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MOLECULAR MECHANISMS
OF REDOX REGULATION IN INFLAMMATION

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The Protective Action of Hsp70 and Hydrogen Sulfide Donors in THP-1 Macrophages in the Lipopolysaccharide-Induced Inflammatory Response by Modulating Endocytosis

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Abstract—Hsp70 and hydrogen sulfide donors reduce inflammatory processes in human and animal cells. The biological action mediated by Hsp70 and H_2S donors (GYY4137 and sodium thiosulfate) depends on their protection kinetics from cell activation by lipopolysaccharides. However, the molecular mechanisms of action of Hsp70 and H_2S are not well understood. We studied the effect of human recombinant Hsp70 and H_2S donors on the formation of reactive oxygen species and tumor necrosis factor-alpha induced in human cells (THP-1) by lipopolysaccharides. Transcriptomic changes occurring in these cells after LPS administration in combination with GYY4137 pretreatment were investigated. The results we obtained showed that Hsp70 and hydrogen sulfide donors reduce inflammatory processes in cells activated by the action of LPS. Hsp70 and H_2S donors differed in the kinetics of the protective action, while hydrogen sulfide donors turned out to be more effective. The role of endocytosis in the mechanisms of protection of cells by H_2S and Hsp70 donors from the action of LPS was studied. It has been found that GYY4137 pretreatment of LPS-exposed cells reduces the LPS-induced induction of various pro-inflammatory genes and affects the expression of genes of various intracellular signaling pathways.

Keywords: GYY4137, STS, Hsp70, LPS, THP-1, TNFα, ROS
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INTRODUCTION

In the etiopathogenesis of a wide range of diseases, the inflammatory processes, the activators of which are often endotoxins (lipopolysaccharides, LPS), play an important role. The main sources of LPS in the body include Gram-negative bacteria that are part of the human microbiome, and the escape of LPS from the intestinal lumen into the bloodstream (endotoxemia) [1] is considered an important pathogenetic factor in gram-negative sepsis, cardiovascular and neurodegenerative diseases, type 2 diabetes mellitus, and others. The development of metabolic endotoxemia is caused by malnutrition and plays an important role in the development of many chronic diseases in animals and humans [2].

LPS circulating in the blood activate various cells of innate immunity (neutrophils, monocytes, macrophages, etc.) [3] through a chain of successive events. First, LPS bind to the LBP and CD14 auxiliary proteins; the signal from them is transmitted to the Toll-like receptor 4 (TLR4) in complex with MD2. After dimerization of the TLR4–MD2 complex on the cell surface, MyD88-dependent signaling occurs, followed by the expression of transcription factors (NF-κB, etc.). TLR4 internalization induces MyD88-independent (TRIF-dependent) endosome signaling and the expression of regulators such as interferon regulatory factor 3 (IRF-3) and, ultimately, type 1 interferons (IFNs). This results in the synthesis of cytokines and chemokines such as IL-1β, IL-6, IL-8, CCL2, and TNFα using MyD88-dependent pathways and CXCL10, CCL5, IFN-β, and nitric oxide via MyD88-independent pathways [4]. Constant replenishment of the pool of pro-inflammatory cytokines leads to weakly expressed systemic chronic inflammation [5].

One of the natural TLR4 ligands is the inducible form of the Hsp70 heat shock protein, which is also responsible for the folding of proteins synthesized in the cell and the refolding of partially denatured proteins under various types of stress, including heat shock [6, 7].

Abbreviations: Hsp70, heat shock protein 70 kDa; LPS, lipopolysaccharide; ROS, reactive oxygen species; TNFα, tumor necrosis factor-alpha; STS, sodium thiosulfate.

It was previously shown that recombinant Hsp70 has an anti-inflammatory effect, inhibits the production of reactive oxygen species (ROS), as well as the expression of the main inflammatory mediators (TNFα, IL-1β, IL-6, IL-8, etc.) by neutrophils and macrophages in response to various bacterial toxins [8–11]. The interaction of endogenous Hsp70 with TLR4 leads to rapid phagocytosis of the formed complex [12]. It is also known that endogenous intracellular Hsp70 inhibits the activity of TLR4, contributing to its ubiquitin-dependent degradation [13].

Another important anti-inflammatory compound is the gas transmitter hydrogen sulfide (H_2S), which is formed in cells as a result of the transsulfurization process that has been described in most prokaryotic and eukaryotic organisms, including humans [14]. In mammals, H_2S is produced by cells with various enzymes, predominantly cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfide transferase (3-MST), but part of H_2S is produced by the gut microbiota. Suppression of endogenous enzymes involved in the formation of H_2S in various diseases, or genetic defects in enzymatic systems of H_2S biosynthesis lead to the development of various pathologies, including oncological, cardiovascular, neurodegenerative, autoimmune (rheumatoid arthritis, and asthma) pathologies [15–18]. In recent years, our studies of the protective properties of recombinant human Hsp70 and various H_2S donors showed that these compounds protect cells from the action of LPS [6, 11, 19, 20]. It is also known that elevated levels of H_2S in cells can lead to the induction of Hsp70 formation [21]. Previously, we studied the effect of exogenous Hsp70 on the functional properties of neuroblastoma cells under the action of various inhibitors of endocytosis [22]. In this work, we studied the effect of the Hsp70 protein on THP-1 cells in the presence of LPS and hydrogen sulfide donors of various natures, GYY4137, and sodium thiosulfate (STS).

