

Recombinant human Hsp70 protects against lipoteichoic acid-induced inflammation manifestations at the cellular and organismal levels

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Abstract It has been previously reported that pretreatment with exogenous heat shock protein 70 (Hsp70) is able to protect cells and animals from the deleterious effects of bacterial lipopolysaccharide (LPS) produced by Gram-negative bacteria. However, the effects of Hsp70 pretreatment on lipoteichoic acid (LTA) challenge resulted from Gram-positive bacteria infection have not been fully elucidated. In this study, we demonstrated that preconditioning with human recombinant Hsp70 ameliorates various manifestations of systematic inflammation, including reactive oxygen species, TNF α , and CD11b/CD18 adhesion receptor expression induction observed in different myeloid cells after LTA addition. Therefore, exogenous Hsp70 may provide a mechanism for controlling excessive inflammatory responses after macrophage activation. Furthermore, in a rat model of LTA-induced sepsis, we demonstrated that

prophylactic administration of exogenous human Hsp70 significantly exacerbated numerous homeostatic and hemodynamic disturbances induced by LTA challenge and partially normalized the coagulation system and multiple biochemical blood parameters, including albumin and bilirubin concentrations, which were severely disturbed after LTA injections. Importantly, prophylactic intravenous injection of Hsp70 before LTA challenge significantly reduced mortality rates. Thus, exogenous mammalian Hsp70 may serve as a powerful cellular defense agent against the deleterious effects of bacterial pathogens, such as LTA and LPS. Taken together, our findings reveal novel functions of this protein and establish exogenous Hsp70 as a promising pharmacological agent for the prophylactic treatment of various types of sepsis.

Keywords Lipoteichoic acid · Lipopolysaccharide · Exogenous heat shock protein 70 · Sepsis models

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Introduction

Despite the wonderful achievements of modern biology and medicine, sepsis remains one of the major causes of hospital mortality (Stearns-Kurosawa et al. 2011). In the USA alone, 750,000 cases of sepsis occur each year, and more than 215,000 patients die of sepsis-induced damage because the known endotoxemia antagonists are only able to partially suppress sepsis symptoms (Martin et al. 2003). The growing frequency of sepsis syndrome observed in the modern world may be partially explained by complications often observed after chemotherapy in cancer patients, organ transplantation, cardiac surgery, and prosthesis use (Friedman et al. 1998; Vincent 2008). Moreover, in 40–60% of all

patients with severe sepsis, acute respiratory distress syndrome often develops, coupled with acute lung dysfunction, which usually results in mortality (Gong et al. 2005; MacCallum and Evans 2005).

In recent years, the proportion of sepsis induced by Gram-positive bacterial infections has increased. In the USA in 2001, more than 52.1% of patients were hospitalized with Gram-positive sepsis (Leaver et al. 2008). Furthermore, there is a tendency toward increased mortality in patients with Gram-positive sepsis and decreased mortality in patients infected with Gram-negative bacteria (Geerdes et al. 1992). Importantly, increased drug resistance has been reported for Gram-positive bacteria, such as *Staphylococcus aureus* (Wang et al. 2003; Leaver et al. 2008).

Pathological processes after bacterial infection are induced by structural components of bacterial cell walls (Gustot 2011). Thus, in Gram-positive bacteria, these components are represented by lipoteichoic acid (LTA; Fischer et al. 1990; Lappin and Ferguson 2009); for Gram-negative flora, these components are lipopolysaccharide (LPS). In the early stages of bacterial infection, structural components of Gram-positive and Gram-negative bacteria interact with specific Toll-like receptors (TLRs) of macrophages, neutrophils, and monocytes (Leaver et al. 2008; Lotz et al. 2004; Detmers et al. 1998).

The appearance of LPS and LTA in the blood leads to activation of multiple intracellular signaling pathways necessary for the rapid change of the target cell functional state to provide an efficient innate immune response. TLR4 and TLR2, in cooperation with the CD14 receptor, recognize Gram-negative or Gram-positive pathogens, respectively. Lipoteichoic acid, which represents the major component of the Gram-positive bacterial cell wall, stimulates mammalian phagocytes by activating the TLR2, CD14, and CD36 receptors (Lotz et al. 2004; Nilsen et al. 2008). LPS activates phagocytes that explore components of the TLR4/CD14/MD2 complex as well as a few other proteins and receptors (Leon et al. 2008). When bacterial pathogens are recognized by TLR2, these receptors often form heterodimers with TLR1 (Takeuchi et al. 2001).

Previously, it has been shown that after LPS challenge, activation of TLR4 results in enhanced synthesis of several transcription factors, including nuclear factor kappa B (NF- κ B), activator protein-1 (AP-1), and interferon response factor-3 (IRF3). LTA also activates a few transcription factors, including NF- κ B and AP-1 (O'Neill 2003). Furthermore, bacterial pathogens activate the synthesis of a few cytokines, such as IL-1 β , tumor necrosis factor (TNF α), and IL-8 and IL-10 (Pinsky et al. 1993; Gogos et al. 2000). Intracellular generation of reactive oxygen species (ROS) serves to maintain homeostasis; therefore, ROS production is strictly controlled. Both LPS and LTA strongly induce the level of ROS production, enhance the expression of CD11b/CD18 receptors, and inhibit neutro-

phils apoptosis (Lotz et al. 2004; Detmers et al. 1998). The development of systematic inflammation response syndrome and the subsequent manifestations of multiple organ dysfunction syndrome (MODS) result in the rapid progression of septic shock, which often leads to death (Bone 1991; Vincent 2008).

Aside from the activation of specialized blood cells involved in the innate immune response, the appearance of pathogens in the blood leads to activation of various SOS systems within the organism, including various heat shock or stress proteins (Hsps). Primarily, Hsps appear to serve as molecular chaperones because they recognize and bind to nascent polypeptide chains and partially folded proteins, preventing their aggregation and misfolding (Nelson et al. 1992). Major chaperones including Hsp70, Hsp90, and small Hsps are often present in the cell at high concentration under normal physiological conditions. Also, their expression may be strongly induced by different forms of stress (Henderson 2010).

Heat shock proteins play an important role in protecting organisms at the cellular level from various adverse environmental stressors, including high temperatures. The 70-kDa stress-induced heat shock protein has been reported to play a major role in such protection by several authors (Tsan and Gao 2004). This protein is found in all animals, and it is composed of two major functional domains. The NH₂-terminal, which is highly conserved in various animals represents ATPase domain, which is able to bind and hydrolyze ATP, whereas the COOH-terminal is the less-conserved domain and is required for polypeptide binding (Young 2010). It was shown that sepsis is accompanied by increased synthesis and accumulation of Hsp70 in myeloid cells, apparently providing cellular tolerance against bacterial endotoxins (Tsan and Gao 2009). Furthermore, it has been shown that extracellular Hsp70 enhances innate immunity (Johnson and Fleshner 2006) and is able to protect cultured cells from various factors (Guzhova et al. 1998).

Previously, we demonstrated that intravenous administration of exogenous Hsp70 significantly reduced LPS-induced mortality in rats (Kustanova et al. 2006) and normalized neutrophil activation that was disrupted by LPS (Yurinskaya et al. 2009). We also showed that administration of Hsp70 preparations from different sources (bull muscle, camel heart, and recombinant human protein expressed in *Spodoptera* cells) efficiently protects blood phagocytes from LPS challenge (Rozhkova et al. 2010).

Herein, we investigate the protective potential of recombinant human rHsp70 at the cellular and organismal levels against the deleterious effects of LTA in a model of "Gram-positive sepsis." In this study, we monitored the effect of the combined action of LTA and rHsp70 on ROS production by neutrophils and monocytes. Because

decreased proinflammatory protein expression, such as TNF α production, is a hallmark of endotoxin tolerance, we monitored TNF α levels in our myeloid cell experiments. Using this model system, we also monitored and recorded CD11b/CD18 receptor levels.

Furthermore, we studied the protective properties of rHsp70 using rats injected with semi-lethal doses of LTA (model of LTA-induced sepsis). In these studies, clear-cut protective effects of prophylactic Hsp70 administration were evidenced by less severe LTA-induced sepsis symptoms. The experiments demonstrated that rHSP70 preconditioning significantly decreases ROS and TNF α production as well as CD11b/CD18 receptor expression in human neutrophils induced by LTA administration. Furthermore, prophylactic rHsp70 injections decreased LTA-induced mortality in rats and partially normalized hemostasis, hemodynamics, and multiple biochemical properties in blood exposed to LTA challenge.

