Endotoxin Contamination in Recombinant Human Heat Shock Protein 70 (Hsp70) Preparation Is Responsible for the Induction of Tumor Necrosis Factor α Release by Murine Macrophages*

Baochong Gao‡ and Min-Fu Tsan

From the Institute for Clinical Research and Veterans Affairs Medical Center, Washington, D. C. 20422

Using commercially available recombinant human heat shock protein 70 (rhHsp70), recent studies have shown that rhHsp70 could induce the production of tumor necrosis factor α (TNFα) by macrophages and monocytes in a manner similar to lipopolysaccharide (LPS) e.g. via CD14 and Toll-like receptor 4-mediated signal transduction pathway. In the current study, we demonstrated that a highly purified rhHsp70 preparation (designated as rhHsp70–1) with a LPS content of 1.4 pg/μg was unable to induce TNFα release by RAW264.7 murine macrophages at concentrations up to 5 μg/ml. In contrast, a less purified rhHsp70 preparation (designated as rhHsp70–2) at 1 μg/ml with a LPS content of 0.2 ng/μg was able to induce TNFα release to the same extent as that induced by 0.2 ng/ml LPS. Failure of rhHsp70–1 to induce TNFα release was not because of defective physical properties since rhHsp70–1 and rhHsp70–2 contained identical hsp70 content as determined by SDS gels stained with Coomassie Blue and Western blots probed with an anti-rhHsp70 antibody. Both rhHsp70 preparations also had similar enzymatic activities as judged by their ability to remove clathrin from clathrin-coated vesicles. Removal of LPS from rhHsp70–2 by polymyxin B-agarose column or direct addition of polymyxin B to the incubation medium essentially eliminated the TNFα-inducing activity of rhHsp70–2. The addition of LPS at the concentration found in rhHsp70–2 to rhHsp70–1 resulted in the same TNFα-inducing activity as observed with rhHsp70–2. The TNFα-inducing activities of rhHsp-3, LPS alone, and LPS plus rhHsp70–1 were all equally sensitive to heat inactivation. These results suggest that rhHsp70 does not induce TNFα release from murine macrophages and that the observed TNFα-inducing activity in the rhHsp70–2 preparation is entirely due to the contaminating LPS.

The 70-kDa heat shock proteins (Hsp70α) are highly conserved proteins expressed both constitutively (Hsc70) and under stressful conditions (Hsp70) in all prokaryotes and eu-
Hsp70, Endotoxin, and Induction of TNFα

assayed for their abilities to bind and hydrolyze ATP. The ESP-555 preparation was the low endotoxin preparation containing ~50 EU (endotoxin units)/mg of rhHsp70 as determined using the Limulus Amebocyte lysate (LAL) assay, which was recommended for use in assays requiring low endotoxin. The NSSP-555 preparation was not tested for endotoxin levels and was used in protein-binding assays. For the purpose of this report, the low endotoxin preparation, ESP-555, was designated rhHsp70–1, whereas the NSSP-555 preparation was designated rhHsp70–2.

Protein-free LPS (from JMS8 E. coli K-12, rough strain) was kindly provided by Dr. John E. Somerville of Bristol-Myers Squibb Co. Before use, LPS was dissolved in sterile, pyrogen-free water, sonicated on ice for 30 s with a sonic dismembrator (Fisher), and diluted with phosphate-buffered saline (Invitrogen). Polymyxin B sulfate (catalog No. P4932, cell culture-tested), phosphoketrate kinase, creatine phosphate, and ATP were purchased from Sigma. Polymyxin B-agarose (Detoxi Gel, catalog No. 20339) was obtained from Pierce. Enhanced chemiluminescence (ECL) Western blotting detection solutions were purchased from Amersham Biosciences.

Cell Culture—RAW 264.7 murine macrophages (from American Tissue Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Subcultures of macrophages were prepared every 2 days by scraping cells into fresh medium.