EXPERIMENTAL

Reagents. We used the culture medium RPMI-1640, fluorescent probe SF7-AM, HEPES, *L*-glutamine, phosphate buffered saline (PBS), Crystal Violet dye, nitroblue tetrazolium (NBT) dye, dye 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), actinomycin D, sodium dodecyl sulfate (SDS), penicillin, streptomycin, dimethyl sulfoxide (DMSO), Trypsin-Versene solution, LPS from *Escherichia coli* O55:B5, GYY4137 (4-Methoxyphenyl)-4-morpholinyl-phosphinodithioic acid, sodium salt (all Sigma-Aldrich, United States); fetal bovine serum (FBS) tested for the presence of endotoxin 0.01 U/mL (HyClone, United States); dinasor (Dyn), amiloride (Am), methyl-beta-cyclodextrin (MBCD), nocodazole (Noc), filipin (Fil) (Sigma-Aldrich); and STS (Dalkhimfarm, Russia).

Isolation of recombinant human Hsp70. Recombinant human Hsp70 with five amino-acid substitutions that increase protein solubility and stability [23] was produced in bacterial culture using a construct based on the pET-14b vector and purified by metal affinity chromatography on Ni-NTA-sepharose and affinity chromatography on ATP-agarose. Additional purification of Hsp70 preparations from residual LPS was carried out on polymyxin- or polylysine-agarose; the degree of purification was controlled by polyacrylamide gel electrophoresis (PAGE) and LAL-test according to the manufacturer's instructions. The purified protein concentration was measured by the Bradford method (Pierce Coomassie (Bradford) Protein Assay Kit).

Cell cultures. The human THP-1 promonocyte line was obtained from the American Type Culture Collection (ATCC, United States); the L-929 mouse fibroblast cell line was obtained from the collection of cell cultures of the Institute of Cytology, Russian Academy of Sciences (St. Petersburg, Russia). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich) containing 10% heat-inactivated FBS, 2 mM *L*-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO_2.

THP-1 cells were differentiated in a culture medium (CM) in 24-well flat-bottomed plates supplemented with 200 nM phorbol-12-myristate-13-acetate (PMA) for 72 h at 37°C in an atmosphere of 5% CO_2, washed with CM, and then used in the experiments; 2 μg/mL Hsp70, 2 mM STS or 200 μM GYY4137 were added to the cells for 120 min, then 1 μg/mL LPS from *E. coli* (serotype O55:B5) and incubated for 24 h at +37°C in an atmosphere of 5% CO_2.

Formation of reactive oxygen species (ROS) in THP-1 cells was determined using the NBT dye [24]. This method reflects the activity of oxygen-dependent cellular metabolism, as the source of ROS. At the end of cultivation, CM was taken from the plates with cells, the cells were washed with PBS, and a 0.1% NBT solution was added (2 h at +37°C and 5% CO_2). The cells were then washed 2 times with PBS, fixed with ethanol, and dried. The resulting intracellular formazan was dissolved in 300 μL of 2 M KOH and 400 μL of DMSO per well. The optical density of the resulting solution was measured at a wavelength of 620 nm using a Uniplan plate reader (Pikon, Russia).

TNFα products were determined by the cytotoxic effect of the samples on target cells, the L-929 mouse fibroblast line [19]. L-929 cells were cultured in 96-well plates (2×10^4 in 100 μL of medium per well) at +37°C and 5% CO_2 for 24 h, then actinomycin D (1 μg/mL) was added to the resulting monolayer, followed by 100 μL of the supernatant of the cells under study. Only CM was added to the control wells. The plates were incubated for 24 h, then the cells were washed with phosphate buffer, stained with Crystal

Violet (Sigma-Aldrich, United States), and cell survival was determined after the crystals were dissolved in 1% SDS solution. Optical density was measured at 595 nm using a Uniplan plate reader (Pikon, Russia). TNFα production was determined by the cytotoxicity index [25].

Cell viability was controlled using the MTT test [19, 20]. The cell viability in experimental samples was at least 96–99%.

Formation of hydrogen sulfide in cells. Formation of H_2S under the action of the GYY4137 and STS hydrogen sulfide donors was recorded on a CytoFLEX flow cytometer (Beckman Coulter, United States) using an SF7-AM fluorescent probe [26]. It was shown that the addition of GYY4137 and STS led to the release of H_2S in THP-1 cells, which fully corresponds to the results presented in [19].