Materials and methods

Production of human rHsp70

In our research, we used human recombinant Hsp70 expressed in armyworm (*Spodoptera frugiperda*) cell culture. The clone containing human Hsp70 cDNA (pBlueScriptSK(+)-hsp70) was a kind gift of Prof. R. Morimoto (Northwestern University). Human hsp70 gene was subcloned in donor plasmid pFastBacHTb-hsp70 at *HindIII* and *XhoI* restriction sites under polyhedrin promoter (Rozhkova et al. 2010; Merkulov et al. 2011). The plasmid was subsequently used in Bac-to-Bac system (Invitrogen) in order to express human Hsp70. Recombinant protein contained six His at its N-end which enables its isolation from the cell extracts using Ni-NTA resin columns according to the manufacturer's instructions (QUIAGEN, Ni-NTA Superflow BioRobot Hand Book). Before Ni-NTA column we routinely performed high salt DEAE SepharoseTM Fast Flow extraction to get rid of insect DNA fragments. Figure 1 shows (a) SDS-electrophoresis of

the resulted human recombinant silver-stained Hsp70 purified using Ni-NTA resin columns and (b) Western blot of the isolated rHsp70 using antibodies against human-Hsp70. The isolated recombinant protein was analyzed for endotoxin content using *Limulus* ameobocyte lysate assay (E-Toxate LAL assay Sigma-Aldrich) and the endotoxin concentration of 50 μ g recombinant Hsp70 sample was below detection (data not shown) essentially as shown by other authors using the same expression and purification systems (Zheng et al. 2010) who also concluded basing on thorough analysis that the recombinant human Hsp70 protein expressed in Sf9 cells is free of endotoxin.

Cell culture

Neutrophils were isolated from the plasma of volunteer donors by Ficoll-verografin gradients as described previously (Boyum 1968). Neutrophil viability was monitored by flow cytofluorimetry (Vinokurov et al. 2006).

Monocytes Human monocytes were isolated by a modified technique (Almeida et al. 2000) using Percoll gradient centrifugation (density 1.064 g/ml) of mononuclear fraction. The purity of isolated monocytes was routinely checked by flow cytofluorimetry by staining the isolated cells with fluorescein isothiocyanate-labeled anti-CD14 antibodies and analyzing the morphology of the cell population. The purity of monocytes was estimated to be about 95%.

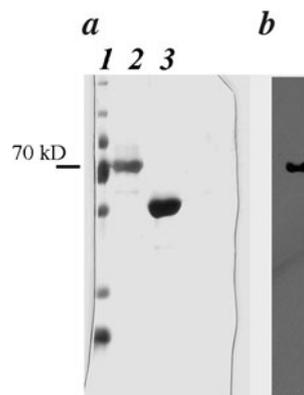
LTA from *S. aureus* (Sigma) was dissolved in sterile phosphate-buffered saline. LPS from *Escherichia coli* O55: B5 (Sigma) was also dissolved in sterile phosphate-buffered saline.

Where indicated, neutrophils in Hanks balanced salt solution (HBSS) medium (1×10^6 cells/ml) were primed with LTA for 30 min at 37°C in the presence of 2% serum (plasma of ten healthy donors that had been stored at -70°C before use). The samples were placed into a 1,250 LKB luminometer (Sweden). The cells were stimulated by 1 μ M formyl-methionine-leucine-phenylalanine (fMLP) in the presence of 35 μ M luminol. Chemiluminescence (CL) was measured at the 10-min mark.

Expression of CD11b and CD18 receptors on the surface of neutrophils was assayed using the corresponding CD11b antibodies conjugated with fluorescein isothiocyanate (Caltag, Burlingame, CA, USA) followed by flow cytometric analysis (Wang et al. 2004).

In the course of apoptosis studies, the isolated neutrophils (10^6 cells/ml) were cultured in RPMI 1,640 medium containing 10% heat-inactivated fetal calf serum (FCS), 1% glutamine, 1% penicillin, and 1% streptomycin at 37°C under 5% CO₂ for 14 h. The level of apoptosis was determined by flow cytofluorimetry, using a fluorescent

Fig. 1 Human recombinant Hsp70 isolated from *Spodoptera* cells using Ni-NTA columns. **a** SDS-PAGE electrophoresis of silver-stained proteins. Lane 1 Molecular weight markers (Page Ruler Plus, Fermentas), lane 2 rHsp70 (700 ng), lane 3 human albumin (2 μ g, Sigma). **b** Western blot of rHsp70 (700 ng) using anti-human Hsp70 monoclonal antibodies (CO2F3A-5, Stress Gen)



probe (1 µg/ml; Hoechst-33258, St. Louis, MO, USA; Vinokurov et al. 2006). Neutrophil viability after the experiments was 96–98%.

The determination of TNF α levels

TNF α assay TNF α production was evaluated based on the cytotoxic effect of neutrophils supernatants using L-929 cells as a target. L-929 cells were obtained from the Russia Tissue Culture Collection, Institute of Cytology, Russian Academy of Sciences. L-929 cells (2×10^4 cells/100 µl) were placed into 96-well flat-bottomed plates and were cultured in RPMI 1,640 medium containing 10% heat-inactivated FCS, 1% L-glutamine, 1% penicillin, and 1% streptomycin at 37°C under 5% CO₂ for 24 h. After 24 h, actinomycin D (Sigma) was added (concentration 1 µg/ml) to the monolayer as well as 100 µl of supernatant of neutrophil cells in each well. Pure medium was added to the control wells. The plates were incubated for 24 h at 37°C under 5% CO₂ and stained with Crystal Violet (Sigma). Survival of the cells was determined after the crystals were completely dissolved in 1% SDS. The absorbance at 595 nm was determined using the UNIPLAN plate reader (ZAO PICON, Moscow, Russia). Triplicate wells were assayed for each condition, and S.D. was determined. Relative levels of TNF α production were determined based on toxicity index as described previously (Pfister et al. 1992).

Animal use Adult male Sprague-Dawley rats weighing 300–350 g were used in all experiments. The animals were maintained in their home cages in a climate-controlled room with a 12:12-h light–dark cycle and had free access to water and food. All procedures involving animals were reviewed and approved by the Animal Care and Use Committee of Branch of Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry.

Measurement of arterial blood pressure and mortality rate

Animals were anesthetized with chloral hydrate 400 mg/kg intraperitoneally and catheterized. Catheters were placed in the inferior vena cava (through the left jugular vein) for administration of drugs and in abdominal aorta (through the left carotid artery) for the monitoring of blood pressure. Gram-positive sepsis was achieved by single administration of *S. aureus* LTA 3 mg/kg as described (Kengatharan et al. 1998).

In the first series of experiments, the antagonizing effect of rHsp70 against endotoxemia caused by *S. aureus* LTA (Sigma) intravenous injections (3 mg/kg) was monitored essentially as described in (Kustanova et al. 2006). Briefly, three experimental groups were formed: rHsp70 was administered 10 min before LTA injections. In the first

group served as a control in this series of experiments animals were injected with 0.9% NaCl ($n=6$); in the second group, animals were injected with LTA ($n=10$); in the third group, animals were injected with LTA 10 min after the administration of rHsp70 ($n=10$).

The experiments were started 24 h after the implantation of catheters. Blood pressure was measured by SR-01 electro manometer. Mean arterial blood pressure (MAP) and heart rate (HR) were monitored for 30 min before the experiments (baseline) and 5 h after LTA and/or Hsp70 administration. Rats were randomly assigned to either LTA treatment groups or Hsp70 and LTA combined treatment groups. All reagents were dissolved in 0.9% NaCl. The volume of the samples injected was equal to 1 ml/kg.

Blood samples for hemostasis parameters and blood characteristics were taken from an arterial catheter before the experiments and 20 min or 5 h after LTA administration.

Registration of hemostasis parameters

The blood samples were centrifuged as described to get the plasma (Kustanova et al. 2006) and the following parameters were monitored using coagulometer “Trombostat” and standard reagents: thromboplastin clotting time, prothrombin clotting time, and fibrinogen concentration. Besides, we monitored the mortality rate of animals in 72 h after LTA administration.

In the plasma, we determined concentration of glucose, albumin, triglycerides, creatinine, bilirubin, and total protein by “Ellipse” biochemical analyzer (AMS, Italy) using standard reagents provided by “Diasys” (Diagnostic Systems GmbH, Germany).