Determination of TNFα Release by Murine Macrophages—Murine macrophages were seeded in 24-well plates at 2.5 × 10⁴ cells/well the day before the experiment. After washing 3 times with the medium, the cells were treated with or without rhHsp70 (0.1–5 μg/ml) and/or LPS (0.1–100 ng/ml) in 250 μl of medium containing 10% fetal bovine serum for 4 h at 37 °C. At the end of the treatment, media were collected and clarified by centrifugation at 10,000 rpm for 5 min in a microcentrifuge (Hermle–Labortechnik, Weihingen, Germany). TNFα content of the media was then determined by a quantitative sandwich enzyme-linked immunosorbent assay using the Quantikine M mouse TNFα immunoassay kit (catalog No. MTA00, R & D Systems, Minneapolis, MN) according to the manufacturer’s recommendation. All experiments were done in duplicate.

In some experiments, rhHsp70 and LPS were heated for 60 min in a boiling water bath before being added to the cells. Likewise, in some experiments macrophages were preincubated with or without polymyxin B sulfate (10 μg/ml) for 30 min at 37 °C before the addition of rhHsp70 or LPS to inactivate LPS.

Measurement of Endotoxin Activity—The endotoxin activities of rhHsp70 and LPS preparations were determined using the LAL assay kit (catalog No.50–648U, BioWhittaker, Walkersville, MD) according to the manufacturer’s recommendation.

Quantification of rhHsp70 Protein by Gel Electrophoresis and Western Blotting—The rhHsp70 preparations and fractions from polymyxin B-agarose column (see “Removal of Endotoxin from rhHsp70–2 Using Polymyxin B–agarose”) were analyzed by SDS-PAGE using 7.5% polyacrylamide gels followed by staining with Coomassie Blue or by Western blotting using an antibody to recombinant human Hsp70 (catalog No. SPA-812, StressGen) followed by ECL detecting system as described previously (25). Densitometric quantification of rhHsp70 of the Coomassie Blue-stained SDS gels or immunoblots were performed using the FluorChem 8000 digital imaging system (Alpha Innotech Corp., San Leandro, CA).

Assay for the Activity of Removing Clathrin from Clathrin-coated Vesicles (Uncoating Reaction)—This was performed as described previously (8). Briefly, the uncoating reaction mixture contained 35 μg/ml (0.5 μM) rhHsp70, bovine clathrin-coated vesicles containing 0.5 μM clathrin (kindly provided by Drs. Lois Greene and Evan Eisenberg of the NHLBI, National Institutes of Health, Bethesda, MD), 1 mM Mg-ATP, and an ATP-regenerating system consisting of 30 units/ml phosphocreatine kinase and 15 mM creatine phosphate. The mixtures were incubated at 25 °C for 10 min, and vesicles in the reaction mixture were removed by centrifugation at 1 × 10⁴ rpm for 6 min in a microcentrifuge (Beckman Instruments). Clathrin released from the coated vesicles in the supernatant was analyzed by SDS-PAGE, Coomassie Blue staining, and densitometric quantification. The results, clathrin-uncoating activities of rhHsp70, were expressed as optical density ratios of clathrin/rhHsp70.

Removal of Endotoxin from rhHsp70–2 Using Polymyxin B–agarose—rhHsp70–2 was removed using purified polymyxin B–agarose (Detoxi Gel, Pierce) according to manufacturer’s recommendation. Briefly, aliquots of 0.5 ml of polymyxin B–agarose were poured into Poly-Prep disposable columns (Bio-Rad) and equilibrated in phosphate-buffered saline. Columns were washed with 5 volumes of 1% sodium deoxycholate followed by 10 volumes of phosphate-buffered saline. 250 μl of rhHsp70–2 at 100 μg/ml was loaded onto each 0.5-ml Detoxi column and incubated at room temperature for 60 min. The column was then eluted with phosphate-buffered saline in 250-μl fractions. The Detoxi column fractions were analyzed by SDS gels, stained with Coomassie Blue, and quantified as described above.

Statistical Analysis—Results were expressed as the mean ± S.D. Levels of significance were determined using a two-tailed Student’s t test (26), and a confidence level of greater than 95% (p < 0.05) was used to establish statistical significance.