Inhibitory assay. The following inhibitors of endocytosis were used in the experiments: amiloride (Am), nocodazole (Noc), filipin (Fil), dynasore (Dyn), and methyl-β cyclodextrin (MBCD). Inhibitors were added to cells 30 min prior to administration of Hsp70, LPS, and hydrogen sulfide donors (GYY4137 and STS) [27]. The optimal and nontoxic concentrations of inhibitors were determined using the predetermined maximum nontoxic concentrations (data not shown).

Registration of the action of Hsp70-Alexa 555 on THP-1 cells. THP-1 cells in CM with 10% FBS were placed in 35 mm Petri dishes with enhanced adhesion (Eppendorf, Germany) and 200 nM PMA was added. Cells were differentiated for 72 h at +37°C in an atmosphere of 5% CO_2, washed with CM, removed from the surface with a Trypsin–Versen solution, and used in the experiments. A suspension of differentiated THP-1 cells (1×10^6/mL) in serum-free CM without phenol red was poured into microtubes of 300 μL, additives (inhibitors) were added and incubated for 30 min at +37°C and 5% SO_2. Then, the cells were centrifuged (washed out of inhibitors) at 250 *g* for 5 min, the pellet was diluted in 300 μL of serum-free CM without phenol red, and Hsp70-Alexa 555 was added at a final concentration of 1 μg/mL. Hsp70 labeled with Alexa 555 (Hsp70-Alexa 555) was kindly provided by B. Margulis (Institute of Cytology Russian Academy of Sciences, St. Petersburg). Samples were incubated at +37°C and 5% CO_2 within 3 h; this time interval was chosen based on the results obtained in [12], and then measured on a CytoFLEX cytometer (Beckman Coulter).

Statistics. Data are presented as the mean ± standard deviation calculated from six independent experiments performed in quadruplicate. Differences between groups were analyzed using Tukey's one-way ANOVA with pairwise comparisons. The normality of the distribution of the analyzed values was checked using the Shapiro–Wilk test. The significance thresholds were as follows, $*p < 0.05$, $**p < 0.01$, $*** p < 0.005$, $**** p < 0.001$, $\# p < 0.001$, and $\#\# p < 0.05$.

RESULTS

The Kinetics of Protection of THP-1 Cells by Heat Shock Protein Hsp70, GYY4137, and STS from LPS-Induced Activation

Incubation of cells with Hsp70 (2 μg/mL), GYY4137 (200 μM), and STS (2 mM) for 24 h results (compared with control cells) in a slight increase in TNFα production by cells, up to 17, 9, and 7 pg/mL respectively (Fig. 1a, *1*). The addition of Hsp70, GYY4137, and STS to cells 2 h before LPS reduced TNFα production (compared to LPS action) by 61, 69, and 65%, respectively. A study of the kinetics of the protective action of the compounds used showed that upon pretreatment of cells for 20 min (before adding LPS), the protection efficiency reaches 50% (Fig. 1b). Of the three compounds, GYY4137 protected cells most effectively from the action of LPS. The efficiency of Hsp70, GYY4137 and STS can be assessed by comparing the ratio of LPS-induced TNFα (or ROS) production by cells to control and the ratio of TNFα production in the presence of Hsp70 (or GYY4137 and STS) and LPS (denoted as the efficiency ratio $\mathbf{K_E}$) to the control. For example, the ratio of TNFα production for LPS was 11.4 (57/5) and for STS, 3.1, respectively. For Hsp70 and GYY4137, the $\mathbf{K_E}$ values are 2.3 and 3.5, respectively.

Endocytosis Inhibitors Reduce Hsp70-Alexa 555 Binding to THP-1 Cells

It has been shown that all used endocytosis inhibitors reduced the binding of Hsp70-Alexa 555 to macrophages compared to control cells. Binding was most effectively reduced by inhibitors of dynasore and MBCD (Fig. 2, MBCD and Dyn) compared to control cells (Fig. 2, CM), ~35%.

The Influence of Hsp70, GYY4137, and STS on LPS-Induced THP-1 Cell Activation in the Presence of Endocytosis Inhibitors

The study of the action of endocytosis inhibitors showed that all inhibitors we used reduced the production of ROS and TNFα by THP-1 cells, as well as LPS-induced production of ROS and TNFα (Figs. 3 and 4, comparison of columns *2* with inhibitors with column *2* with LPS). The maximum decrease in ROS production was caused by MBCD and dynasore. Amiloride, nocodazole, and filipin inhibited ROS production to a lesser extent. MBCD, dynasore, and amiloride slightly reduced the production of ROS for Hsp70, GYY4137, and STS compared to the effects of these compounds without inhibitors. In the presence of inhibitors the $\mathbf{K_E}$ for Hsp70, GYY4137, and STS approaches one.

