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SHORT COMMUNICATION

Encapsulated Hsp70 decreases endotoxin-induced production of ROS and TNFα in human phagocytes

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Abstract Human heat shock protein Hsp70 was experimentally inserted into polyelectrolyte microcapsules. Encapsulated recombinant Hsp70 was studied in terms of its effects on neutrophil apoptosis, the production of reactive oxygen species, and the secretion of tumor necrosis factor alpha by promonocytic THP-1 cells. It was found that encapsulated Hsp70 effectively inhibits neutrophil apoptosis, unlike free exogenous protein used in solution. In THP-1 cells, encapsulated and free Hsp70 reduced LPS-induced tumor necrosis factor alpha production with a similar efficiency. Encapsulated Hsp70 reduces LPS-induced reactive oxygen species production by neutrophils in the course of its release from the microcapsules but not as much as free Hsp70. Thus, the polyelectrolyte microcapsules can be used as containers for the effective delivery of Hsp70 to neutrophils and monocytes to significantly improve the functioning of the innate immune system.

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Introduction

Human blood phagocytes (neutrophils and monocytes) play a key role in various diseases accompanied by inflammation (McCracken 2014; Venet et al. 2013). Under normal non-pathological conditions, neutrophils persist in the blood for less than 24 h, and, thereafter, constitutive apoptosis is activated in these cells (McCracken 2014). However, in the case of inflammation, the production of reactive oxygen species (ROS) is strongly induced in neutrophils, leading to the damage of various tissues in humans (Thieblemont et al. 2016). The damage is also enhanced due to the extended life span of neutrophils in the affected tissues resulting from apoptosis inhibition (lack of normal neutrophil clearance). Thus, apoptosis inhibition occurs in the case of conditions such as bacterial infections (both gram-positive and gram-negative) and heart diseases (Sheth et al. 2001; Power et al. 2002; Garlichs et al. 2004).

Endotoxins (LPS) representing the cell walls of gram-negative bacteria rapidly activate blood phagocytes, leading to a dramatic increase in ROS and various adhesive molecules in the cell membrane, as well as the synthesis and secretion of various cytokines and inflammation mediators (Alves-Filho et al. 2008). The resulting free cytokines activate cells from various tissues and organs, inducing multiple metabolic, hormonal, and neuroendocrine changes in the organism and leading to the development of endotoxin shock with a high level of lethality (Rietschel et al. 1996; Blanco et al. 2008; Martin et al. 2003). The interaction of LPS with TLR4 receptors represents

the key event underlying LPS-induced neutrophil activation (Stack et al. 2012).

Heat shock protein 70, encoded by the HSPA1A gene in humans, is a key component of the machinery protecting cells from various pathological and stress conditions, including inflammation (Qu et al. 2015; Radons 2016). Briefly, Hsp70 binds partially unfolded or misfolded proteins and either assists in their refolding or directs them to a safe disposal (Duncan et al. 2015). Hsp70 may also have additional functions, including acting as regulatory or cytokine-like molecules (Multhoff and Hightower 2011; Radons 2016). The activity of endogenous Hsp70 appears insufficient in many pathological states, notably in various neurodegenerative disorders (Pratt 2015; Radons 2016).

Normally, Hsp70 is a cytoplasmic protein. However, it may exit the cell via unusual mechanisms, different from regular secretory processes. Frequently, Hsp70 exits the cell under stress conditions and/or in transformed cells. The usefulness of exogenously produced or artificially induced Hsp70 has been demonstrated in several model systems accompanied by inflammation (Ekimova et al. 2010; Hoshino 2011; Bobkova et al. 2014; Bobkova et al. 2015).

Thus, exogenous Hsp70 significantly decreased the mortality in a rat model of endotoxin shock (Kustanova et al. 2006). The exogenous Hsp70 reduces the generation of ROS by neutrophils under the influence of LPS and lipoteichoic acid (LTA) (Rozhkova et al. 2010; Vinokurov et al. 2012).

There are various lines of evidence demonstrating that exogenous Hsp70 may interact with the cells using TLR4 or TLR2 receptors (Asea et al. 2002). It was also shown that Hsp70 may decrease the level of TLR2 and TLR4 mRNAs induced by the presence of LPS (Yurinskaya et al. 2015). Based on all these data, Hsp70 has emerged as a promising drug that can be used to protect cells and organisms as a whole from the action of bacterial pathogens. However, presently, the exact molecular mechanism underlying the protective effect of exogenous Hsp70 is not known. It is known that exogenous Hsp70 may exhibit both pro- and anti-inflammatory effects. It efficiently decreases LPS-induced TNFα secretion; however, in the absence of LPS, it may slightly increase the secretion of this cytokine by phagocytes (Evdonin and Medvedeva 2009). Furthermore, it has been recently demonstrated that exogenous Hsp70 undergoes rapid proteolysis after entering the cells (Yurinskaya et al. 2015).

