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Extracellular Heat Shock Protein-70 Induces Endotoxin Tolerance in THP-1 Cells

Rajesh Aneja,* Kelli Odoms,† Katherine Dunsmore,† Thomas P. Shanley,‡ and Hector R. Wong†

Recent data suggest that heat shock protein-70 (HSP-70), an intracellular protein, can exist in the extracellular compartment and signal through the CD14/TLR4 pathway. In this study, we tested the hypothesis that extracellular HSP-70 induces endotoxin (LPS) tolerance. Using human monocyte cell line (THP-1), initial dose-response experiments were conducted to determine a subthreshold concentration of HSP-70 that does not induce NF-κB activity. Differentiated THP-1 cells were preconditioned with subthreshold concentration (0.03 μg/ml HSP-70) for 18 h, followed by LPS stimulation (1 μg/ml) for 4 h. Preconditioning with HSP-70 decreased subsequent LPS-mediated NF-κB-dependent promoter activity and was accompanied by significant decreases of supernatant TNF levels. Furthermore, human monocytes isolated from human volunteers, subsequently preconditioned with HSP-70, demonstrated LPS tolerance as evidenced by abrogated supernatant TNF levels. Additional experiments were conducted to exclude the possibility of endotoxin contamination of HSP-70 by boiling HSP-70 at 100°C for 1 h or preconditioning with equivalent concentrations of endotoxin as present in the HSP-70 preparation. These experiments indicated that induction of tolerance was not secondary to endotoxin contamination. Neutralization experiments with an anti-HSP-70 Ab confirmed the specificity of HSP-70 in tolerance induction. Preconditioning with HSP-70 attenuated cytosolic degradation of inhibitor κB and inhibited activation of inhibitor κB kinase following LPS stimulation. HSP-70 preconditioning decreased phosphorylation of the p65 subunit of NF-κB following LPS stimulation. These data suggest a novel role for extracellular HSP-70 in modifying mononuclear cell responses to subsequent LPS challenge. The Journal of Immunology, 2006, 177: 7184–7192.

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3 Abbreviations used in this paper: HSP, heat shock protein; IKK, IkB kinase.

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Materials and Methods

Cell culture

The human acute monocytic leukemia cell line, THP-1, was purchased from American Type Culture Collection. For all experiments, cells first underwent a differentiation step by treatment with IFN-γ (100 U/ml) for 3 h.

Primary human monocytes were isolated from buffy coats obtained from plasma of volunteer donors by the Ficoll-Hypaque procedure as described previously (14). The Monocyte Isolation Kit II (Miltenyi Biotech) was used to obtain a pure monocyte population using the manufacturer’s recommendations. Monocytes were plated at a density of 0.5 × 10⁶ cells.

Cells were cultured in RPMI 1640 medium containing 10% FBS, kanamycin, 2-2-ME, and 2% glutamine (pH 7.35). Where indicated, stimulation

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was performed with 1 \mu g/ml LPS (Escherichia coli, serotype O55:B5; Sigma-Aldrich).