RESULTS

Induction of TNFα Release by rhHsp70 and LPS—The reported induction of proinflammatory cytokine release by rhHsp70 was similar to the effect of LPS, i.e. mediated through the CD14 and TLR-4 receptor complex signal transduction pathway (12, 15–17). Because induction of manganese superoxide dismutase (MnSOD) by LPS is also dependent on CD14 and TLR-4 (27), we wanted to know whether rhHsp70 could also induce MnSOD. To avoid the potential confounding effect of LPS, we first studied the highly purified, low endotoxin preparation of rhHsp70, i.e. rhHsp70–1. We observed to our surprise that rhHsp70–1 not only did not induce manganese superoxide dismutase but also failed to induce TNFα production by RAW264.7 murine macrophages (data not shown). Failure to induce TNFα release from macrophages by rhHsp70–1 was in marked contrast to the reported effect of rhHsp70. For this reason, we decided to study the effects of both rhHsp70–1 and the less purified rhHsp70–2 preparation to determine whether rhHsp70 could in fact induce TNFα production by macrophages.

As shown in Fig. 1A, rhHsp70–1 at 1 μg/ml, as compared with control, did not cause an increase in TNFα release by murine macrophages. In contrast, rhHsp70–2 at the same concentration induced a marked increase in TNFα release to a similar extent as induced by 100 ng/ml LPS. Dose response studies revealed that rhHsp70–1 at concentrations up to 5 μg/ml failed to induce TNFα release. However, rhHsp70–2 at a concentration as low as 0.1 μg/ml induced a marked release of TNFα (Fig. 1B). Likewise, LPS at a concentration as low as 0.1 ng/ml induced a marked release of TNFα from murine macrophages (Fig. 1C).

Endotoxin Contents and Functional Properties of rhHsp70–1 and rhHsp70–2—There were two possible interpretations for the above observations. First, rhHsp70 had no effect on the TNFα release by macrophages; the observed effect of rhHsp70–2 was due to some contaminant(s) present in the rhHsp70–1 preparation. Second, rhHsp70 did have TNFα-inducing effect; failure of rhHsp70–1 to induce TNFα release by macrophages was due to the presence of functionally inactive rhHsp70 in the rhHsp70–1 preparation. To distinguish these two possibilities, we determined the functional characteristics as well as endotoxin contents of the rhHsp70–1 and rhHsp70–2 preparations.

As shown in Fig. 2, A and B, SDS-PAGE and immunoblot analyses of rhHsp70–1 and rhHsp70–2 revealed that both preparations were identical in rhHsp70 protein content, molecular weight, and ability to interact with an anti-rhHsp70 antibody. In addition, both preparations had similar molecular chaperone activities as determined by the uncoating of clathrin-coated vesicles (Fig. 2C). In contrast, rhHsp70–2 contained a markedly higher content of endotoxin than rhHsp70–1 as determined using the LAL assay. As shown in Fig. 2D, the endotoxin activity of rhHsp70–1 was 4.1 ± 0.2 EU/mg, that of rhHsp70–2 was 577 ± 74.2 EU/mg, and that of E. coli LPS was (2.9 ± 0.7) × 10⁵ EU/mg (n = 3). Thus, the endotoxin content in the rhHsp70–2 preparation was 140 times higher than that in the rhHsp70–1 preparation. The calculated equiv-
To determine whether the contaminating LPS in the rhHsp70 preparations was sufficient to cause the observed TNF-\(\alpha\)-inducing activity, we first used polymyxin B-agarose (Detoxi gel) to remove LPS from the rhHsp70 preparations at the indicated concentrations for 4 h. TNF-\(\alpha\) concentration in conditioned media was then determined. A, TNF-\(\alpha\)-inducing activities of the rhHsp70 preparations and LPS. B, dose response of rhHsp70-induced TNF-\(\alpha\) release by murine macrophages. Values in A and C represent the means \(\pm\) S.D. of 3–6 experiments. *, \(p < 0.05\) (versus control).