Therefore, it is necessary to develop a technique that makes possible the delivery of recombinant Hsp70 directly to the target cells and extends its lifespan in the cells after delivery. Biodegradable polyelectrolyte microcapsules (PEMC), as well as liposomes, are often used for the delivery of biologically active substances to the targets (De Koker et al. 2009; LaVan et al. 2003; Kastl et al. 2013; Liu et al. 2014; Gao et al. 2016).

Encapsulated proteins have several apparent advantages over proteins in solution. When administered using injections or orally, the microcapsules containing drugs can be readily transported to a desired site, while the drugs themselves are thereby protected from enzymatic degradation and oxidation. Additionally, under appropriate conditions, microcapsules can be introduced directly into the cells. The formation of specific microcapsule structures may be used to ensure their slow degradation and drug release into the site of destination. Furthermore, the capsules are eventually digested in the organism by specialized cells such as macrophages and dendritic cells (De Koker et al. 2009; Szarpak et al. 2010).

In the present study, we investigated the action of encapsulated human Hsp70 on the endotoxin-induced activation of human neutrophils and monocytes exploring biodegradable polyelectrolyte capsules and demonstrated the high protective potential of this approach.

Materials and methods

Isolation of human recombinant Hsp70

In our research, we used human Hsp70 LPS-free expressed in armyworm (*Spodoptera frugiperda*) cells (Rozhkova et al. 2010). The clone containing human Hsp70 cDNA (pBlueScriptSK+-Hsp70) used to make expression constructs was a kind gift from Professor R. Morimoto (Northwestern University). The purity of the Hsp70 preparations from *Spodoptera* cells was confirmed by polyacrylamide gel electrophoresis followed by staining with Coomassie blue and immunoblotting using monoclonal 3B5 anti-Hsp70 and N69 anti-Hsc70 antibodies. The protein concentration was measured according to Bradford's protocol (Bradford 1976).

Cell cultures

Neutrophils

Neutrophils were isolated from the peripheral blood of healthy volunteer donors using the method of differential centrifugation on a two-layer gradient of ficoll–verografin (1.119 and 1.077 g/ml) (Rozhkova et al. 2010). The purity of the isolated neutrophils was 98–99 %. Neutrophil viability was monitored by fluorescence microscopy (Yurinskaya et al. 2015).

To control the functional activity of the isolated neutrophils, the percentage and phagocytosis index in the presence of microcapsules were determined. It was shown that, after 30 min of incubation with microcapsules, the percentage of phagocytosis was 40 ± 2; however, in the presence of LPS, this value reached 60 ± 2 %. The phagocytosis index was 3 ± 1 microcapsules per cell. To monitor FITC-labeled PEMC phagocytosis by neutrophils, fluorescence inverted microscope (Keyence, Japan) and confocal microscope (Leica, Germany) were used.

During the apoptosis studies, the isolated neutrophils (1×10^6 cells/ml) were cultured in RPMI-1640 medium containing 5 % heat-inactivated fetal calf serum (FCS), certified to have an endotoxin activity of 0.01 endotoxin units/ml (HyClone, Logan, UT, USA), 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere supplemented with 5 % CO_2 for 16 h. The neutrophils were combined with PEMC at a 1:2 ratio in the absence or presence of LPS. The level of apoptosis was determined by fluorescence microscopy, using a fluorescent probe (5 μg/ml; Hoechst-33342, St. Louis, MO, USA) (Yurinskaya et al. 2014). The percentage of apoptotic cells was calculated as a fraction of propidium iodide (PI)-negative (30 μM) cells with fragmented DNA to the total number of cells (100 %). To monitor apoptosis, at least 20 fields of view were analyzed, each of which contained 250–350 cells.

The viability of the cells was controlled as described using propidium iodide (Otsuki et al. 2003). The neutrophil viability after the experiments was 96–98 %.

Monocytes

Human promonocytic cell line, THP-1, was obtained from the American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium containing 10 % heat-inactivated fetal calf serum (FCS), 1 % glutamine, 1 % streptomycin, and 100 units/ml of penicillin at 37 °C under 5 % CO_2. For differentiation, THP-1 cells (1×10^6 cells in 2 ml) in culture medium were placed into 24-well flat-bottomed plates, and 200 nM phorbol-12-myristate-13-acetate was added. The cells were grown for 72 h at 37 °C under 5 % CO_2, washed with culture medium, and used in the experiments.