**rHSP-70**

HSP-70 cDNA was isolated from a puC-HSP-70 vector (StressGen Biotechnologies) using EcoRI restriction enzyme. The EcoRI restriction fragment containing the HSP-70 cDNA was cloned into the EcoRI site of the pQE vector (Qiagen). Sense orientation was confirmed by sequencing analysis at the University of Cincinnati DNA core facility. The E. coli strain M15 (pREP4) (Invitrogen Life Technologies) transformed with the HSP-70 expression plasmid was grown for 16 h at 37°C in Luria-Bertani broth supplemented with 100 mg/ml ampicillin. These cultures were diluted 100-fold with fresh Luria-Bertani medium and cultured at 37°C for 4 h while shaking at 250 rpm. Protein expression was induced by the addition of 1 M isopropyl \(\beta\)-D-thiogalactoside to a final concentration of 1.0 mM for 4 h while shaking at 37°C. The induced cells were lysed and sonicated in lysis buffer (50 mM NaH\(_2\)PO\(_4\)-Cl, 300 mM NaCl, 10 mM imidazole, and 1 \mu g/ml lysozyme). Cell debris was removed by centrifugation, and the cell extracts were then loaded into a Ni-NTA resin column. The column was washed and the rHSP-70 eluted with elution buffer (50 mM NaH\(_2\)PO\(_4\)-Cl, 300 mM NaCl, and 250 mM imidazole). The protein was further purified using an endotoxin binding column (Pierce), according to the manufacturer’s instructions. Endotoxin levels were determined using *Limulus* amoebocyte lysate assay (BioWhittaker) before and after the endotoxin binding column. Independent verifications of endotoxin levels after passage through the endotoxin column were also performed by submitting rHSP-70 samples to Charles River Laboratory for endotoxin measurements.

**Model of LPS tolerance with HSP-70**

THP-1 cells cultured in RPMI 1640 medium containing 10% heat-inactivated FBS for 12 h were incubated with IFN-\(\gamma\). The following day, cells were washed with medium and treated with 0.03 \mu g/ml HSP-70 in RPMI 1640 medium, and then incubated at 37°C for 18 h. At that point, the cells were washed with RPMI 1640 medium and then stimulated with 1 \mu g/ml LPS for the indicated time points. The chosen 18-h time point between the preconditioning stimulus with HSP-70 and the LPS stimulus was based on previous literature in which the preconditioning stimulus was a low dose of LPS (13).

**Transient transfections and luciferase assays**

A NF-\(\kappa\B-thc-luciferase reporter plasmid was used to measure LPS-dependent activation of NF-\(\kappa\B-thc. The plasmid (\(5'\)-GTTGAGATTCCTCTTGAG-3') corresponding to the consensus NF-\(\kappa\B-thc binding site was labeled with \([\alpha^{32}P]ATP using T4 polynucleotide kinase (Invitrogen Life Technologies) and purified in Bio-Spin chromatography columns (Bio-Rad). For each sample, 10 \mu g of nuclear proteins was preincubated with EMSA buffer (12 mM HEPES (pH 7.9), 4 mM Tris-HCL (pH 7.9), 25 mM KCl, 5 mM MgCl\(_2\), 1 mM EDTA, 1 mM DTT, 50 ng/ml poly d(C), 125 mM glycerol, and 0.2 mM PMSF) on ice for 10 min before the addition of the radiolabeled oligonucleotide probe for an additional 10 min. Protein-nucleic acid complexes were resolved using a nondenaturing polyacrylamide gel consisting of 5% acrylamide (acrylamide-to-biacrylamide ratio of 29:1) and run in 0.5x TBE (45 mM Tris-HCL, 45 mM boric acid, and 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**

Western blot analyses were performed as described previously (15). Briefly, whole cell lysates containing 50 \mu g of protein were boiled in equal volumes of loading buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% 2-2-ME). Proteins were separated electrophoretically on 8–16% Tris-glycine gradient gels (Novex) and subsequently transferred to nitrocellulose membranes (Novex) using the Novex Xcell MiniGel system. For immunoblots, membranes were blocked with 5% nonfat dried milk in TBS for 1 h. Primary Ab against I\(\kappa\B (Santa Cruz Biotechnology) was applied at 1/1000 dilution overnight. After washing twice with TBS containing 0.05% Tween 20, secondary Ab (peroxidase-conjugated goat anti-rabbit IgG; StressGen Biotechnologies) was applied at 1/10,000 dilution for 1 h. Blots were washed in TTBS twice for 10 min, incubated in commercial ECL reagents (Amersham Biosciences), and exposed to photographic film.

