immunoprecipitation (depletion of Hsp72 from HSCM) reverses the ability of HSCM to attenuate LPS (1 μ g/mL)-mediated activation of NF- κ B

THP-1 cells were transiently transfected with an NF- κ B-luciferase reporter plasmid, incubated with either CM or HSCM $\pm$ LPS for 4 hours, then harvested for luciferase assay. As indicated, some cells underwent treatment with CM or HSCM that had been previously treated with anti-Hsp72 or isotype control. Data represent the mean $\pm$ SEM of 5 individual experiments with each condition carried out in duplicate within each experiment. * $P < 0.05$ versus CM + LPS.