Determination of ROS level with NBT

The level of ROS in neutrophils was determined using nitro blue tetrazolium (NBT). The cells were placed into 24-well plates (Sarstedt, Germany) with increased adhesion at 2×10^6 cells per well in 1 ml of HBSS and kept for 2 h; thereafter, HBSS was replaced with culture media containing 5 % FCS, and then 1 μg/ml Hsp70 or capsules with or without Hsp70 were added. The cells were incubated for 10 min at room temperature, and then LPS was added. Thereafter, the cells were incubated for either 30 min or 16 h at 37 °C with 5 % CO_2. Subsequently, the culture medium was removed, and the cells were incubated in 0.1 % NBT solution for 2 h at 37 °C and 5 % CO_2. The cells were then washed in phosphate buffer, fixed with methanol, and dried.

The formed intracellular formazan was dissolved in 240 μl of 2 M KOH and 280 μl of DMSO. The optical density was determined at 620 nm (Wang et al. 2014) using a Uniplan plate reader (ZAO PICON, Moscow, Russia).

The determination of the TNFα levels

TNFα assay TNFα production was evaluated based on the cytotoxic effect of THP-1 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-1640 medium containing 10 % heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5 % CO_2 for 24 h. After 24 h, actinomycin D (1 μg/ml) was added to the monolayer as well as 100 μl of the supernatant of THP-1 cells in each well. Pure medium was added to the control wells. The plates were incubated for 24 h at 37 °C under 5 % CO_2 and were stained with crystal violet. 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. Triplicate wells were assayed for each condition, and the S.D. was determined. The relative levels of TNFα production were determined based on the toxicity index as described previously (Pfister et al. 1992). The specificity of the TNFα toxic effect was checked using the reaction of neutralization by appropriate monoclonal antibodies (StressMarq, Canada).

Production of polyelectrolyte microcapsules

Preparation of $CaCO_3$ microspherulites Into a 1 M aqueous $CaCl_2$ solution, an equivalent volume of 1 M aqueous Na_2CO_3 solution was rapidly poured under stirring (350 rpm). After a 30-s stirring, the suspension was left for 5–7 min until complete clarification of the supernatant. The resulting $CaCO_3$ particles were centrifuged for 30 s at 4000 g, washed three times with twice distilled water, and used for the preparation of polyelectrolyte microcapsules. The growth of microspherulites was controlled using a Nikon Eclipse E200 light microscope (Japan); the size distribution of the particles was 4.5–5 μm (Volodkin et al. 2004).

Preparation of polyelectrolyte microcapsules The capsules were prepared on $CaCO_3$ cores 3–5 μm in diameter by the successive adsorption of dextran sulfate (DS) (2 mg/ml) and poly-L-arginine (poly (Arg)) (2 mg/ml) in 0.5 M NaCl as described in (She et al. 2010). Each polyelectrolyte layer was applied for 15 min; after which, capsules were centrifuged and washed three times with 0.5 M NaCl. If particle aggregation occurred during the adsorption of polyelectrolytes, a suspension of microspherulites was exposed for 1–3 s to ultrasound. After the application of the three layers of DS and three layers of poly (Arg), $CaCO_3$ spherulites were dissolved in 0.2 M EDTA, pH 7.0. After the complete solution of the carbonate matrix, the polyelectrolyte microcapsules were washed with water and were stored as a suspension at 4 °C.

Microcapsules with the architecture [DS/poly (Arg)]3 were used in the work.

Incorporation of the protein into hollow polyelectrolyte microcapsules A suspension of polyelectrolyte microcapsules was centrifuged for 30 s at 4000 g, and the precipitate was resuspended in 450 μL of cold H_2O containing Hsp70 or Alexa555-Hsp70 or FITC-Hsp70 at a concentration of 50 μg/ml, followed by incubation for 30 min at 5 °C. The microcapsules were centrifuged for 30 min at 4000×*g* and were washed with cold deionized water. Next, the microcapsules containing the protein were diluted with a necessary amount of H_2O (Kochetkova et al. 2013). On average, approximately 1 pg of Hsp70 per microcapsule was encapsulated. The encapsulated human Hsp70 exhibited the required stability and was present within the capsules for at least 1–2 weeks after incorporation.