**IkB kinase (IKK) assay**

Treated cells were washed with PBS containing 1 mM PMSF, 100 mM Na\(_2\)VO\(_4\), 2 mM PPNP, and 210 mM/ml aprotinin (Sigma-Aldrich). The procedure for IKK has been described previously (15). Briefly, cells were scraped and centrifuged at 3000 rpm for 5 min. The pellet was resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM PMSF, 100 \mu M Na\(_2\)VO\(_4\), 2 mM PPNP, and 1 M MgCl\(_2\) and pelleted. NF-\(\kappa\B proteins were extracted after treatment, cellular proteins were extracted and analyzed for luciferase activity according to the manufacturer’s instructions. Endotoxin levels were determined using *Limulus* amoebocyte lysate assay (BioWhittaker) before and after the endotoxin binding column.

**ELISA**

TNF-\(\alpha\) concentrations were measured in the medium of treated cells using a commercially available sandwich ELISA (BioSource International). All procedures were performed as recommended by the manufacturer.

**Nuclear protein extraction**

All nuclear protein extraction procedures were performed on ice with ice-cold reagents. A total of 5 X 10\(^5\) THP-1 cells per condition was seeded into tissue-culture flasks the day before transfection. Cells were treated with HSP-70 for 18 h before incubation with LPS for 1 h. Cells were washed twice with PBS and harvested by scraping. Cells were pelleted in 1 ml of PBS at 6000 rpm for 5 min. The pellet was washed twice with PBS and resuspended in lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl\(_2\), 0.2% v/v Nonidet P-40, 1 mM DTT, and 0.1 mM PMSF). The suspension was incubated on ice for 5 min and centrifuged at 48°C at 6000 rpm. The supernatant was discarded, and one cell pellet volume of extraction buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl\(_2\), 25% v/v glycerol, 1 mM DTT, and 0.5 mM PMSF) was added to the supernatant pellet and incubated on ice for 15 min. Nuclear proteins were isolated by centrifugation at 14,000 rpm at 48°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.
210 mU/ml aprotinin. Immunoprecipitation of the cell extract was performed using anti-IKK Ab (Santa Cruz Biotechnology). Protein AG agarose beads (Santa Cruz Biotechnology) were added to the cell extract Ab to pull down the immunoprecipitated IKK. The pellet was washed; resuspended in kinase assay buffer containing 100 μM ATP, 6 μg of GST-IκBα as substrate, and 0.5 μl of [γ-32P]-labeled ATP; and incubated for 30 min at 30°C. The reaction was stopped in an ice bath, and electrophoresis was performed in a Novex MiniCell System for 90 min at 140 volts. The gel was dried on a Bio-Rad gel drying apparatus and exposed overnight and analyzed using a PhosphorImager screen and ImageQuant software (Molecular Dynamics). The protein loading on the IKK assay was normalized with a Western blot for an IKK-α Ab on the immunoprecipitated IKK.

Cell viability

Cell viability after 18-h exposure to HSP-70 and followed by LPS treatment was determined by trypan blue exclusion.

Statistical analysis

Differences in TNF-α levels and luciferase activity between the experimental groups were evaluated by one-way ANOVA and Student-Newman-Keuls test. Values of $p \leq 0.05$ was considered statistically significant.

Results

Dose-response curve

In initial experiments, we sought to establish a threshold concentration at which extracellular HSP-70 did not induce activation of NF-κB. Differentiated THP-1 cells transfected with the 3× NF-κB luciferase reporter plasmid were exposed to increasing (1/2) log concentrations of rHSP-70 ranging from 0.01 to 1 μg/ml for 4 h. The minimum concentration that induced NF-κB activation was 0.1 μg/ml. Cells exposed to 0.01 or 0.03 μg/ml HSP-70 did not show significant induction of the NF-κB-dependent luciferase reporter plasmid. (data not shown).