The above findings strongly suggest that the observed TNF-\(\alpha\)-inducing effect of rhHsp70 was due to the contaminating LPS. If this were the case, one would expect that the addition of LPS at a concentration found in rhHsp70 preparations to the incubation media would result in the same TNF-\(\alpha\)-inducing activity.
polymyxin B (C) column fractions were determined by LAL assay.
PBLPS to the rhHsp70–gels and densitometric scanning. B column fractions were determined by Coomassie Blue-stained SDS from the column, PB-1 and PB-2. rhHsp70 concentrations in polymyxin through a polymyxin B-agarose column. Two fractions were collected rhHsp70/H9251 rose on the endotoxin activity and TNF α-inducing activities of LPS and rhHsp70–1. The endotoxin in rhHsp70–inducing activity of 0.5 g/ml rhHsp70 was determined. The values in the bar graphs shown in B and C represent the means ± S.D. of three experiments.

Results presented in the current study demonstrated that rhHsp-70 did not induce TNFα release from murine macrophages and that the TNFα-inducing activity of the rhHsp70–2 resulted in a similar TNFα-inducing activity as that of 0.2 ng/ml LPS or 1 μg/ml rhHsp70–2.

**Effect of Heat Inactivation on TNFα-Inducing Activities of LPS and rhHsp70–2**—One of the characteristics of LPS is its relative heat resistance. Previous studies show that the TNFα-inducing activity of rhHsp70 was heat-sensitive, whereas that of LPS was heat-resistant (12, 15–17). It was concluded that the TNFα-inducing activity of rhHsp70 could not have been due to the effect of contaminating LPS. As shown in Fig. 6, we also demonstrated that the TNFα-inducing activity of rhHsp70–2 at 1 μg/ml, but not that of LPS at 100 ng/ml, was sensitive to prior heating in a boiling water bath for 60 min.

The demonstrated heat sensitivity of rhHsp70–2 in inducing TNFα release appeared in contradiction to our above conclusion that the TNFα-inducing activity of rhHsp70–2 was due to the contaminating LPS. However, the following evidence suggests that LPS at low concentrations, such as 20 ng/ml, is heat-sensitive and that heat sensitivity by itself is not sufficient to distinguish whether an observed effect is due to LPS.

As shown in Fig. 7A, the addition of 0.2 ng/ml LPS to rhHsp70–1 (1 μg/ml) resulted in a TNFα-inducing effect similar to that of 0.2 ng/ml LPS or 1 μg/ml rhHsp70–2 (as previously demonstrated in Fig. 5). However, the TNFα-inducing effect of the combination of LPS and rhHsp70–1 was as heat-sensitive as that of the rhHsp70–2. Analysis of rhHsp70 using SDS-PAGE revealed that heat inactivation removed most of Hsp70 from the rhHsp70–1 and rhHsp70–2 solutions (Fig. 7B). Thus, it was possible that heat inactivation removed the rhHsp70 along with LPS from the solution, resulting in the observed loss of TNFα-inducing activity.

To eliminate the potential confounding effect of rhHsp in the above-observed heat sensitivity of LPS, we heated LPS alone at two concentrations, 20 ng/ml and 20 μg/ml, and then determined the endotoxin activity and TNFα-inducing activity of 0.2 ng/ml LPS before and after heating. As shown in Fig. 8, heating 20 ng/ml LPS in a boiling water bath for 60 min markedly reduced its endotoxin activity as well as its TNFα-inducing activity. Likewise, heating LPS at 20 μg/ml also reduced its endotoxin activity and TNFα-inducing activity, but to a lesser degree. Thus, LPS at low concentrations is highly heat-sensitive.