Confocal microscopy

Neutrophils were placed on round 12-mm Menzel cover glasses (Menzel-Glaser, Germany), were covered by 0.01 % poly-L-lysine solution into 4-well plates (Nunc, Thermo Scientific, Denmark) at a concentration of 2×10^6 cells/ml in Hanks' balanced salt solution (HBSS), and were kept for 2 h in a CO_2 incubator to allow cells to attach. After 2 h, HBSS was replaced with 5 % culture medium, and PEMC were added with the inclusion of FITC-Hsp70 at a ratio of cells/capsules of 1:2. The mixture of cells and capsules was incubated for 30 min or 16 h at 37 °C with 5 % CO_2. After incubation, the cover glasses were washed twice with PBS and were fixed in 4 % formaldehyde for 30 min at room temperature in the dark. The cells were washed in PBS and were mounted with a ready-to-use permanent mounting medium containing an anti-fading reagent and sodium azide (DAKO, Glostrup, Denmark, S3023) for confocal microscope analysis.

THP-1 cells at a concentration of 0.4×10^6 cells/ml in Nunc™ Lab-Tek™ II Chamber Slides (Nunc, USA) were differentiated by adding 200 nM PMA for 72 h in a culture medium containing 10 % FCS at 37 °C and 5 % CO_2. Then cells were washed, 10 % FCS was replaced with 5 % culture medium, and PEMC were added with the inclusion of Alexa555-Hsp70 at a ratio of cells/capsules of 1:2. The mixture of cells and capsules was incubated for 16 h at 37 °C with 5 % CO_2. After incubation, the cells were washed twice with PBS and were fixed in 4 % formaldehyde for 30 min at room temperature in the dark. The cells were washed in PBS and were mounted with a ready-to-use permanent mounting medium containing an anti-fading reagent and sodium azide (DAKO, Glostrup, Denmark, S3023) for confocal microscope analysis.

Laser Scanning Leica TCS SP5 confocal microscope (Leica, Mannheim, Germany) was used to observe the cells stained with FITC-Hsp70 or Alexa555-Hsp70. Images were acquired with HCX PL APO Lambda Blue 63 × 1.4 Oil UV (Leica) and were collected with Leica Application Suite Advanced Fluorescence (LAS AF) ver. 2.6.4.8702 (Leica). The accumulated data were analyzed using Image J and SigmaPlot programs.

Statistical analysis

For statistical analysis (calculations of mean values, standard deviations, and Student's *t* tests), statistical functions of Microsoft Excel were used. Statistical inference was based on levels of significance where $p \leq 0.05$.

Results and discussion

Previously, we have demonstrated that protection against LPS-induced ROS production by the administration of recombinant Hsp70 is realized within 5–10 min (Rozhkova et al. 2010). However, it was also demonstrated that exogenous Hsp70 is not stable after administration and can be rapidly degraded within the cells (Yurinskaya et al. 2015).

Based on these data, we carried out the comparison of the protective effect of exogenous Hsp70 in solution with Hsp70 encapsulated in PEMC under the same conditions (Fig. 1). Thus, the cells were incubated with these preparations of Hsp70 for 10 min; after this, LPS was added where necessary, and the cells were incubated for 30 min at 37 °C and 5 % CO_2 (Rozhkova et al. 2010). After this, the level of ROS was determined as described in the M&M.

The analysis demonstrated that a 30-min incubation of neutrophils with LPS resulted in significant induction of ROS (~200 %) in the cells. Exogenous Hsp70 efficiently decreased LPS-induced ROS production by neutrophils (Fig. 1a, H+L). Interestingly, the addition of empty capsules, as well as capsules loaded with Hsp70, leads to approximately the same induction in the ROS level (Fig. 1a, MC and Fig. 1a, MC-H). The effect of LPS in terms of ROS induction in the presence of empty PEMC was equal to that induced by the addition of solo LPS added to neutrophils (Fig. 1a, MC+L). In this series of experiments, encapsulated Hsp70 did not decrease LPS-induced activation of neutrophils (Fig. 1a, MC-H+L) and, hence, had no protective effect.

The analysis of confocal microscope images clearly demonstrated that, while PEMC with encapsulated Hsp70 undergo phagocytosis by neutrophils (Fig. 1b), the entrapped Hsp70 is not released from the capsules during this time period (30 min).

Based on the accumulated data, it is possible to conclude that both empty PEMC and capsules containing Hsp70 significantly increase ROS production by neutrophils probably due to the phagocytosis of PEMC by the cells. However, in these experiments, the capsules loaded with Hsp70 failed to exhibit