Statistics

All data were analyzed statistically using the Statistics for Windows program (Microsoft, Redmond, WA, USA). Other analyses were carried out using the Wilcoxon test with the analysis of variance (ANOVA 2 method) and the Duncan range test. Statistical inference was based on levels of significance where $P < 0.05$.

Results

The effect of rHsp70 on LTA-induced increase of ROS production in human neutrophils and monocytes

In the first series of experiments, we compared the effect of human recombinant Hsp70 (rHsp70) on ROS production generated by human neutrophils in response to fMLP (Fig. 2). Prophylactic incubation of the cells with LTA

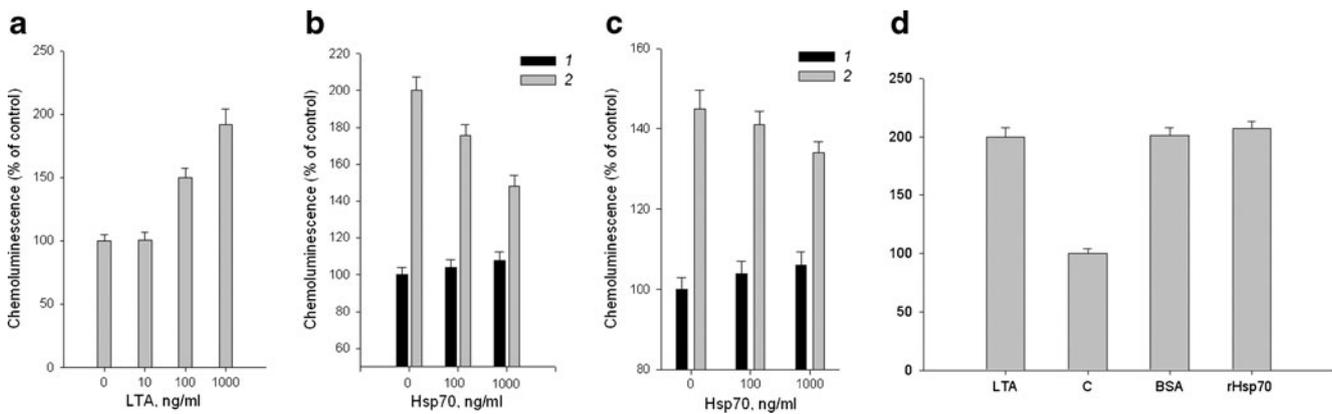


Fig. 2 Effect of LTA and rHsp70 addition on ROS production in neutrophils (**a**, **b**) and monocytes (**c**). *1* Without LTA, *2* With LTA. LTA was added 5 min after addition of rHsp70, and fMLP (1 μ M) was added 25 min later. The concentration of neutrophils was 10^6 cells/ml, and the concentration of monocytes was 5×10^5 cells/ml. **d** Effect of bovine albumin (BSA) and heat-denatured Hsp70 on ROS production in neutrophils. LTA was added 5 min after addition of denatured

rHsp70 (1 μ g/ml) or BSA (1 μ g/ml) and fMLP (1 μ M) was added 25 min later. *C* chemoluminescence of control untreated cells. rHSP70 was boiled at 100°C for 20 min and tested for the influence on ROS production. The concentration of LTA in **b**, **c**, and **d** is 1 μ g/ml. The results are expressed as the percentage of the control. Mean \pm standard error of six experiments is shown ($P < 0.01$)

30 min before fMLP addition (Fig. 2a) significantly increased the CL value in comparison with the control in dose-dependent manner at a range of LTA concentrations (10 ng/ml to 1 μ g/ml). The CL level of neutrophils at 37°C in the absence of LTA was taken as a control in our experiments. rHsp70 slightly increased ROS level in comparison to the control cells (Fig. 2b). Addition of polymyxin B (20 μ g/ml) to neutrophils and monocytes treated with LTA in the absence of rHsp70 did not decrease CL in the cells; these data indicate that the LTA used was not contaminated with LPS (data not shown).

Administration of rHsp70 5 min before LTA addition significantly attenuated LTA-induced CL in comparison with those cells to which only LTA was applied (Fig. 2b). In order to check whether the protective antitoxic effect of exogenous rHsp70 represents a specific feature of neutrophils, we performed similar experiments with human monocytes and observed virtually identical results. Figure 2b, c summarizes this data, demonstrating a dose-dependent protective effect of rHsp70 in terms of LTA-induced ROS production in neutrophils and monocytes. Control experiments exploring heat-inactivated rHsp70 or BSA (Fig. 2d) demonstrated that pretreatment of the neutrophils with these agents does not modify LTA-induced ROS level.

Special experiments were performed to elucidate the effect of time interval between rHsp70 administration and LTA addition on ROS production in neutrophils (Fig. 3). The administration of Hsp70 5 min before LTA addition significantly attenuated LTA-induced CL in comparison with the series where only LTA has been applied (Fig. 3), and hence, basing on this analysis, we routinely added rHsp70 5 min before LTA challenge.

The effect of rHsp70 on LTA-induced increase of TNF α production in neutrophils

In the second series of experiments, we monitored the effect of rHsp70 preconditioning on TNF α production in neutrophils induced by LTA or LPS administration. In the absence of both bacterial toxins, rHsp70 administration induced a slight increase in TNF α production in control neutrophils (Fig. 4). However, after addition of both bacterial toxins, a strong induction of TNF α production was evident (Fig. 4). Characteristically, the induction was more pronounced when LPS was added (150 and 50 μ g/ml, respectively). Preconditioning of neutrophils with rHsp70 at

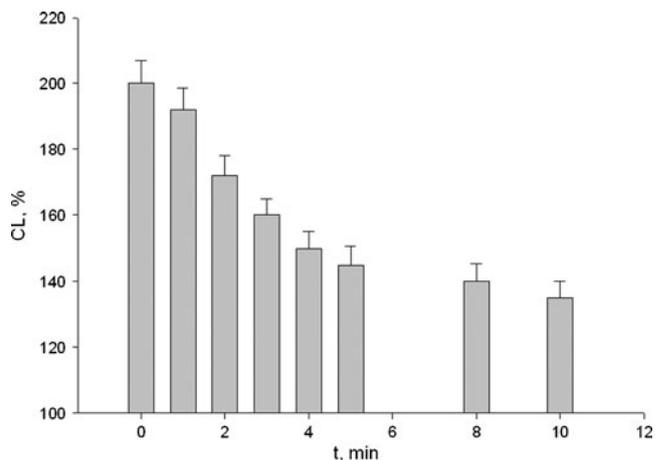


Fig. 3 Effect of time interval between rHsp70 administration and LTA addition on ROS production in neutrophils (10^6 cells/ml). LTA (1 μ g/ml) was added to the cells after 1, 2, 4, 8, or 10 min of incubation with rHsp70 (1 μ g/ml), and fMLP (1 μ M) was added to each tube 30 min later. The mean \pm standard error of six independent experiments is shown ($P < 0.01$)

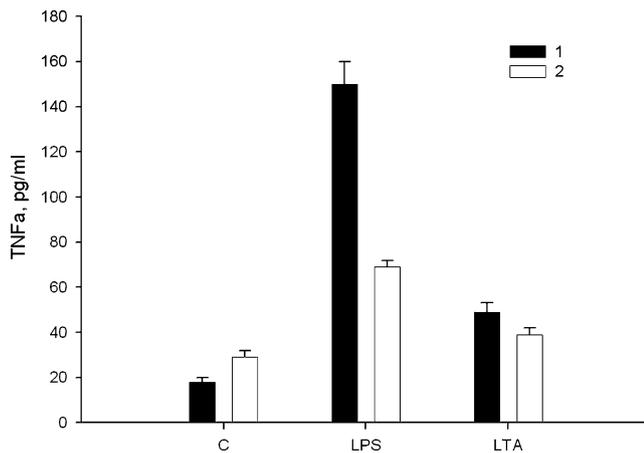


Fig. 4 Effect of rHsp70 (5 $\mu\text{g/ml}$) and the two bacterial toxins (LPS and LTA) on TNF α production in neutrophils (5×10^6 cells/ml). 1 Without rHSP70, 2 With rHSP70. C control. The concentration of LTA and LPS was 1 $\mu\text{g/ml}$. The mean \pm standard error of six independent experiments is shown ($P < 0.01$)

5 $\mu\text{g/ml}$ for 5 min led to a significant decrease in TNF α production after administration of both toxins (LPS, 47 pg/ml ; LTA, 32 pg/ml ; Fig. 4).