HSP-70 pretreatment induces tolerance in differentiated THP-1 cells

After establishing the threshold concentration of HSP-70 that induced NF-κB activation, we sought to determine whether tolerance can be induced with a subthreshold concentration of extracellular HSP-70 (0.03 μg/ml). Differentiated THP-1 cells were transiently transfected with a 3× NF-κB promoter luciferase reporter plasmid and exposed to the experimental conditions. LPS treatment induced NF-κB promoter activity in THP-1 cells (42.5 ± 1.3-fold induction above baseline). Preconditioning with 0.03 μg/ml HSP-70 for 18 h before LPS stimulation significantly attenuated NF-κB-dependent promoter activity following LPS stimulation. (42.5 ± 1.3 vs 26.4 ± 3.3 luciferase fold induction, $p < 0.05$; Fig. 1A).

To determine comparable degree of LPS tolerance with LPS preconditioning, we exposed differentiated, 3× NF-κB luciferase reporter plasmid-transfected THP-1 cells to varying concentrations of LPS (0.01–100 ng/ml) followed by LPS stimulation with 1 μg/ml. The lowest LPS concentration that trended toward induction of

**FIGURE 1.** A, Luciferase assay demonstrating the effect of HSP-70 preconditioning on LPS-mediated NF-κB promoter activity. Differentiated THP-1 cells were transfected with a 3× NF-κB promoter luciferase reporter plasmid and preconditioned with 0.03 μg/ml HSP-70 for 18 h, as indicated. Cells were then exposed to 1 μg/ml LPS for 4 h. Data represent mean ± SEM of four separate experiments with each condition performed in triplicate. (*, $p < 0.05$ vs LPS alone). B, Differentiated THP-1 cells were transfected with a 3× NF-κB promoter luciferase reporter plasmid and preconditioned with varying concentrations of LPS for 18 h, as indicated. Cells were then exposed to 1 μg/ml LPS for 4 h. Data represent mean ± SEM of four separate experiments with each condition performed in triplicate (*, $p < 0.05$ vs LPS alone).
tolerance was 1 ng/ml; however, significant tolerance could be attained only after pretreatment with LPS concentrations higher than 1 ng/ml (i.e., 10 and 100 ng/ml). Cells preconditioned with LPS (10 ng/ml) demonstrated significant attenuation of NF-κB-dependent promoter activity following LPS stimulation (36.13 ± 4.5 vs 25.67 ± 1.15 luciferase fold induction, p < 0.05; Fig. 1B), consistent with the classically described phenomenon of endotoxin tolerance. These data indicate that preconditioning with HSP-70 induced tolerance to subsequent LPS-mediated activation of NF-κB in a manner similar to that of classic endotoxin tolerance.

**TNF-α levels in supernatant**

Decreased TNF-α expression has been considered a hallmark of LPS tolerance (16). We determined the effect of extracellular HSP-70 on LPS-mediated production of the proinflammatory cytokine, TNF-α. Culture supernatants were assayed for TNF-α expression via ELISA. As shown in Fig. 2, preconditioning with 0.03 μg/ml HSP-70 significantly attenuated LPS-induced TNF-α levels, compared with cells that were treated with LPS alone (701.2 ± 26.7 vs 811 ± 28.4 pg/ml, p < 0.05). By decreasing the HSP-70 concentration used for preconditioning the THP-1 cells, the TNF-α levels in the supernatant were higher, illustrating a dose-response curve. THP-1 cells preconditioned with 0.01 μg/ml HSP-70 demonstrated higher TNF-α levels, compared with THP-1 cells preconditioned with 0.03 μg/ml HSP-70 (765.2 ± 24 vs 811 ± 28.4 pg/ml; Fig. 2).