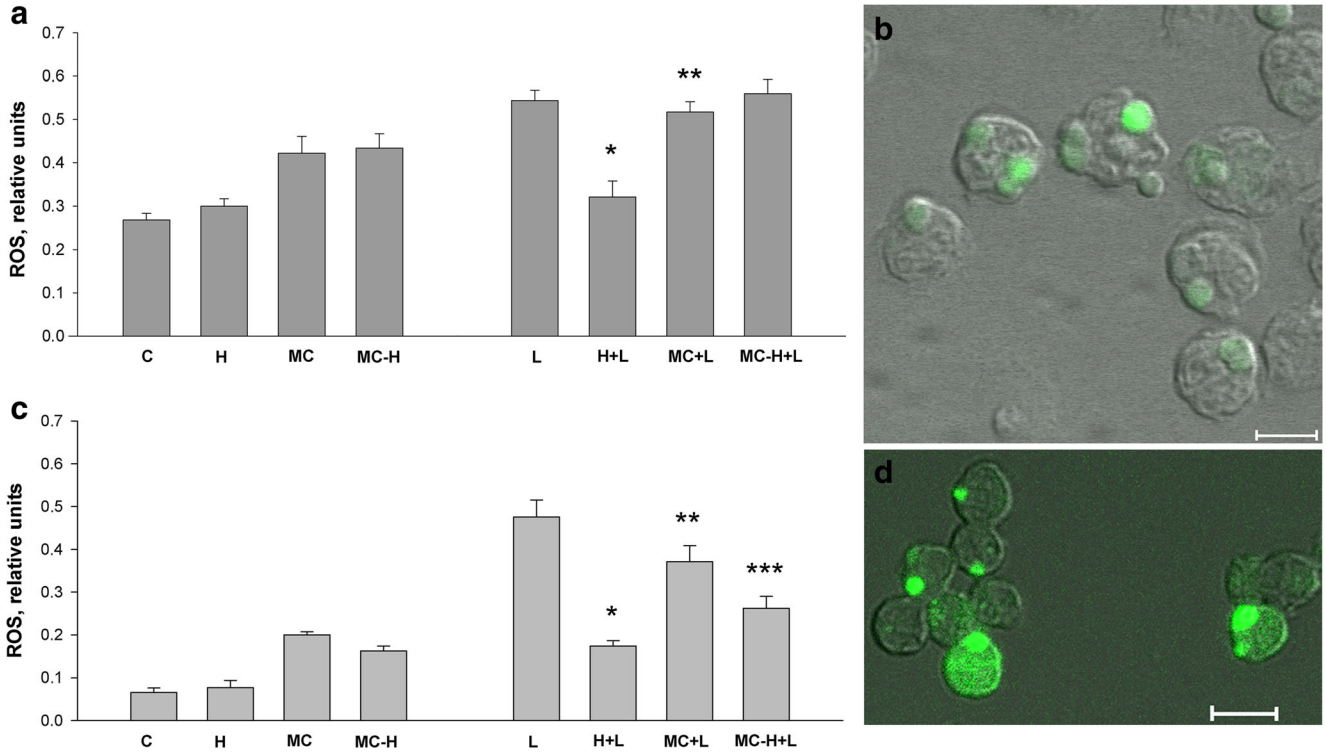

Fig. 1 Effect of encapsulated Hsp70 on LPS-induced ROS production by human neutrophils within 30 min (**a**, **b**) or 16 h (**c**, **d**). *C*—control non-treated neutrophils, *L*—100 ng/ml LPS, *H*—1 μg/ml Hsp70, *H+L*—1 μg/ml Hsp70 + 100 ng/ml LPS, *MC*—PEMC, *MC+L*—PEMC + 100 ng/ml LPS, *MC-H*—encapsulated Hsp70, *MC-H+LPS*—encapsulated Hsp70 + 100 ng/ml LPS. The results are presented as M ± SD; $n = 10$ for each group. **a** $*p < 0.005$, $**p < 0.05$. **c** $*p < 0.0025$, $**p < 0.05$, $***p < 0.01$. **a**, **c** NBT staining of neutrophils. **b**, **d** Confocal microscopy images of neutrophils after incubation with PEMC containing FITC-labeled Hsp70. **b** *Bar* 5 μm. **d** *Bar* 10 μm

any protective effect in neutrophils in terms of LPS-induced ROS production apparently because this time period (30 min) is not sufficient for encapsulated protein to be released from the capsules.

The images obtained by confocal microscopy clearly show that microcapsules containing Hsp70 undergo phagocytosis in the cells, but the encapsulated protein is not released from the capsules during the 30-min period (Fig. 1b), explaining the absence of a protective effect of capsules containing Hsp70 on LPS-induced ROS production by neutrophils.

Along these lines, at the next stage, we demonstrated that the process of Hsp70 release from the capsules usually starts 12–14 h after the start of incubation of neutrophils with microcapsules, a finding that is apparently due to the gradual degradation of the capsule membranes and appearance of released Hsp70 in the cytoplasm (Fig. 1d). These results fully corroborate the data of other authors on the uptake of antigens delivered by polyelectrolyte microcapsules to dendritic cells (De Koker et al. 2009). Based on these data, we carried out experiments to monitor LPS-induced ROS production by neutrophils during 16 h of incubation with microcapsules.