Endocytosis inhibitors influence the products of TNFα cells more significantly than ROS production (Fig. 4). Thus, in the presence of all inhibitors, LPS-

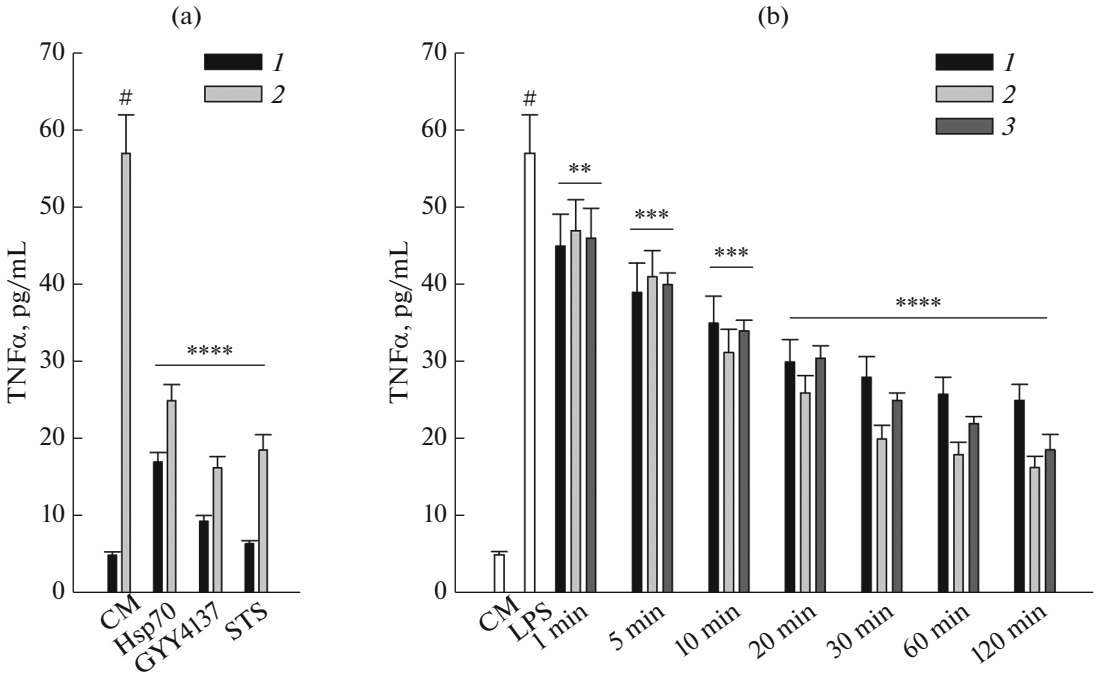

Fig. 1. The effects of Hsp70, GYY4137, and STS on LPS-induced TNFα production by THP-1 cells. (a): (*1*) production of TNFα by cells in the absence of LPS; (*2*), production of TNFα by cells in the presence of LPS. (b): (*1*) 2 μg/mL Hsp70; (*2*) 200 μM GYY4137; (*3*) 2 mM STS. (*1*, *2* and *3*) sequential addition of one of the compounds (Hsp70, GYY4137, and STS) to the cells, and then (in the range of 1–120 min) LPS. The concentration of LPS in cell samples was 1 μg/mL.

induced TNFα production decreases by more than 2 times (Fig. 4, bars *2* with inhibitors versus column *2*, CM). In the presence of inhibitors in all cell samples, $\mathbf{K_E}$ is in the interval of ~1.3–1.5. The MBCD inhibitors dynasore, amiloride, and filipin reduced TNFα production in cell samples with Hsp70, GYY4137, and STS, compared with the action of these compounds without inhibitors (Fig. 4, *1*–*5*).

DISCUSSION

TLR4 is activated by LPS with the participation of LBP (LPS binding protein) and CD14 and sequentially triggers two signaling cascades. The first cascade involving the TIRAP and MyD88 adapter proteins is induced in the plasma membrane, while the second cascade is activated by the attachment of the TRAM and TRIF adapter proteins to the duplex complex (TLR4–MD2) in early endosomes after endocytosis of the TLR4 receptor [28]. The membrane form of CD14 is anchored in lipid microdomains of the cell membrane next to TLR4 molecules [29].

One of the first cell responses to the proinflammatory effect of LPS is the induction of ROS synthesis followed by the synthesis of TNFα and a number of other proinflammatory cytokines [30]. The main source of ROS in macrophages is NADPH oxidase [31], whose activation mechanism that involves Toll-like receptors (TLRs) remains poorly understood; however, it is known that NADPH oxidase, at least in part, switches on signaling pathways involving various protein kinases [31, 32].