**Preconditioning with a comparable concentration of LPS present in the HSP-70 preparation fails to induce tolerance in THP-1 cells**

The concentration of endotoxin in 0.03 μg/ml HSP-70 was 4.08 × 10⁻⁹ μg/ml as measured using the *Limulus* amoebocyte lysate assay. To test the assumption that tolerance is conferred by HSP-70 and not endotoxin contamination of rHSP-70, cells were preconditioned with either HSP-70 or 4.08 × 10⁻⁹ μg/ml LPS. Similar to the previous experiments, we observed that LPS markedly increased NF-κB promoter activity, and this was significantly attenuated in THP-1 cells preconditioned with HSP-70 (39.5 ± 0.1 vs 26.9 ± 1.6 luciferase fold induction, p < 0.05; Fig. 3). We observed lack of tolerance in cells preconditioned with equal concentration of LPS (as is present in HSP-70) because there was no decrease in NF-κB promoter activity after the stimulating dose of LPS (39.5 ± 0.02 vs 43.0 ± 2.15 luciferase fold induction). THP-1 cells preconditioned with equal amounts of LPS as present in HSP-70 also demonstrated lack of tolerance as measured by TNF-α levels, which was comparable with cells that were not preconditioned (786.17 ± 30 vs 811 ± 28.4 pg/ml; Fig. 2). Together, these data suggest that tolerance conferred after preconditioning with HSP-70 is a property of HSP-70 and not of the trace amount of endotoxin present in the HSP-70 preparation.

**Preconditioning with boiled HSP-70 fails to induce tolerance in differentiated THP-1 cells**

We conducted additional experiments to exclude endotoxin contamination as the cause of tolerance to LPS. HSP-70 protein was denatured by boiling at 100°C for 60 min. Differentiated THP-1 cells were pretreated with unboiled or boiled HSP-70 at 0.03 μg/ml for 18 h and then treated with LPS similar to experiments mentioned above. We observed that NF-κB promoter activity was comparable in THP-1 cells preconditioned with boiled HSP-70 and cells treated with LPS alone (33.2 ± 3.3 vs 36.2 ± 5.2 luciferase fold induction) compared with cells that were not preconditioned (786.17 ± 30 vs 811 ± 28.4 pg/ml). These data indicate that preconditioning with boiled HSP-70 fails to induce tolerance in THP-1 cells.

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**FIGURE 2.** ELISA results demonstrating the effects of HSP-70 pretreatment on TNF-α levels in the supernatants of differentiated THP-1 cells. Studies were performed in 12-well plates at a density of 1 × 10⁶ cells. Control cells were maintained in a basal growth medium. Cells were preconditioned with HSP-70 (either 0.01 or 0.03 μg/ml) for 18 h before the addition of LPS 1 μg/ml for 4 h. Another set of cells was treated with the measured LPS concentration in 0.03 μg/ml HSP-70 (4.08 × 10⁻⁹ μg/ml) for 18 h followed by LPS stimulation. Data represent the mean ± SEM of four separate experiments with each condition performed in triplicate (*, p < 0.05 vs LPS alone).

**FIGURE 3.** Effect of preconditioning with HSP-70 or equivalent amounts of endotoxin present in HSP-70 on LPS-mediated NF-κB promoter activity. Differentiated THP-1 cells were transfected with 3× NF-κB promoter luciferase reporter plasmid and preconditioned with HSP-70 or the measured LPS concentration in 0.03 μg/ml HSP-70 (4.08 × 10⁻⁹ μg/ml) for 18 h. Cells were then exposed to 1 μg/ml LPS for 4 h. Data represent mean ± SEM of four separate experiments with each condition performed in triplicate (*, p < 0.05 vs LPS alone).
fold induction; Fig. 4). The cells treated with unboiled HSP-70 demonstrated tolerance as evidenced by decreased NF-κB promoter activity, compared with cells treated with LPS alone (19.8 ± 2.1 luciferase fold induction, *p < 0.05). As a further control, we also boiled LPS at a concentration of 10 ng/ml, a concentration of LPS that induces classic endotoxin tolerance. Boiled LPS (10 ng/ml) retained the capacity to induce tolerance (33.2 ± 3.3 vs 21.2 ± 2.2 luciferase fold induction, *p < 0.05; Fig. 4). These data demonstrate that denaturing HSP70 through boiling abolishes the ability to induce tolerance, while boiling a concentration of LPS that is known to induce tolerance has no effect. Collectively, these data provide further evidence that endotoxin contamination of the HSP-70 preparation is not accountable for our observations.