In 16 h, the neutrophil LPS-induced activation (absolute value) was decreased compared with the level observed after 30 min of activation (Fig. 1c, H+L). However, the protective action of exogenous free Hsp70 is preserved (Fig. 1c, L). Notably, in the presence of LPS, neutrophil activation by "empty" capsules increases approximately twofold (Fig. 1c, MC+L). ROS production in the presence of PEMC containing encapsulated Hsp70 is equal to the ROS level induced by empty PEMC. It is evident that encapsulated Hsp70 more efficiently decreases LPS-induced ROS production (Fig. 1c, MC-H+L) compared with the effect of empty PEMC (Fig. 1c, MC+L). The observed protective action of PEMC loaded with recombinant Hsp70 is apparently due to the gradual release of the protein from the capsules into the cytoplasm.

It is well known that ROS production plays an important role in apoptosis regulation in many cell types, including human neutrophils (Krüger et al. 2014). We have shown that the interaction of empty microcapsules with human neutrophils leads to a significant increase in ROS production (Fig. 1), which may enhance the constitutive apoptosis level as observed in the case of phagocytosis of bacteria (Watson et al. 1996). It is evident from Fig. 2 that LPS addition inhibited neutrophil apoptosis by almost twofold essentially as demonstrated by other authors (Ocaña et al. 2008; Antoine et al. 2013). On the other hand, recombinant Hsp70 at a concentration of 1 μg/ml did not modify the apoptosis level in neutrophils and decreased the inhibitory effect of LPS.

Interestingly, while empty capsules enhanced apoptosis by 12–15 % (Fig. 2, MC), in the presence of empty

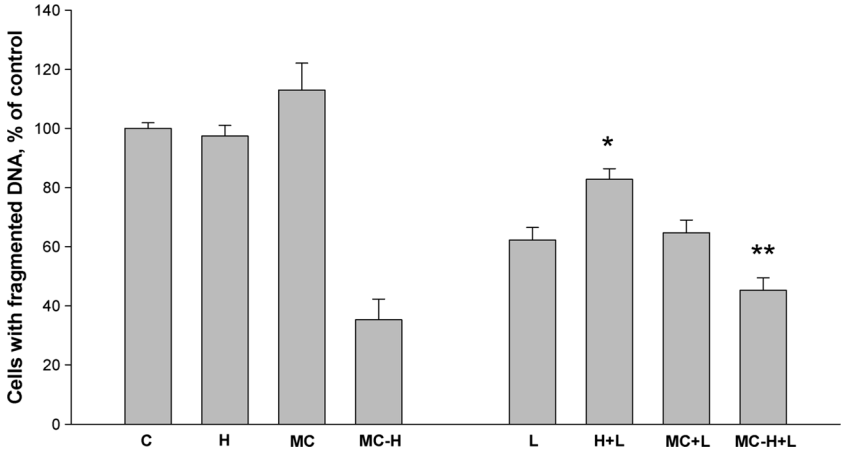

Fig. 2 Effect of microcapsules on neutrophil apoptosis. The microcapsules were incubated with neutrophils for 16 h. The description of the groups given under the abscissa axis is the same as in Fig. 1. The results are presented as M ± SD; $n = 8$ for each group. $^{*}p < 0.01$, $^{**}p < 0.01$. Neutrophil apoptosis was determined as ratio of live cells (from the total number) based on fragmented DNA analysis after staining with fluorescent probes (Hoechst 33342 and propidium iodide)

microcapsules and LPS, we observed significant apoptosis inhibition equal to 40 % (Fig. 2, MC+L). The administration of encapsulated Hsp70 resulted in more pronounced inhibition of neutrophil apoptosis (up to 65 %) than the administration of free recombinant Hsp70 (Fig. 2, MC-H). However, the combined effect of Hsp70-contained microcapsules and LPS resulted in less-pronounced neutrophil apoptosis inhibition (Fig. 2, MC-H+L). The encapsulated Hsp70 released from the capsules into the cytoplasm may inhibit apoptosis more efficiently than "free" exogenous Hsp70 probably due to its more prolonged action occurring during its gradual release from the capsules.

The synthesis of proinflammatory cytokines such as tumor necrosis factor (TNFα) represents one of the major manifestations of the phagocyte response to LPS stimulation (Buttenschoen et al. 2010). The performed experiments using THP-1 cells and capsules containing Alexa555-labeled Hsp70 demonstrated that, in these cells, the encapsulated protein is released from PEMC approximately after 16 h of incubation (Fig. 3a).