Study of the kinetics of the protective effect of Hsp70 and H_2S donors showed that hydrogen sulfide donors reduce cell activation more significantly under the action of LPS than Hsp70 (Fig. 1b). At the same time, both Hsp70 and H_2S in the absence of LPS increased the production of TNFα by cells compared to the control (Fig. 1a, column *2*). The Hsp70 protein had the maximum activation (17 pg/mL), and the STS protein had the minimum activation (6.5 pg/mL). The lower protective effect of Hsp70 (compared to STS) is probably due to its small pro-inflammatory effect. The slight increase in TNFα production by cells under the action of GYY4137 (9.4 pg/mL) may be due to the fact that GYY4137 is able to increase the expression of proteins of the Hsp70 family [19].

We have shown (Fig. 3) that Hsp70 and hydrogen sulfide donors significantly reduce ROS production. These results are also confirmed by the data of our transcriptomic analysis, according to which LPS causes an increase in the synthesis of several subunits of NADPH oxidase: membrane gp67 and two cytoplasmic subunits, p47phox and p67phox [19]. At the same time, GYY4137 reduces the level of three subunits in the NADPH oxidase complex. This seems to be the main mechanism of cell protection from LPS-induced ROS production. In bacterial infection,

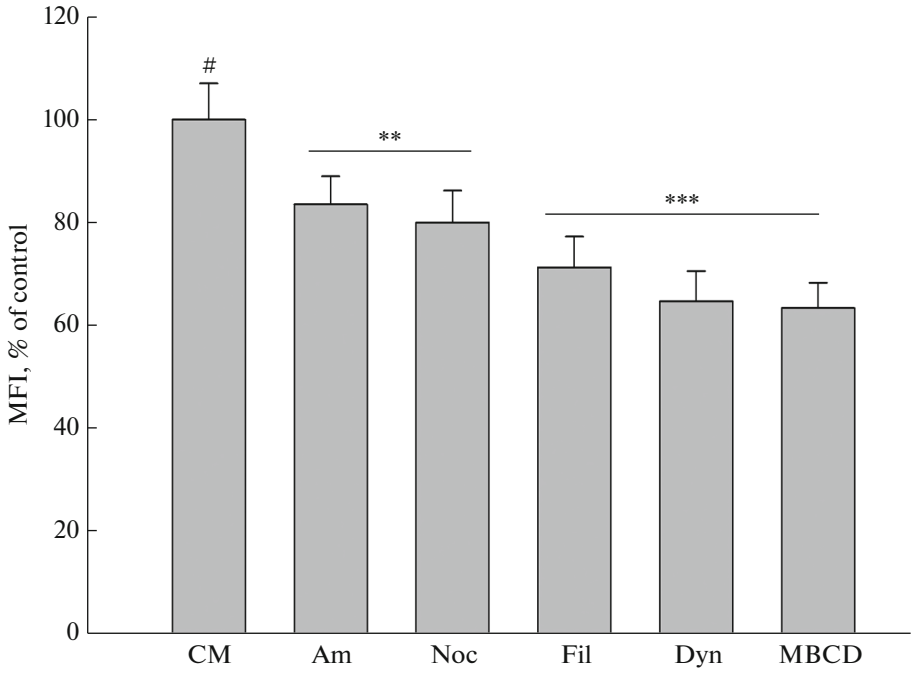

Fig. 2. The effect of endocytosis inhibitors on Hsp70-Alexa 555 binding to THP-1 cells. MFI is the median fluorescence intensity. CM, culture medium; Am, 100 μM amiloride; Noc, 1 μM nocodazole; Fil, 1 μM filipin; Dyn, 40 μM dynasore; MBCD, 2 mM methyl-β-cyclodextrin. Inhibitors were added to cells 30 min before Hsp70-Alexa 555. MFI of cells in culture medium is taken as 100%, all other values are presented as a percentage of the specified value. The total cell culture time was 24 h. $N = 6$, $^{*}p < 0.01$, $^{**}p < 0.005$ compared to CM.