HSP-70 Ab blocks induction of LPS tolerance by HSP-70

In a separate group of experiments, we determined whether the simultaneous addition of HSP-70 and HSP-70 Ab blocks induction of LPS tolerance. In concurrence with our previous experiments, we observed that NF-κB promoter expression was significantly attenuated in differentiated THP-1 cells preconditioned with HSP-70, compared with cells treated with LPS alone (43.2 ± 1.4 vs 21.2 ± 1.8 luciferase fold induction, *p < 0.05; Fig. 5). This induction of tolerance can be blocked by the simultaneous addition of HSP-70 and an anti-HSP-70 Ab during preconditioning, compared with cells preconditioned with HSP-70 alone (41.4 ± 1.4 vs 21.2 ± 1.8 luciferase fold induction, *p < 0.05). In contrast, the addition of an irrelevant Ab (anti-β-actin Ab) was unable to block the induction of tolerance by HSP-70 (25.2 ± 2.0 vs 41.4 ± 1.4 luciferase fold induction, *p < 0.05). Collectively, these data, along with the data from previous experiments, confirm that pretreatment with HSP-70 specifically induces tolerance to LPS in differentiated THP-1 cells.

HSP-70 pretreatment induces tolerance in primary human monocytes

Primary human monocytes isolated from volunteer donors were preconditioned with 0.03 μg/ml HSP-70, for 18 h before LPS stimulation. Monocytes preconditioned with HSP-70 demonstrated
HSP-70 preconditioning inhibits LPS-mediated NF-κB p65 subunit phosphorylation

As NF-κB activation acts as a control point for proinflammatory gene expression, we next determined the effect of preconditioning with HSP-70 on LPS-mediated NF-κB DNA binding using EMSA. Treatment with LPS alone increased DNA binding of NF-κB, compared with control cells (Fig. 7A). DNA binding of NF-κB in cells preconditioned with HSP-70 was comparable to cells treated with LPS alone (Fig. 7A). We also examined the composition of the NF-κB dimers by supershift analyses of the protein complex bound to DNA. Nuclear proteins purified from THP-1 cells were incubated with Ab for p65 and p50 (Fig. 7B). Preconditioning with HSP-70 did not affect the NF-κB subunit composition in response to LPS.

Our data thus far demonstrate a discrepancy between NF-κB activity (luciferase assay) and NF-κB DNA binding (EMSA). Other authors have demonstrated the role of dynamic posttranslational modifications that occur after the release of NF-κB from IκB. One such modification is phosphorylation of the p65 subunit of NF-κB that is required to generate a fully active NF-κB complex (17). As shown in Fig. 8, lane 2, treatment with LPS alone markedly increased phosphorylation of p65, compared with untreated control cells. HSP-70 preconditioning inhibited LPS-mediated phosphorylation of the p65 subunit of NF-κB (Fig. 8, lane 3). In summary, preconditioning with HSP-70 does not affect NF-κB DNA binding or NF-κB subunit composition, but does decrease phosphorylation of the p65 subunit of NF-κB.

HSP-70 pretreatment inhibits LPS-mediated degradation of IκB

As IκB-α is an inhibitor protein of NF-κB, that binds NF-κB and retains it in the cytoplasm, we examined IκB-α degradation by Western blot analyses. Treatment with LPS alone resulted in degradation of IκB-α, compared with cells treated with medium alone (Fig. 9, lane 2). Preconditioning with HSP-70 resulted in preservation of IκB-α, compared with cells treated with LPS alone (Fig. 9, lane 3).