**DISCUSSION**
preparation was entirely due to the contaminating LPS. This conclusion was derived from the following evidence. First, the highly purified rhHsp70 preparation (rhHsp70–1) with an LPS content of 1.4 pg/μg was unable to induce TNFα release from macrophages at concentrations up to 5 μg/ml, whereas the less purified rhHsp70 preparation (rhHsp70–2), at 1 μg/ml, with a LPS content of 0.2 ng/μg was able to induce TNFα release to the same extent as that induced by 0.2 ng/ml LPS (Figs. 1 and 2D). Second, failure of rhHsp70–1 to induce TNFα release was not due to defective physical properties since rhHsp70–1 and rhHsp70–2 contained identical Hsp70 content as determined by SDS gels stained with Coomassie Blue and Western blots probed with an anti-rhHsp70 antibody (Fig. 2, A and B). Both rhHsp70 preparations also had similar enzymatic activities as judged by their ability to remove clathrin from clathrin-coated vesicles (Fig. 2C). Third, removal of LPS from rhHsp70–2 by polymyxin B-agarose column or direct addition of polymyxin B to the incubation medium essentially eliminated the TNFα-inducing activity of rhHsp70–2 (Figs. 3 and 4). Fourth, the addition of LPS at the concentration found in rhHsp70–2 to rhHsp70–1 resulted in the same TNFα-inducing activity as observed with rhHsp70–2 (Fig. 5).

Using rhHsp70 obtained from the same source as the one we used in the current study (StressGen Biotechnologies Corp.), a number of laboratories reported that rhHsp70 markedly induced TNFα production by monocytes and macrophages (12, 15–17). However, with the exception of one report by Dybdahl et al. (12), none of the reports indicated which rhHsp70 preparation from StressGen was used in the studies. Using the less purified preparation of rhHsp70 (NSP-555/SPP-755 or rhHsp70–2), Dybdahl et al. (12) report that their rhHsp70 preparation had an endotoxin activity of about 100 EU/mg. They demonstrated that the rhHsp70 preparation could induce TNFα and interleukin-6 production by murine macrophages and human monocytes at rhHsp70 concentrations of 3–10 μg/ml. The endotoxin activities present in 3–10 μg/ml rhHsp70,
contaminating LPS from rhHsp70—almost completely eliminates the TNF heat-sensitive.
the observed TNF/H9251 effect on TNF macrophages, heat inactivation of LPS appeared to have no
ing activity of LPS was heat-resistant. Results in Fig. 8 showed
80% decrease in TNF/H9251 induction. Results in Fig. 1C showed, however, LPS at 0.1 ng/ml was sufficient to induce significant TNFα release from murine macrophages. Thus, even if heating in boiling water for 60 min inactivated more than 99% of LPS at 100 ng/ml, there would still be sufficient active LPS left to induce TNFα release, giving the impression that TNFα-inducing activity of LPS was heat-resistant. Results in Fig. 8 showed that heating LPS at 20 ng/ml, the same concentration found in 100 μg/ml rhHsp70—2 when it was heated, resulted in an 80% decrease in TNFα-inducing activity. This explains why the observed TNFα-inducing activity of rhHsp70—2 was heat-sensitive.

In the current study, we demonstrated that removal of the contaminating LPS from rhHsp70—2 by polymyxin B-agarose column or by preincubation of macrophages with polymyxin B almost completely eliminates the TNFα-inducing activity of rhHSP70—2. In contrast, others observed no significant effect of preincubation or co-incubation with polymyxin B on cytokine-inducing activity of the rhHsp70 (12, 15, 17). The reason for this discrepancy is not clear. The effect of polymyxin B may depend on the concentrations of polymyxin B and rhHsp70 and the amount of contaminating LPS present in the final incubation medium. Further studies are necessary to clarify this discrepancy.

In addition to rhHsp70, recombinant chlamydial Hsp60 and recombinant human Hsp60 have all been shown to have cytokine-inducing effects (29—32). Hsp70, Hsp90, and gp96 isolated from mouse liver are also shown to induce cytokine production (33), although at concentrations 1—2 orders of magnitude higher than the recombinant Hsp70s. In view of the current study, further investigation is required to determine whether these observed cytokine-inducing effects of heat shock proteins (Hsps) was indeed due to the Hsps themselves or the contaminination of LPS and/or bacterial lipoproteins in heat shock protein preparations.

REFERENCES
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