In the course of our studies, we also determined TNFα production by THP-1 cells after LPS administration (Fig. 3b) for 16 h. It was shown that, while THP-1 cells produce 8 pg/ml TNFα under normal conditions, LPS administration leads to a dramatic increase in TNFα production, reaching 65 pg/ml (Fig. 3b, L). The incubation of THP-1 cells with either empty or Hsp70-containing capsules leads to a significant increase in the TNFα level, which reaches 27 pg/ml (Fig. 3b, MC) and 25 pg/ml (Fig. 3b, MC-H), respectively.

The observed increase in this cytokine level probably results from the interaction of the microcapsules with phagocytes similar to that observed after the interaction of neutrophils with bacteria (Ocaña et al. 2008).

When the cells were incubated with empty microcapsules in the presence of LPS, we observed a slight decrease in the

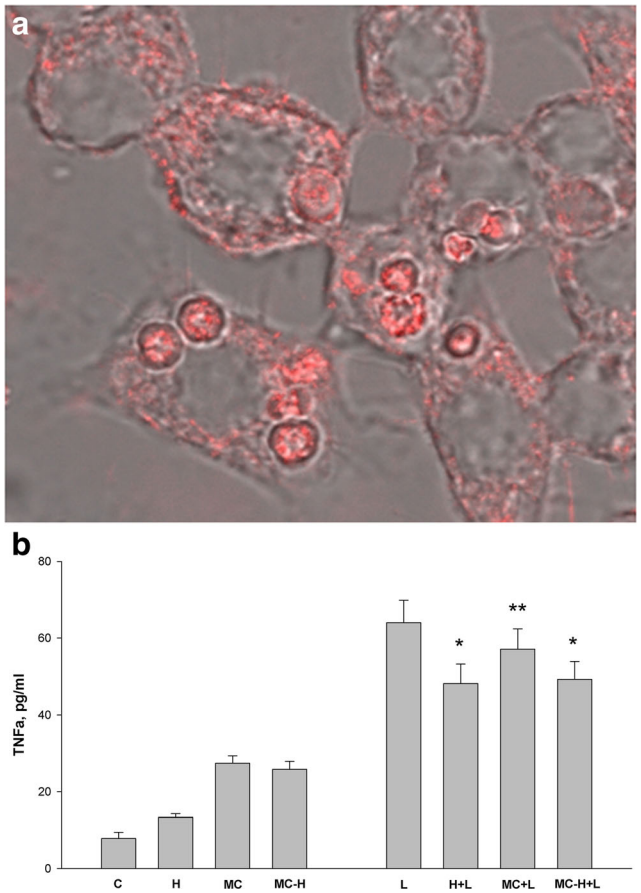

Fig. 3 Effect of microcapsules on TNFα secretion by THP-1 cells. The cells were kept with capsules for 16 h. **a** Confocal microscopy of the cells containing PEMC loaded with Alexa555-labeled Hsp70. *Bar* 10 μm. **b** TNFα secretion by THP-1 cells. The description of the groups given under abscissa axis is the same as in Fig. 1. LPS concentration—1 μg/ml. Hsp70 concentration—2 μg/ml. The ratio microcapsules/cells—2:1. The results are presented as M ± SD; $n = 4$ for each group. $^{*}p < 0.05$, $^{**}p < 0.01$

TNFα level (Fig. 3b, MC+LPS) compared with experiments where only LPS was added probably due to the absorption of endotoxin on the walls of the capsules. Interestingly, in these series of experiments, encapsulated Hsp70 exhibited a pronounced protective effect in terms of decreasing LPS-induced TNFα production close to that of free exogenous Hsp70 (Fig. 3b, MC-H+L).

It is well known that innate immunity cells such as blood phagocytes (neutrophils and monocytes) represent the major protective barrier of an organism against the invasion of various pathogens. This protective role depends primarily on their ability to undergo phagocytosis and the production of reactive oxygen species (ROS) strongly activated by bacterial endotoxins (Mayadas et al. 2014; Kruger et al. 2015).

Our results clearly show that recombinant human Hsp70 added in solution (Fig. 1a, H+L) within 30 min significantly reduced the LPS-induced production of ROS in neutrophils (Fig. 1a, H+L). These data are in agreement with our previous results obtained by another method demonstrating that the protective effect of LPS and LTA activation is usually realized within 5–10 min (Rozhkova et al. 2010; Vinokurov et al. 2012). During this brief period, exogenous Hsp70 only begins to enter the cells as has been previously demonstrated for several cell lines using ^{125}I-labeled Hsp70 (Yurinskaya et al. 2015); hence, it can hardly interact with intracellular signaling pathways due to its low concentration. Therefore, exogenous Hsp70 likely interacts with the TLR4 and TLR2 (Asea et al. 2002), representing the major mechanism underlying the protective effect of Hsp70 from LPS-induced ROS production by neutrophils. The interaction of Hsp70 with neutrophil NADPH oxidase represents another possible mechanism underlying its protective effect (Chen et al. 2012).