HSP-70 pretreatment inhibits LPS-induced IKK activity

IKK is the primary kinase that phosphorylates IκB-α and triggers the activation of the NF-κB/IκB pathway. We next determined the effect of HSP-70 preconditioning on IKK activity. As shown in Fig. 10, lane 2, LPS increased IKK activity above the activity in untreated control cells. Consistent with the IκB-α data above, HSP-70 preconditioning inhibited the LPS-mediated increase of IKK activity (Fig. 10, lane 3).

Discussion

HSP-70 has traditionally been considered as an exclusively intracellular protein that serves a variety of chaperone and cytoprotective functions (18–20). Recent studies have cited that HSP-70 may be present in the extracellular compartment of patients with trauma and sepsis (7–9). The significance of the elevated levels of HSP-70 in these patients is not clear. An increasing number of studies have indicated that extracellular HSP-70 may have a role in innate immunity. Asea et al. (21) have demonstrated that extracellular HSP-70 can elicit a potent proinflammatory immune response in human monocytes by signaling through TLR4/CD14 pathway similar to the canonical TLR4 ligand, LPS.

Previously, it has been shown that, in response to repeated endotoxin challenge, cells appear to have a reduced response to subsequent LPS challenges, a phenomenon referred to as LPS tolerance. We report in this study the novel finding that preconditioning with HSP-70, at a concentration that does not appear to activate NF-κB, induces tolerance to LPS in THP-1 cells, thus suggesting a unique functional role, in the context of innate immunity, for low concentrations of extracellular HSP-70. In addition, human monocytes isolated from human volunteers and subsequently preconditioned with HSP-70 demonstrated LPS tolerance as evidenced by abrogated supernatant TNF-α levels. Thus, our observations in the THP-1 cell line are also operative in primary human monocytes.

LPS tolerance has been described in vivo with reduced LPS lethality in mice pretreated with low-dose LPS (22, 23). These data have been corroborated in vitro with a reduced capacity of monocytes isolated from septic patients to release proinflammatory cytokines in response to further LPS stimulation (24–26). Other synonyms used for tolerance include hyporesponsiveness, reprogramming, desensitization, adaptation, refractoriness, and adaptation. The phenomenon of tolerance is not a global down-regulation of signaling pathway but a reprogramming of the cellular signaling pathways (27). LPS-tolerant monocytes/macrophages demonstrate impaired activation of intracellular signaling pathways (e.g., NF-κB, IKK) and subsequent decreased proinflammatory gene transcription and protein production, including TNF-α, IL-β, and IL-6.
Because decreased proinflammatory protein expression (i.e., TNF-α production) is a hallmark of LPS tolerance, we examined TNF-α levels in our experimental paradigm. We found decreased TNF-α levels in the supernatant levels of cells preconditioned with HSP-70. We also demonstrated a decrease in IKK activation and subsequent cytosolic preservation of IκBα.

The LPS tolerance literature has reported attenuation in DNA binding of NF-κB and an increase of NF-κB p50 homodimers in tolerant monocytes (28). We were struck by the finding that, while HSP-70 pretreatment inhibited IKK activation and preserved IκBα following LPS stimulation, it had no effect on the DNA binding of NF-κB. There was also no increase in the NF-κB p50 homodimers in tolerant THP-1 cells as evaluated by supershift analyses.

For a maximal NF-κB transcriptional response, the NF-κB complex must undergo additional posttranslational modification involving site-specific phosphorylation. It has been suggested that phosphorylation of p65 subunit of NF-κB is involved in activation of NF-κB. This key phosphorylation event occurs at multiple phosphoacceptor sites in different domains of p65 that are targeted in a redundant manner, by different kinases and different inciting stimuli (29, 30). Our study demonstrates that preconditioning with HSP-70 decreases LPS-induced p65 phosphorylation with apparent consequences on NF-κB-dependent gene expression. The mechanisms involved in the regulatory phosphorylation of DNA binding subunits of NF-κB have been described with other inducible transcription factors, such as AP-1 and CREB. Similar to our study, another study had demonstrated that p38 MAPK inhibitor, SB203580, does not interfere with induced nuclear translocation and DNA binding of NF-κB, but significantly inhibits NF-κB-dependent gene expression (31).