Preconditioning with rHsp70 significantly modifies LTA-induced expression of CD11b/CD18 receptors in neutrophils

It is well-known that bacterial endotoxins can significantly increase the level of various adhesion receptors on neutrophils. Thus, bacterial LPS induce the expression of β_2 -integrins in the membrane of neutrophils and increase the level of CD11b/CD18 receptors (Detmers et al. 1998). Therefore, in the third series of experiments, we investigated the effect of rHsp70 pretreatment on the expression of CD11b/CD18 receptors after LTA addition.

Importantly, while rHsp70 only slightly induced the expression of the receptors studied (data not shown) LTA administration at a 1- $\mu\text{g/ml}$ concentration significantly induced the expression of these receptors in neutrophils (Fig. 5). These data corroborate the results of other authors (Carratelli et al. 1996). The pretreatment of neutrophils with an rHsp70 preparation (1 $\mu\text{g/ml}$ for 5 min) before LTA addition was quite efficient at significantly decreasing LTA-induced expression of CD11b/CD18 receptors (Fig. 5).

Hsp70 pretreatment does not significantly modify LTA-induced inhibition of neutrophil apoptosis

It is well-known that during sepsis in animals and in vitro, bacterial endotoxins significantly inhibit apoptosis of neutrophils, which leads to clearance disturbance and may induce inflammation (Power et al. 2002). Therefore, in the fourth series of experiments, we monitored the effect of

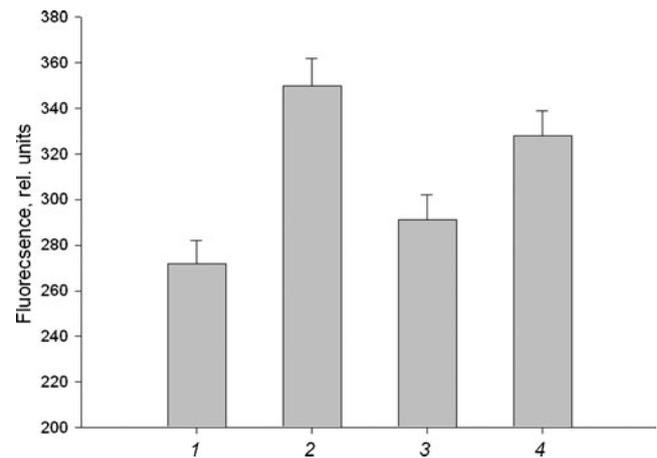


Fig. 5 Expression of CD11b/CD18 receptors in neutrophils (10^6 cells/ml). 1 Control cells, 2 LTA (1 $\mu\text{g/ml}$), 3 rHsp70 (2 $\mu\text{g/ml}$), 4 Neutrophils preincubated with rHsp70 (2 $\mu\text{g/ml}$) before LTA addition (1 $\mu\text{g/ml}$). The mean \pm standard error of six independent experiments is shown ($P < 0.01$)

rHsp70 preparations on normal neutrophil apoptosis after LTA addition. The experiments demonstrated that 5 $\mu\text{g/ml}$ LTA inhibited apoptosis of neutrophils by almost 32% (Fig. 6). This value coincides with the results of other authors (Lotz et al. 2004). In these series of experiments, apoptosis of neutrophils not treated with LTA was considered to be 100%. Characteristically, 5 $\mu\text{g/ml}$ rHsp70 also slightly (18%) inhibited neutrophil apoptosis (Fig. 6). Furthermore, preconditioning with 5 $\mu\text{g/ml}$ Hsp70 with subsequent administration of LTA (Fig. 6) leads to a 30% inhibition of endotoxin-induced apoptosis in neutrophils. Therefore, in our experiments rHsp70 preconditioning does not significantly normalize apoptosis disturbed by LTA administration.

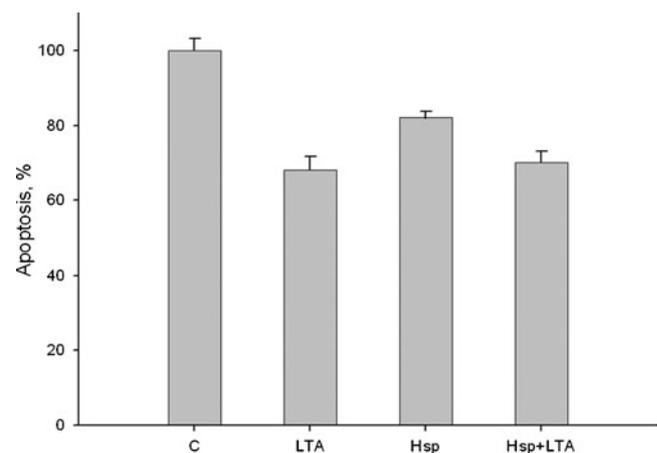


Fig. 6 Effect of LTA (5 $\mu\text{g/ml}$) and rHsp70 (5 $\mu\text{g/ml}$) on neutrophil (1×10^6 cells/ml) apoptosis. C control cells. LTA—5 $\mu\text{g/ml}$ LTA; Hsp—5 $\mu\text{g/ml}$ rHSP70; Hsp + LTA—neutrophils preincubated with 5 $\mu\text{g/ml}$ rHSP70 before LTA addition (5 $\mu\text{g/ml}$). Time of neutrophil cultivation was 18 h. The results are expressed as the percentage of the control. Mean \pm standard error of six experiments is shown ($P < 0.001$)

Animal study

The effect of rHsp70 on LTA-induced mortality

Previously, our experiments demonstrated that administration of exogenous mammalian Hsp70 before LPS challenge significantly reduces the mortality rate and improves several parameters of hemostasis and hemodynamics in rats caused by *E. coli*- and *Salmonella typhimurium*-derived LPS injections (Kustanova et al. 2006; Rozhkova et al. 2010; Rozhkova et al. 2011).

Herein, we continue our experiments investigating the protective effect of rHsp70 with another toxin (LTA) isolated from the Gram-positive bacteria, *S. aureus*. In the experiments described below, we used recombinant, inducible human Hsp70 expressed in *Spodoptera* cells. In the experiments described below, after intravenous administration of LTA, the mortality of experimental animals at 72 h reached 50% (Fig. 7). However, prophylactic rHsp70 injection 10 min before LTA challenge effectively protected animals, and the level of mortality in this group did not differ significantly from that of the group injected with a physiological solution (0.9% NaCl; Fig. 7). Therefore, prophylactic administration of rHsp70 showed clear-cut protective effects and ameliorated the deleterious effects of LTA at the organismal level.

Normalizing effect of prophylactic administration of rHsp70 on parameters of hemostasis and hemodynamics

Intravenous LTA injection into the rat model resulted in significant decreases (hypotension) of MAP within the treatment time course (5 h). During the first hour after LTA administration, a significant drop of more than 20% in comparison with the baseline value was observed (Fig. 8a), followed by a partial recovery 2 h after LTA challenge. It is

evident from Fig. 8a that the prophylactic administration of rHsp70 before the LTA had a significant normalizing effect on MAP. In this series of experiments, MAP values differed from the experiments in which only LTA was used within two time intervals (35–90 and 275–285 min; Fig. 8a).

It is well-known that bacterial toxins may strongly affect HR and induce tachycardia. Along these lines, significant increases in HR after LTA injection were observed to peak first at 30–50 min (Fig. 8b). Characteristically, the increased HR after LTA injection was preserved during the entire period of observation (5 h; Fig. 8b). rHsp70 introduced before LTA injection significantly attenuated the first increased HR peak, but it was not very effective during the later stages. However, in general, the severity of tachycardia in the animals after prophylactic rHsp70 treatment was reduced in comparison with the LTA-only injected group (Fig. 8b).