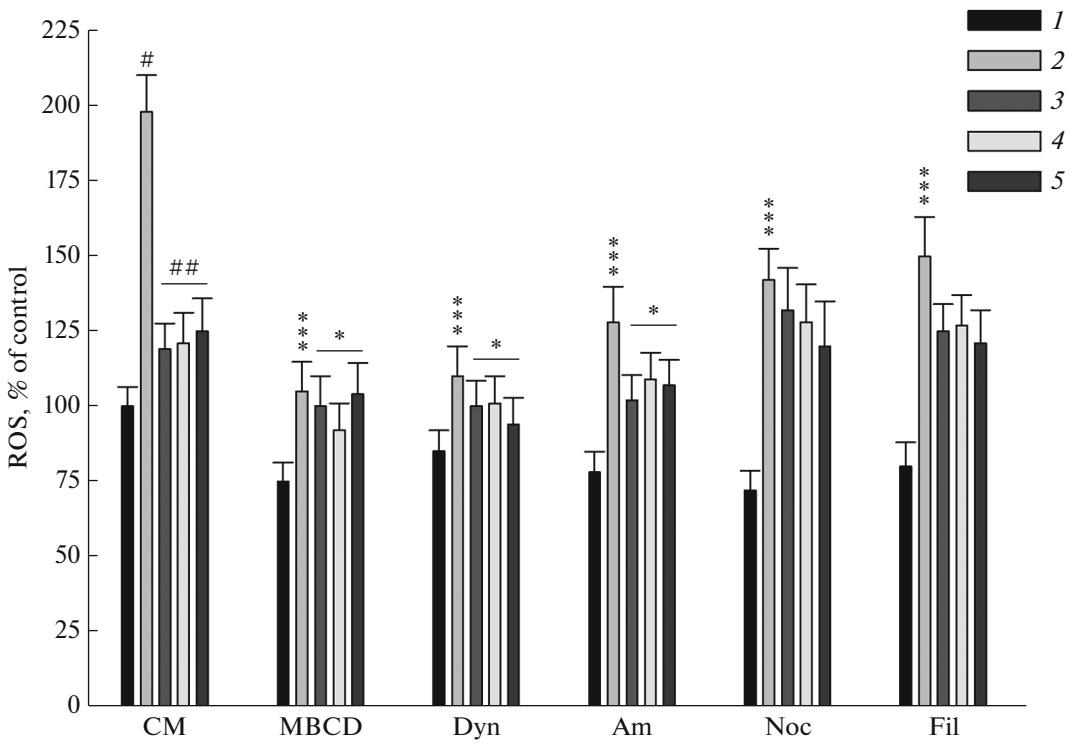

Fig. 3. The effect of endocytosis inhibitors on ROS production by THP-1 cells in the presence of Hsp70 (2 μg/mL), GYY4137 (200 μM), STS (2 mM), and LPS (1 μg/mL). CM, culture medium; MBCD, 2 mM methyl-β-cyclodextrin; Dyn, 40 μM dynasore; Am, 100 μM amiloride; Noc, 1 μM nocodazole; Fil, 1 μM filipin. (1), cells in the absence of LPS, Hsp70, GYY4137 and STS; (*2*), addition of LPS; (*3*), addition of Hsp70; (*4*), addition of GYY4137. (*5*), adding STS. Inhibitors were added 30 min before Hsp70, GYY4137 and STS; with the latter cells were incubated for 2 h and LPS was added. ROS production by THP-1 cells in control was taken as 100% (CM, column *1*), all other values are presented as a percentage of the specified value. The total cell culture time was 24 h. We compared # with *** and ## with *.

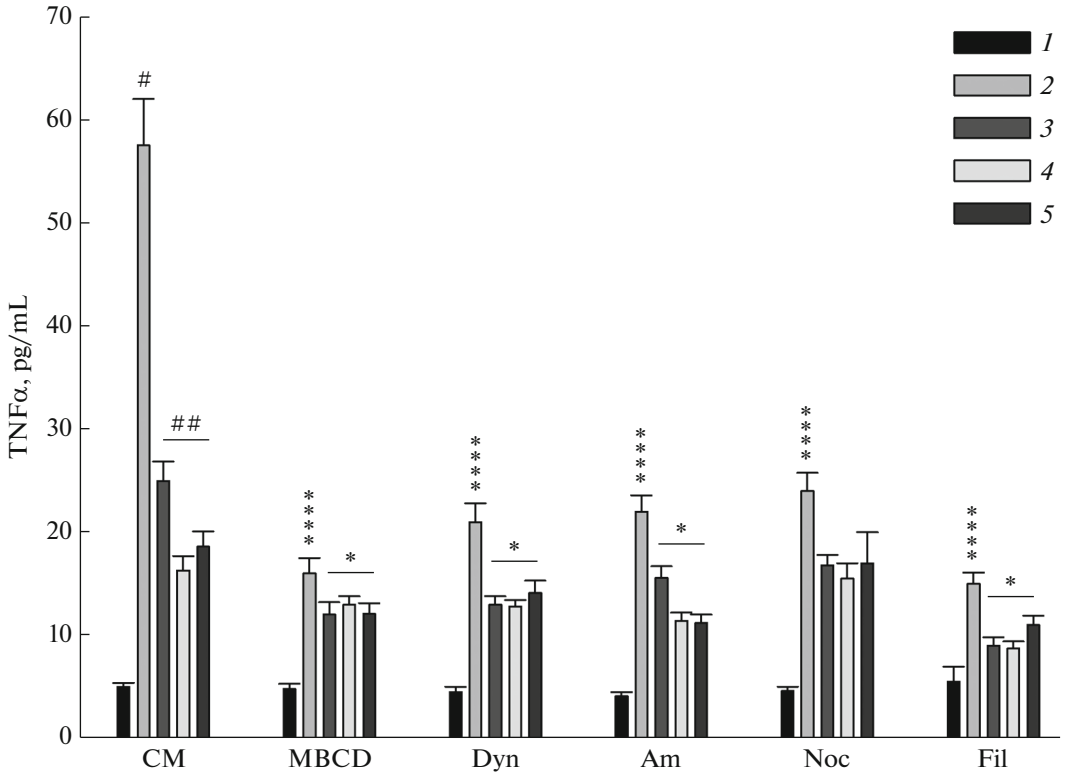

Fig. 4. The effect of endocytosis inhibitors on TNFα production by THP-1 cells. Designations as in Fig. 3. We compared # with **** and ## with *.