A review of the heat shock literature reveals controversy regarding the potential endotoxin contamination of the rHSP-70 and its role as a confounding factor in TLR4-dependent signaling. Gao and Tsan (32) suggested that the observed TNF-α-inducing activity in the rHSP-70 preparation is due entirely to the contaminating LPS. We addressed this important concern by performing multiple tolerance experiments using boiled HSP-70, preconditioning with LPS.
known LPS concentrations in the HSP-70 preparation, and simultaneous addition of HSP-70 Ab along with HSP-70. All these experiments lend credence to the theory that it is HSP-70 that induces tolerance to LPS and not the endotoxin contamination of HSP-70.

It is also not clear how HSP-70 is released into the extracellular space. It has been speculated that the presence of this protein in the extracellular compartment may indicate cell death and possibly represents a danger signal (33). The release of HSP-70 is presumed to be either an active process of secretion by cells or release from necrotic cells. It has been shown that, while necrotic cells can release HSP-70, apoptotic cells cannot release HSP (34). It has also been postulated that both inducible and constitutive HSP, once released into the extracellular compartment by a pathological cell death, act as danger signals by activating dendritic cells (35). Regardless of the mechanism of HSP-70 release, our current data suggest that another functional consequence of extracellular HSP-70 is to modulate the response of the mononuclear cell to LPS.

**FIGURE 9.** Representative autoradiograph of Western blot analysis for IκB-α demonstrating the effect of HSP-70 preconditioning on IκB-α degradation following LPS stimulation. Control cells were maintained in basal growth medium. THP-1 cells were preconditioned with HSP-70 (0.03 μg/ml) for 18 h before the addition of LPS (1 μg/ml) for 30 min. The gel is representative of three experiments with similar results. Relative densitometric analysis of IκB-α content is shown in the graph (*, p < 0.05 vs LPS alone).

**FIGURE 10.** Representative autoradiograph of IKK assay demonstrating the effect of preconditioning with HSP-70 on LPS-mediated activation of the IKK complex. Control cells were maintained in basal growth medium. THP-1 cells were preconditioned with HSP-70 (0.03 μg/ml) for 18 h before the addition of LPS (1 μg/ml) for 5 min. The gel is representative of three experiments with similar results. Relative densitometric analysis of IKK activity is shown in the graph (p < 0.05 vs LPS alone).
Since the first description of endotoxin tolerance (36), there has been immense curiosity about the potential pathways involved in mediating endotoxin tolerance. Although tremendous progress has been made, the precise pathway for inducing LPS tolerance is unclear. However, it is clear that the tolerance phenomenon is a complex orchestrated counter regulatory response to inflammation (13). There is also an ongoing debate about the clinical relevance and usefulness of endotoxin tolerance. Although endotoxin tolerance attenuates the severity of infections and ischemia reperfusion damage, conversely it can increase the sensitivity of patients with systemic inflammation response syndrome to nosocomial infections (37).

In conclusion, we have demonstrated that in vitro subthreshold concentrations of HSP-70 (that do not induce NF-κB activation) can induce LPS tolerance. Potential mechanisms involved in HSP-70-mediated LPS tolerance include decreased IKK activity, preservation of IκBα, and decreased phosphorylation of p65. These data are further strengthened by supporting data indicating that LPS contamination of the HSP-70 preparation does not account for our observations and that primary human monocytes can also be rendered tolerant by preconditioning with HSP-70. Collectively, these data suggest a novel role for low concentrations of extracellular HSP-70 in modulating the innate immune response.

Disclosures

The authors have no financial conflict of interest.

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