The experiments performed enable us to conclude that recombinant human Hsp70 exhibits antiseptic properties similar to those previously described for Hsp70 isolated from bovine muscle used in an LPS model of sepsis (Kustanova et al. 2006). Therefore, it is worthwhile to explore its protective properties at the cellular level in an attempt to better understand mechanisms underlying such protection.

rHsp70 regulates coagulation system disturbed by LTA challenge

It is well-known that disseminated intravascular coagulation syndrome characterized by systemic intravascular activation of coagulation represents one of the most severe sepsis manifestations, and it is often lethal (McCabe et al. 1983). It is evident from Fig. 9a, b that LTA administration results in significant disturbances of practically all components of the coagulation system. Thus, 20 min after LTA injection, a hypercoagulopathy developed in which thromboplastin

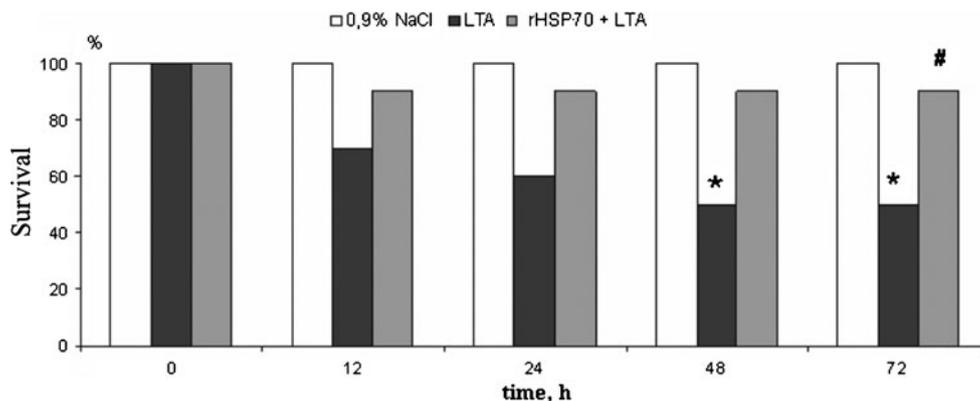
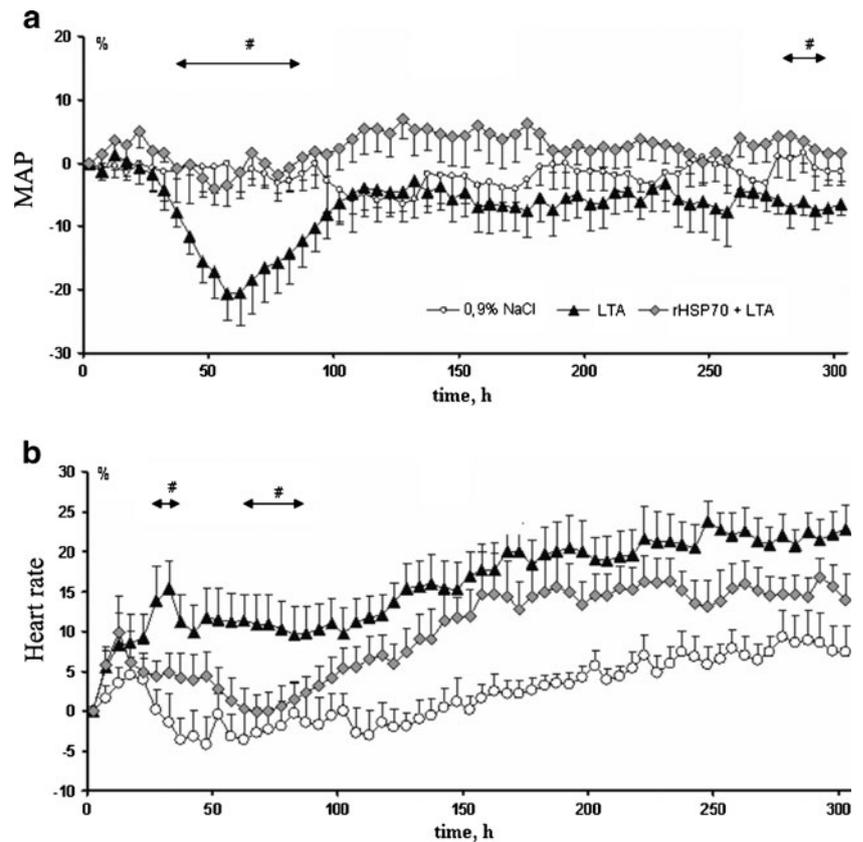


Fig. 7 Effects of rHsp70 on LTA-induced mortality in experimental groups: white column indicates 0.9% NaCl ($n=6$), black column indicates LTA from *S. aureus* (3 mg/kg; $n=10$), gray column indicates LTA added after rhHSP70 pretreatment (266 $\mu\text{g}/\text{kg}$; $n=10$). # $P<0.05$

(using a chi-square test). The results are expressed as the percentage of the LTA group; # $P<0.05$ (using a chi-square test) when the NaCl group was taken as 100%. The abscissa—time after LTA addition (in hours); the ordinate—percentage of surviving animals

Fig. 8 Changes in the **a** mean arterial pressure (MAP) and **b** heart rate after intravenous injection of 0.9% NaCl, LTA, and LTA after rHsp70 pretreatment. Asterisk indicates statistically significant differences ($P < 0.05$ using Kruskal–Wallis) in relation to the LTA group



clotting time (TCT) and prothrombin clotting time (PCT) were significantly decreased. Moreover, these important parameters of hemostasis remain decreased in comparison to basal levels even at 20 h after LTA administration (Fig. 9). Prophylactic administration of rHsp70 had clear-cut protective effects on both coagulation parameters (TCT and PCT) and effectively normalized (decreased) them upon LTA injection (Fig. 9).

Fibrinogen concentration in the plasma represents another important parameter usually modified by bacterial toxins; hence, it can be used as a marker of the acute phase of inflammation (Helling et al. 2010). In our case, 20 min after LTA injection, the fibrinogen level was slightly increased, perhaps due to intensive synthesis of this protein in the liver during sepsis development. However, 5 h after LTA challenge, the fibrinogen level dropped significantly, apparently due to its depletion in the process of intensive blood coagulation (Fig. 9). Preventive administration of Hsp70 has a significant protective effect on fibrinogen level increased after LTA injection (Fig. 9).

Effect of rHsp70 preparation on biochemical parameters of blood under conditions of endotoxemia induced by LTA

The development of endotoxemia induced by LTA injections has been investigated by monitoring the major

biochemical characteristics of blood. Biochemical analysis demonstrated that prophylactic Hsp70 injection did not have any significant effect on glucose and total protein serum levels slightly modified by LTA administration (Fig. 10a, b). On the other hand, Hsp70 pretreatment was quite effective decreasing triglycerides (TAG) and bilirubin concentrations that were drastically enhanced after LTA injection (Fig. 10c, f). Alternatively, prophylactic Hsp70 injection does not significantly modify creatinine concentrations strongly enhanced by LTA (Fig. 10e). Interestingly, the concentration of albumin in contrast to other registered biochemical parameters was significantly decreased after LTA injection, perhaps due to catabolic processes prevailing over anabolic ones (Fig. 10d). In the latter case, Hsp70 administration was also quite effective at normalizing albumin level decreased after LTA injection (Fig. 10d).

Discussion

Intravenous administration of bacterial cell wall components into animals is a widely used model of sepsis (De Kimpe et al. 1995; Zanotti-Cavazzoni and Goldfarb 2009). This approach enables the investigation of both the early processes of sepsis development and the initial response of target cells to the pathogens used.

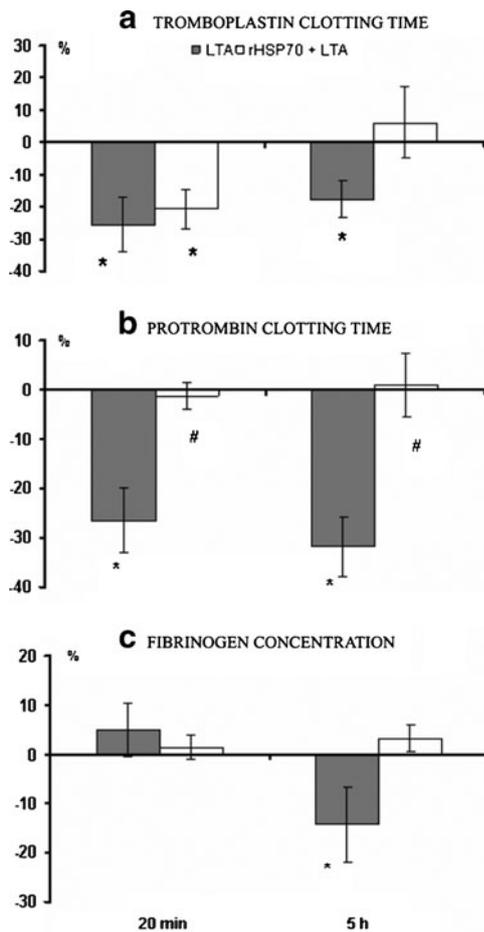


Fig. 9 Changes of hemostatic parameters due to intravenous administration of LTA depending on administration of rHsp70 preparations (**a** thromboplastin clotting time, **b** prothrombin clotting time, **c** fibrinogen concentration). Gray column indicates LTA and while column indicates rHsp70 + LTA. * $P < 0.05$ using Wilcoxon test relative to baseline values; # $P < 0.05$ using Mann-Whitney test in relation to the LTA group

In recent years, abundant evidence has appeared implicating Hsps, particularly Hsp70, in the modification the immune response after introduction of various bacterial pathogens into the vasculature, including LTA and LPS (Johnson and Fleshner 2006; Yurinskaya et al. 2009; Rozhkova et al. 2010; Wheeler et al. 2011).