NADPH oxidase is involved in the mechanisms of bacterial death in phagosomes. Transcriptome analysis showed [19] that LPS increases the level of cathepsin and Fcγ receptor. This receptor, together with phospholipase D and ARF6, can be involved in the activation of NADPH oxidase [33]. In our experiments, the expression of phospholipase D and ARF6 did not change under the influence of LPS and GYY4137, while GYY4137 reduced the level of FcαR, FcγR, and cathepsin present in phagolysosomes. Phagolysosomes contain only cathepsin. The inhibitor dynasore reduced the protection of cells from LPS by H_2S donors and Hsp70, as well as the total production of ROS and TNFα (Figs. 3, 4, Dyn). The decrease in the production of ROS and TNFα by cells under the action of dynasore may be due to the relationship of NADPH oxidase [34] and TLR4 [35] with cell membrane clathrin.

Another mechanism for reducing the levels of ROS and TNFα in our experiments (Figs. 1, 3, and 4) may be a decrease in the level of cellular TLR4 in the presence of Hsp70 and H_2S donors [19, 20].

LPS can not only activate NADPH oxidase, but also switch macrophage metabolism from oxidative phosphorylation to glycolysis and the pentose phosphate pathway. Inhibition of the pentose phosphate pathway suppresses the LPS-induced increase in NOX2 activity and is associated with a significant decrease in the expression of NADPH oxidase mRNA [36]. According to transcriptome analysis, LPS and GYY4137 affect the expression of enzymes involved in the regulation of glycolysis and gluconeogenesis. It has been established [19] that LPS increases the expression of galactose mutarotase, hexokinase 1, fructose-1,6-bisphosphatase, and bisphosphoglycerate mutase. GYY4137 reduces the expression of the first three enzymes and does not affect the expression of bisphosphoglycerate mutase. These data suggest that under the action of LPS, the metabolism of THP-1 cells switches from oxidative phosphorylation to glycolysis. GYY4137 stabilizes these processes.

In our experiments, Hsp70 effectively protected cells from LPS-induced production of ROS and TNFα (Figs. 1, 3, and 4). One of the mechanisms of this protection may be the direct interaction of Hsp70 with NADPH oxidase, followed by degradation of this enzyme [37, 38]. It has been shown that Hsp70, using TLR4, can suppress the induction of NOX3 [39].

TLR4 plays a key role in the activation of macrophages under the action of LPS [4]. Our earlier transcriptomic analysis of THP-1 cells [19] showed that LPS increases the expression level of MyD88 and activates the NF-kB signaling pathway, in which TRAF6 (TNF receptor associated factor 6) plays an important role. Under the action of LPS, the expression of TRAF6 in THP-1 cells does not change, but it signifi-

cantly decreases in the presence of GYY4137 [19]. LPS also causes an increase in TRIF expression (TIR-domain-containing adapter-inducing interferon-β) and the adapter kinase RIP1 (receptor-interacting protein 1) associated with the regulation of signaling pathways involving MAP kinases, as well as with the regulation of programmed cell death [40]. According to transcriptome analysis data [19], the expression of chemokines and pro-inflammatory cytokines (INFα, IL-1β, IL-6, and IL-8) significantly increases in the presence of LPS [19, 20, 22].

Exogenous Hsp70 interacts with target cells through a number of cellular receptors, the main of which are TLR4 and TLR2, as well as LOX-1, SREC-1, and CLEVER-1 (Scavanger receptors, SR group), as well as CD40 (TNF receptor family), CD91 (low density lipoprotein receptor) and CCR5 (chemokine receptor) [41]. In addition, recombinant Hsp70 can enter cells by interacting with cell membrane lipids [42]. By binding to receptors, Hsp70 enters cells during the internalization of receptors. It has been shown that Hsp70 can also enter cells via clathrin-independent endocytosis [43]. LPS can enter cells via receptor-dependent and clathrin-dependent endocytosis [44, 45], as well as using LBP, HMGB1 (high-mobility group protein B1), and RAGE (Receptor of advanced glycation end products) [4].