After longer periods of incubation with medium containing exogenous Hsp70, its concentration within the cells is increased with kinetics depending on the expression of TLR2 and TLR4 characteristic for a given cell line (Yurinskaya et al. 2015). However, Hsp70 undergoes rapid proteolysis after entering the cells (Yurinskaya et al. 2015). Our recent analysis also clearly demonstrated that recombinant Hsp70 undergoes very rapid degradation in the mouse brain after intranasal administration and is not detectable in 1 h after the injection (Yurinskaya et al. 2015). Notably, in our previous investigation where we used intravenous injections of Hsp70 into rats, the concentration of the injected protein in the blood also decreased very rapidly, and more than 90 % of injected Hsp70 was eliminated from the blood within 20 min after injection (Kustanova et al. 2006). Furthermore, the rapid disappearance of Hsp70 from the blood flow has been documented by other authors (Fleshner and Johnson 2005).

Our results presented here demonstrated that while encapsulated Hsp70 failed to exhibit any protective effect within 30 min against LPS-induced ROS production, the protection is evident after 16 h and the time necessary for encapsulated Hsp70 to be released from the degrading capsules. It is well known that the cultivation of neutrophils for more than 16 h results in the activation of constitutive apoptosis and, hence, the decrease of many intracellular enzyme activities (Luo et al. 2008; McCracken et al. 2014). This phenomenon probably accounts for the decrease in ROS production observed in the experimental cells treated by exogenous Hsp70 (Fig. 1c, H) as well as in the control untreated cells (Fig. 1c, C).

However, in the presence of LPS, we failed to observe the decrease in the ROS level because LPS induces ROS production and effectively inhibits neutrophil apoptosis. Importantly, exogenous Hsp70 was shown to decrease the level of LPS-induced TLR2 and TLR4 mRNA expression, a finding that may also contribute to its protective effect (Yurinskaya et al. 2015).

Our experiments presented herein clearly demonstrated that encapsulated Hsp70 can inhibit apoptosis to a greater extent than free exogenous Hsp70 (Fig. 2, MC-H and Fig. 1, H). The observed difference may be explained by the gradual release and, hence, the prolonged action of encapsulated Hsp70 in the cells in contrast to soluble exogenous Hsp70 that rapidly undergoes proteolysis after entering the cells (Yurinskaya et al. 2015).

Notably, encapsulated Hsp70 inhibited neutrophil apoptosis to a lesser degree when LPS was present. ROS production in the presence of PEMC and LPS was also lower (Fig. 1c, MC+L) than that in the case of LPS addition without microcapsules (Fig. 1c, L). This may be due to the sorption of LPS and decrease in its concentration and, hence, ROS induction.

The secretion of various cytokines, including TNFα, represents the later stage (compared with ROS induction) of the phagocyte response to the introduction of LPS and other pathogens into the cells (Ciallella et al. 2005; Gresnigt et al. 2012). Importantly, the accumulated data clearly demonstrate that both free and encapsulated human Hsp70 efficiently reduced TNFα secretion by promonocytic cells (THP-1) used in this study. Furthermore, we observed some increase in TNFα secretion after the introduction of empty capsules and capsules containing Hsp70, probably resulting from the phagocytosis of PEMC by the cells.

It is known that PEMC can be successfully absorbed by various phagocytic cells such as dendrite cells and macrophages (De Koker et al. 2009; Kastl et al. 2013). Besides, PEMC may penetrate into non-phagocytic cells (e.g., liver cells, fibroblasts, cancer cells, etc.) by endocytosis (Javier et al. 2008). Targets of PEMC are numerous and include different cancer cells and thrombocytes (Javier et al. 2008; Takacova et al. 2016). The delivery of PEMC to the target organs or tissue may be achieved by various means including peritoneal injections (De Koker et al. 2009; Federici et al. 2015).

Conclusion

In the last decade, recombinant Hsp70 has emerged as a promising agent exhibiting beneficial effects in various forms of neurodegeneration and cancer, and, hence, there is an urgent need to develop ways of its safe and efficient delivery to various targets. Exploring different cells we demonstrated that encapsulated Hsp70 efficiently decreased LPS-induced ROS and TNFα production, and, thus, PEMC with encapsulated eHsp70 represents a promising candidate for this purpose.

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