Interestingly, recently two truncated forms of human Hsp70 were generated and it was demonstrated that in human THP-1 mononuclear cells, C terminus Hsp70 induced tolerance to subsequent LPS stimulation, whereas N terminus Hsp70 did not induce tolerance (Wheeler et al. 2011).

Herein, we continued our studies at the cellular level and investigated the protective role of exogenous human Hsp70 administration prior to LTA challenge using human phagocytes (neutrophils and monocytes) as a model of early pathogen response. Moreover, we investigated the prophylactic role of exogenous Hsp70

during the later stages of sepsis development exploring animal model of LTA-induced sepsis.

It is well-known that bacterial pathogens interact with target myeloid cells via special receptors. Increased ROS concentration represents one of the first indications of endotoxemia (Victor et al. 2005). We showed that exogenous Hsp70 exhibits a maximal protective effect when applied 5–7 min before LTA administration by monitoring ROS level at different time intervals (Fig. 3). The results obtained are in general in agreement with the results of our previous studies on LPS-induced activation of human neutrophils (Rozhkova et al. 2010).

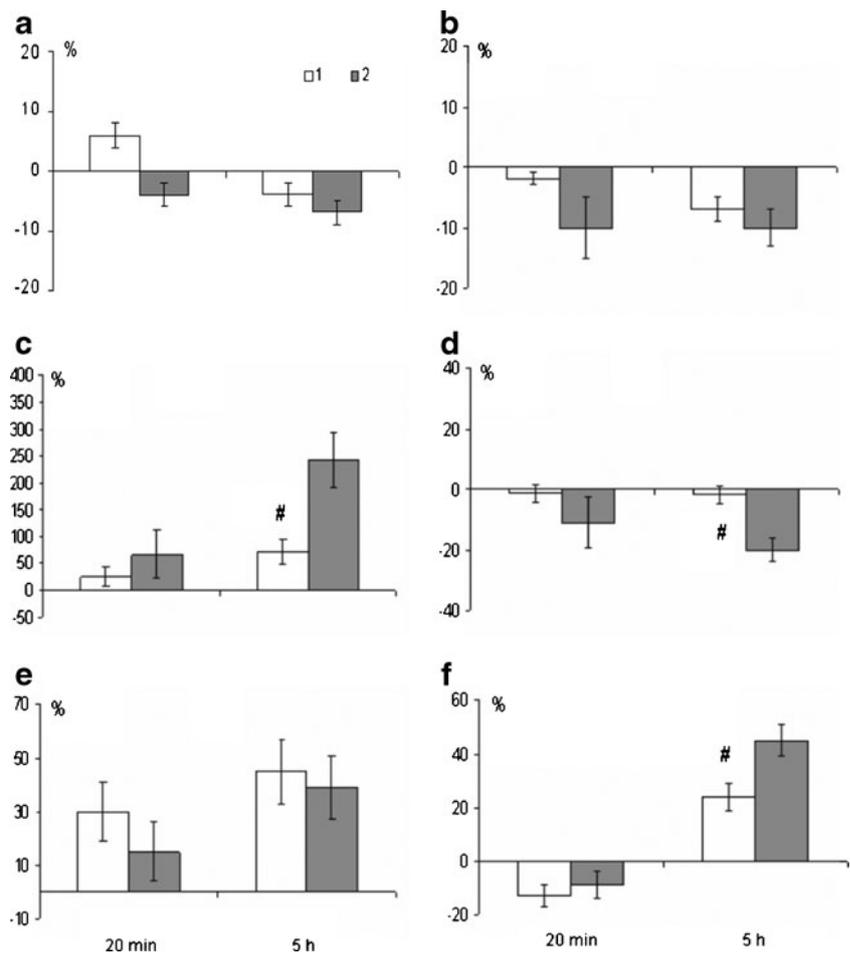
However, in our experiments, LTA could increase ROS levels in myeloid cells up to 200% within a concentration range of 0.01 to 1 $\mu\text{g/ml}$ (Fig. 2a). On the other hand, LPS administration resulted in approximately the same ROS concentration increase when much lower concentrations of the toxin were applied (1–20 ng/ml; Lotz et al. 2004; Rozhkova et al. 2010). It is noteworthy that although the delivery of both toxins (LTA and LPS) occurs via CD14 receptors (Lotz et al. 2004; Leon et al. 2008), only about 7,000 receptors can be activated in neutrophils, while in monocytes, more than 100,000 of these receptors can be expressed (Antal-Szalmás 2000). Furthermore, both LTA and LPS administration to myeloid cells results in the enhanced expression of CD11b/CD18 adhesion receptors (Carratelli et al. 1996), as well as enhanced LPS activity (Detmers et al. 1998). Exogenous rHsp70 used in our experiments was able to significantly increase the level of CD11b/CD18 receptors (Fig. 5). Similarly, prophylactic administration of exogenous Hsp70 in our study significantly attenuated LTA-induced TNF α production in neutrophils (Fig. 4).

It was shown previously that endogenous Hsp70 induced by temperature elevation can significantly increase the expression of TLR2 and TLR4 in macrophages and monocytes (Zhou et al. 2005). Coupled together, these data implicate Hsp70 in the activation of TLR4- and TLR2-dependent responses to bacterial pathogen challenge. Importantly, p38MAP kinase participates in myeloid cell activation induced either by Hsp70 or by LPS challenge (Tsan and Gao 2009; Zhou et al. 2005). It was also shown that LTA addition increases the production of nitric oxide by means of phosphatidylinositol 3-kinase, Akt, and p38MAPK (Kao et al. 2005).

It was also shown that inhibition of neutrophil apoptosis by LTA is regulated mainly via the activation of TLR2, CD14, and CD36 receptors (Lotz et al. 2004), while Hsp70 is able to interact with target cells by means of the CD14/TLR2 and CD14/TLR4 complexes (Tsan and Gao 2004).

Previously, we demonstrated that exogenous Hsp70 (1 $\mu\text{g/ml}$) may slightly inhibit apoptosis in neutrophils and significantly decrease LPS-induced neutrophil apopto-

Fig. 10 The percent change of biochemical parameters of animal blood, including glucose (a), total protein (b), triglycerides (c), albumin (d), creatinine (e), and bilirubin (f) concentrations after intravenous injection of the following: 1 rHsp70 (dose 266 $\mu\text{g}/\text{kg}$) + LTA relative to baseline values (before introduction of the agents) or 2 LTA from *S. aureus* (dose 3 mg/kg). # $P < 0.05$ (using Mann–Whitney test). The abscissa—time after introduction of LTA; the ordinate—changes in biochemical parameters



sis when the toxin was used at concentrations of 100 ng/ml (Yurinskaya et al. 2009; Rozhkova et al. 2010). On the other hand, while LTA addition significantly decreases neutrophil apoptosis at the concentrations used in the present study rHsp70 pretreatment did not exhibit a significant protective effect in this case but just a tendency to normalize apoptosis (Fig. 6). The differences in the level and pattern of protection demonstrated by exogenous Hsp70 regarding LPS and LTA challenge may be explained by action via different pathways on neutrophils activation. Thus, LPS interacts with TLR4, while LTA interacts with TLR2 (Lotz et al. 2004; Leon et al. 2008). In both cases, the protective effect of Hsp70 is likely realized by the modification of TLR2 and TLR4 expression in target myeloid cells (Zhou et al. 2005). Therefore, our experiments demonstrated that preconditioning of cells with human rHsp70 has a significant protective effect and decreases the LTA-induced production of ROS and $\text{TNF}\alpha$. Moreover, exogenous Hsp70 significantly inhibits the expression of CD11b/CD18 receptors on human neutrophils, which are enhanced by LTA administration (Fig. 5). The accumulated data enable us to speculate that potential mechanisms underlying the protective antiseptic action of Hsp70 preparations used in this study are likely

realized at the receptor–membrane complex level in blood phagocytes, which represent the major target of LTA action.