A study of the effect of endocytosis inhibitors showed that the most effective inhibitor of Hsp70-Alexa 555 endocytosis was MBCD. The ability of other inhibitors to reduce Hsp70-Alexa 555 endocytosis changed in the following order: amiloride < nocodazole < filipin < dynasore < MBCD) (Fig. 2). This is associated with a significant inhibition of the production of ROS and TNFα by cells in the presence of an inhibitor of lipid microdomains, MBCD (Figs. 3 and 4). It should be noted that MBCD does not completely suppress the production of ROS and TNFα in the presence of LPS and Hsp70, which is apparently due to the penetration of LPS and Hsp70 into the cell by pathways not associated with receptors. In addition, LPS-induced activation of TLR4 enhances the synthesis of fatty acids, which are converted into sphingolipids that are part of lipid microdomains [46].

Transcriptomic analysis of the effect of LPS and GYY4137 on signaling pathways regulating endocytosis showed that these compounds act on both clathrin-dependent and clathrin-independent endocytosis. LPS increases the level of MHC1, END1 (participates in the recycling of receptors [47]), CHMP5, MVB12, SWIP, SNX5, and CPML and reduces the levels of Ras, TGFβ receptors, etc. GYY4137 slightly increases TGFβR and Smad (2/3) and decreases cPML. It has been established that the formation of the LPS-TLR4 complex induces the inactivation of signals from the TGFβ receptor (TGFβR–Smad (2/3) transcription factors). The efficiency of signal transduction from TGFβR to Smad (2/3) increases upon attachment of adapter proteins cPML and SARA to TGFβR [48]. The signaling pathway from the TGF-β receptor can be activated by oxidative stress, which further leads to the activation of signaling pathways involving p38 MAPK. It has been shown that H_2S can effectively inhibit the NF-κB, TGFβ, and IL-4/STAT6 signaling pathways, as well as reduce ROS production by suppressing NOX2/4 [49].

LPS reduces the level of GPCR and β-arrestin, while GYY4137 increases their level. We also found that LPS increases the level of caveolin and Src tyrosine kinase, which play an important role in the pathways of clathrin-independent endocytosis, while GYY4137 reduces the level of caveolin and Src tyrosine kinase [19]. Philipin, an inhibitor of caveolin-dependent endocytosis, virtually abolished cell protection by H_2S donors and Hsp70 from LPS-induced ROS production. At the same time, filipin reduced the formation of ROS induced by LPS by almost 30%. Interestingly, filipin reduced TNFα production (associated with LPS) by almost 6 times (Fig. 4, Fil). Caveolae are also associated by signaling pathways with MAP kinases, Akt, Rac small GTPases, etc. All these signaling pathways are to some extent associated with the activating action of LPS. In our experiments, filipin, an inhibitor of caveolin-dependent endocytosis, reduced the protective effect of Hsp70 and GYY4137 by disrupting the structure of caveolae and affecting the diversity of intracellular signaling pathways associated with caveolin-dependent endocytosis [50].

The macropinocytosis inhibitor amiloride reduces both the activation of LPS cells and the protective effect of Hsp70 and hydrogen sulfide donors (Fig. 3, Am). An important role in macropinocytosis is played by NADPH oxidase, inhibition of which suppresses macropinocytosis, as well as Na^+/H^+, an exchanger that is involved in the regulation of actin levels. Amiloride affects NF-kB signaling pathways and reduces TNFα production by cells, which correlates with the data in [51].

An inhibitor of tubulin-dependent endocytosis, nocodazole, reduced LPS-induced production of ROS (by ~20%) and TNFα (by ~2.5 times). At the same time, the inhibitor significantly reduced the protective effect of Hsp70 and hydrogen sulfide donors (Figs. 3 and 4, Noc), as in [52].

The study of the effects of endocytosis inhibitors on the production of ROS and TNFα by THP-1 cells in the presence of LPS, Hsp70, and hydrogen sulfide donors showed that all these inhibitors reduce the production of ROS and TNFα by cells. All inhibitors reduce TNFα production by cells in the presence of LPS, Hsp70, and hydrogen sulfide donors. MBCD, dinasor, and amiloride reduce ROS production in the presence of LPS, Hsp70, and hydrogen sulfide donors.

Thus, Hsp70 and hydrogen sulfide donors reduce inflammatory processes in cells activated by LPS.

Hsp70 and H_2S donors differ in the kinetics of the protective action, while hydrogen sulfide donors turned out to be more effective. The role of endocytosis inhibitors in the mechanisms of cell protection by H_2S donors was studied and Hsp70 by the action of LPS. It has been established that GYY4137 pretreatment of LPS-exposed cells increases the LPS-mediated induction of pro-inflammatory genes and reduces the expression of genes for such intracellular signaling pathways as endocytosis, phagocytosis, glycolysis, the NF-kB signaling pathway, signaling pathways involving TNFα receptors, TLR4, and signaling pathways. tyrosine kinase pathways (PI-3K, MAPK, Src, etc.).

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COMPLIANCE WITH ETHICAL STANDARDS

This work was carried out without involving human participants and animals as objects of research.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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