In the second part of our study, we investigated the protective action of exogenous Hsp70 using an animal model of LTA-induced sepsis. In the first 5 h after LTA injection, drastic hemostatic and hemodynamic disturbances were observed (Fig. 8). However, prophylactic administration of human exogenous Hsp70 partially normalized tachycardia, decreased MAP (Fig. 8) and significantly reduced LTA-induced mortality in rats (Fig. 7).

Notably, the endogenous Hsp70 actively synthesized in all cells after heat shock also effectively inhibits bacterial pathogen-induced production of $\text{TNF}\alpha$ and other cytokines, and it appears to be responsible for normalization of the cardiovascular and coagulation systems that are strongly affected by bacterial endotoxins (Ding et al. 2001; Suganuma et al. 2002; Nakada et al. 2005). Hsp70 preconditioning also normalized all hemostatic parameters, including fibrinogen concentration, disturbed by LTA challenge. Additionally, prophylactic addition of rHsp70 improved the prothrombin and thromboplastin clotting times, which were also strongly affected when only LTA was added (Fig. 9).

Analysis of the biochemical parameters of blood after LTA addition, in contrast to LPS challenge, revealed less drastic deviations from the control values for the most parameters monitored. These results corroborate the conclusion of De Kimpe et al., who stated that LTA-induced sepsis is rarely accompanied by MODS, as opposed to sepsis induced by Gram-negative bacteria (LPS; De Kimpe et al. 1995).

It is known that bilirubin concentration serves as a marker for liver dysfunction, and it is usually increased during sepsis (Hawker 1991). Furthermore, decreased glucose concentration in the blood is a sign of gluconeogenesis disturbances, which usually accompany increased insulin secretion often observed in the case of sepsis (Hirasawa et al. 2009). The enhanced TAG level apparent during sepsis is a consequence of lipid metabolism failure (Baue 1991) while kidney dysfunction often results in an increased serum creatinine level (Carbonell et al. 2004).

Both types of sepsis are accompanied by hypoproteinaemia and hypoalbuminemia due to drastic decreases of total protein and albumin in the blood of the patients. Characteristic deviations of the above-mentioned parameters are evident in LTA-induced sepsis model studied herein (Fig. 10b, d). This phenomenon indicates that catabolic processes are most pronounced in the organism, and kidney and liver dysfunction is probable.

Creatinine level was significantly enhanced in animals after intravenous LTA injections (Fig. 10c), which indicates acute kidney dysfunction, and was typical for bacterial infection (De Kimpe et al. 1995). Prophylactic administration of rHsp70 turned out to be not effective in ameliorating subsequent creatinine increases induced by LTA induction (Fig. 10e). On the other hand, prophylactic rHsp70 administration partially normalizes the concentrations of TGA, albumin, and bilirubin disturbed by LTA challenge (Fig. 10c, d, f).

Our findings suggest that Hsp70 administration prior to LTA challenge has a strong protective effect attenuating most of its harmful consequences at the cellular and organismal levels. However, the detailed mechanisms underlying this protection need to be further investigated.

Concluding remarks

Our experiments demonstrated that preconditioning of various myeloid cells with human rHsp70 free of endotoxin has a significant protective effect and decreases the manifestation of LTA-induced inflammation including production of ROS, TNF α , and CD11b/CD18 adhesion receptors.

Furthermore, in animal LTA-sepsis model prophylactic administration of rHsp70 has clear-cut protective effects, decreases mortality of rats, normalizes hemostatic and hemodynamic characteristics of their blood, coagulation

system, and many other cellular parameters usually disrupted by LTA challenge. Taken together, our findings reveal novel functions of exogenous Hsp70 and establish recombinant human Hsp70 as a promising pharmacological agent for the prophylactic treatment of various types of sepsis.

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References

- Almeida MC, Silva AC, Barral A, Barral Netto M (2000) A simple method for human peripheral blood monocyte isolation. *Mem Inst Oswaldo Cruz* 95:221–223
- Antal-Szalmas P (2000) Evaluation of Cd14 in host defence. *Eur J Clin Invest* 30:167–179
- Baue AE (1991) Nutrition and metabolism in sepsis and multisystem organ failure. *Surg Clin North Am* 71:549–565
- Bone R (1991) The pathogenesis of sepsis. *Ann Int Med* 115:457–469
- Boyum A (1968) Separation of leukocytes from blood and bone marrow. *J Lab Invest* 22:77–85
- Carbonell N, Blasco M, Ferreres J, Blanquer J, García-Ramón R, Mesejo A, Miguel A (2004) Sepsis and SOFA score: related outcome for critically ill renal patients. *Clin Nephrol* 62:185–192
- Carratelli CR, Nuzzo I, Bentivoglio C, Galdiero M (1996) CD11a/CD18 and CD11b/18 modulation by lipoteichoic acid, N-acetylmuramyl-alpha-alanyl-D-isoglutamine, muramic acid and protein A from *Staphylococcus aureus*. *FEMS Immunol Med Microbiol* 16:309–315
- De Kimpe SJ, Thiemermann C, Vane JR (1995) Role for untracellular platelet-activating factor in the circulatory failure in a model of gram-positive shock. *Br J Pharmacol* 116:3191–3198
- Detmers PA, Zhou D, Polizzi E, Thieringer R, Hanlon WA, Vaidya S, Bansal V (1998) Potential role of membrane internalization and vesicle fusion in adhesion of neutrophils in response to lipopolysaccharide and TNF. *J Immunol* 161:1921–1929
- Ding XZ, Fernandez-Prada CM, Bhattacharjee AK, Hoover DL (2001) Over-expression of hsp-70 inhibits bacterial lipopolysaccharide-induced production of cytokines in human monocyte-derived macrophages. *Cytokine* 16:210–219
- Fischer W, Mannsfeld T, Hagen G (1990) On the basic structure of poly(glycerophosphate) lipoteichoic acids. *Biochem Cell Biol* 68:33–43
- Friedman G, Silva E, Vincent JL (1998) Has the mortality of septic shock changed with time. *Crit Care Med* 26:2078–2086
- Hawker F (1991) Liver dysfunction in critical illness. *Anaesth Intensive Care* 19:165–181
- Helling H, Schenk HJ, Pindur G, Weinrich M, Wagner B, Stephan B (2010) Fibrinolytic and procoagulant activity in septic and haemorrhagic shock. *Clin Hemorheol Microcirc* 45:295–300
- Henderson B (2010) Integrating the cell stress response: a new view of molecular chaperones as immunological and physiological homeostatic regulators. *Cell Biochem Funct* 28:1–14
- Hirasawa H, Oda S, Nakamura M (2009) Blood glucose control in patients with severe sepsis and septic shock. *World J Gastroenterol* 15:4132–4136
- Geerdes HF, Ziegler D, Lode H, Hund M, Loehr A, Fangmann W, Wagner J (1992) Septicemia in 980 patients at a university

- hospital in Berlin: prospective studies during 4 selected years between 1979 and 1989. *Clin Infect Dis* 15:991–1002
- Gogos CA, Drosou E, Bassaris HP, Skoutelis A (2000) Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J Infect Dis* 181:176–180
- Gong MN, Thompson BT, Williams P, Pothier L, Boyce PD, Christiani DC (2005) Clinical predictors of and mortality in acute respiratory distress syndrome: potential role of red cell transfusion. *Crit Care Med* 33:1191–1198
- Gustot T (2011) Multiple organ failure in sepsis: prognosis and role of systemic inflammatory response. *Curr Opin Crit Care* 17:153–159
- Guzhova IV, Arnold AC, Darieva ZA, Kinev AV, Lasunskaya EB, Nilsson K, Bozhkov VM, Voronin AP, Margulis BA (1998) Effects of exogenous stress protein 70 on the functional properties of human promonocytes through binding to cell surface and internalization. *Cell Stress Chaperones* 3:67–77
- Johnson JD, Fleshner M (2006) Releasing signals, secretory pathways, and immune function of endogenous extracellular heat shock protein 72. *J Leukoc Biol* 79:425–434
- Kao SJ, Lei HC, Kuo CT, Chang MS, Chen BC, Chang YC, Chiu WT, Lin CH (2005) Lipoteichoic acid induces nuclear factor-kappaB activation and nitric oxide synthase expression via phosphatidylinositol 3-kinase, Akt, and p38 MAPK in RAW 264.7 macrophages. *Immunology* 115:366–374
- Kengatharan K, De Kimpe S, Robson C, Foster S, Thiemermann C (1998) Mechanism of gram-positive shock: identification of peptidoglycan and lipoteichoic acid moieties essential in the induction of nitric oxide synthase, shock, and multiple organ failure. *J Exp Med* 188:305–315
- Kustanova GA, Murashev AN, Karpov VL, Margulis BA, Guzhova IV, Prokhorenko IR, Grachev SV, Evgen'ev MB (2006) Exogenous heat shock protein 70 mediates sepsis manifestations and decreases the mortality rate in rats. *Cell Stress Chaperones* 11:276–286
- Lappin E, Ferguson AJ (2009) Gram-positive toxic shock syndromes. *Lancet Infect Dis* 9:281–290
- Leaver A, Burke-Gaffney A, Evans TW (2008) Gram-positive and gram-negative sepsis: two disease entities? In J.-L. Vincent (Ed.) *Yearbook of intensive care and emergency medicine* (volume 2008). doi:10.1007/978-3-540-77290-3
- Leon CG, Tory R, Jia J, Sivak O, Wasan KM (2008) Discovery and development of toll-like receptor 4 (TLR4) antagonists: a new paradigm for treating sepsis and other diseases. *Pharmaceutical Res* 25:1751–1761
- Lotz S, Aga E, Wilde I, van Zandbergen G, Hartung T, Solbach W, Laskay T (2004) Highly purified lipoteichoic acid activates neutrophil granulocytes and delays their spontaneous apoptosis via CD14 and TLR2. *J Leuk Biol* 75:467–477
- McCabe WR, Treadwell TL, De Maria A Jr (1983) Pathophysiology of bacteremia. *Am J Med* 75(1B):7–18
- MacCallum NS, Evans TW (2005) Epidemiology of acute lung injury. *Curr Opin Crit Care* 11:43–49
- Martin GS, Mannino DM, Eaton S, Moss M (2003) The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 348:1546–1554
- Merkulov VA, Plekhanova TM, Zverev A, Karpov VL, Evgen'ev MB, Kadykova ON, Gordeev EV, Petrov AA, Kovtun AL, Makhlaï AA, Mironov AN (2011) Production of 70 kDa recombinant human heat shock protein in baculovirus expression system and assessment of its antiviral activity. *Zh Mikrobiol Epidemiol Immunobiol* 1:54–60
- Nakada J, Matsura T, Okazaki N, Nishida T, Togawa A, Minami Y, Inagaki Y, Ito H, Yamada K, Ishibe Y (2005) Oral administration of geranylgeranylacetone improves survival rate in a rat endotoxin shock model: administration timing and heat shock protein 70 induction. *Shock* 24:482–487
- Nelson RJ, Ziegelhoffer T, Nicolet C, Werner-Washburne M, Craig EA (1992) The translation machinery and 70 kd heat shock protein cooperate in protein synthesis. *Cell* 71:97–105
- Nilsen NJ, Deininger S, Nonstad U, Skjeldal F, Husebye H, Rodionov D, von Aulock S, Hartung T, Lien E, Bakke O, Espevik T (2008) Cellular trafficking of lipoteichoic acid and Toll-like receptor 2 in relation to signaling: role of CD14 and CD36. *J Leukoc Biol* 84:280–291
- O'Neill LA (2003) Therapeutic targeting of Toll-like receptors for inflammatory and infectious diseases. *Curr Opin Pharmacol* 3:396–403
- Pfister H, Hennet T, Jungi TW (1992) Lipopolysaccharide synergizes with tumour necrosis factor-alpha in cytotoxicity assays. *Immunology* 77:473–476
- Pinsky MR, Vincent JL, Deviere J, Alegre M, Kahn RJ, Dupont E (1993) Serum cytokine levels in human septic shock. Relation to multiple-system organ failure and mortality. *Chest* 103:565–575
- Power C, Fanning N, Redmond HP (2002) Cellular apoptosis and organ injury in sepsis: a review. *Shock* 18:197–211
- Rozhkova E, Yurinskaya M, Zatssepina O, Garbuz D, Karpov V, Surkov S, Murashev A, Ostrov V, Margulis B, Evgen'ev M, Vinokurov M (2010) Exogenous mammalian extracellular HSP70 reduces endotoxin manifestations at the cellular and organism levels. *Ann N Y Acad Sci* 1197:94–107
- Rozhkova EA, Zatssepina OG, Iurinskaia MM, Vinokurov MG, Evgen'ev MB (2011) The effect of extracellular recombinant human heat shock protein 70 (Hsp70) on protein pattern observed after endotoxin-induced macrophage activation. *Mol Biol (Mosk)* 45:386–390
- Stearns-Kurosawa DJ, Osuchowski MF, Valentine C, Kurosawa S, Remick DG (2011) The pathogenesis of sepsis. *Annu Rev Pathol* 6:19–48
- Suganuma T, Irie K, Fujii E, Yoshioka T, Muraki T (2002) Effect of heat stress on lipopolysaccharide-induced vascular permeability change in mice. *J Pharmacol Exp Ther* 303:656–663
- Takeuchi O, Kawai T, Muhlratt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K, Akira S (2001) Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol* 13:933–940
- Tsan MF, Gao B (2004) Cytokine function of heat shock proteins. *Am J Physiol Cell Physiol* 286:C739–C744
- Tsan MF, Gao B (2009) Heat shock proteins and immune system. *J Leukoc Biol* 85:905–910
- Victor VM, Rocha M, Esplugues JV, De la Fuente M (2005) Role of free radicals in sepsis: antioxidant therapy. *Curr Pharm Des* 11:3141–3158
- Vincent J-L (2008) *Yearbook of intensive care and emergency medicine* (volume 2008). doi:10.1007/978-3-540-77290-3
- Vinokurov MG, Yurinskaya MM, Suslikov AV, Pechatnikov VA, Grachev SV (2006) Dynamics of the Fas- and stress-induced apoptosis of human neutrophils under the action of endotoxins. *Dokl Biol Sci* 410:355–357
- Wang JE, Dahle MK, McDonald M, Foster SJ, Aasen AO, Thiemermann C (2003) Peptidoglycan and lipoteichoic acid in gram-positive bacterial sepsis: receptors, signal transduction, biological effects, and synergism. *Shock* 20:402–414
- Wang YH, Wang WY, Liao JF, Chen CF, Hou YC, Liou KT, Chou YC, Tien JH, Shen YC (2004) Prevention of macrophage adhesion molecule-1 (Mac-1)-dependent neutrophil firm adhesion by taxifolin through impairment of protein kinase-dependent NADPH oxidase activation and antagonism of G protein-mediated calcium influx. *Biochem Pharmacol* 67:2251–2262

- Wheeler DS, Dunsmore KE, Denenberg AG, Muething L, Poynter SE, Wong HR (2011) Biological activity of truncated C-terminus human heat shock protein 72. *Immunol Lett* 135:173–179
- Young JC (2010) Mechanisms of the Hsp70 chaperone system. *Biochem Cell Biol* 88:291–300
- Yurinskaya MM, Vinokurov MG, Zatcepin OG, Garbuz D, Guzhova IV, Rozhkova EA, Suslikov AV, Karpov VL, Evgen'ev MB (2009) Exogenous heat shock proteins (HSP70) significantly inhibit endotoxin-induced activation of human neutrophils. *Dokl Biol Sci* 426:298–301
- Zanotti-Cavazzoni SL, Goldfarb RD (2009) Animal models of sepsis. *Crit Care Clin* 25:703–719
- Zheng H, Nagaraja G, Kaur P, Asea E, Asea A (2010) Chaperone function of recombinant Hsp72 produced in insect cells using baculovirus expression system is retained. *J Biol Chem* 285:349–356
- Zhou J, An H, Xu H, Liu S, Cao X (2005) Heat shock up-regulates expression of Toll-like receptor-2 and Toll-like receptor-4 in human monocytes via p38 kinase signal pathway. *Immunology* 114:522–530