HSP70 in human polymorphonuclear and mononuclear leukocytes: comparison of the protein content and transcriptional activity of *HSPA* genes

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Received: 25 July 2016 / Revised: 10 October 2016 / Accepted: 11 October 2016 / Published online: 25 October 2016
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Abstract Cell-type specific variations are typical for the expression of different members of the HSP70 family. In circulating immune cells, HSP70 proteins interact with units of signaling pathways involved in the immune responses and may promote cell survival in sites of inflammation. In this work, we compared basal HSP70 expression and stress-induced HSP70 response in polymorphonuclear and mononuclear human leukocytes. The intracellular content of inducible and constitutive forms of HSP70 was analyzed in relation to the transcriptional activity of *HSPA* genes. Hyperthermia was used as the stress model for induction of HSP70 synthesis in the cells. Our results demonstrated that granulocytes (mainly neutrophils) and mononuclear cells differ significantly by both basal HSP70 expression and levels of HSP70 induction under hyperthermia. The differences were observed at the levels of *HSPA* gene transcription and intracellular HSP70 content. The expression of constitutive Hsc70 protein was much higher in mononuclear cells consisting of monocytes and lymphocytes than in granulocytes. At the same time, intact neutrophils showed increased expression of inducible Hsp70 protein compared to mononuclear cells. Heat treatment induced additional expression of *HSPA* genes in leukocytes. The most pronounced increase in the expression was observed in polymorphonuclear and mononuclear leukocytes for *HSPA1A/B*. However, in granulocytes, the induction of the transcription

of the *HSPA8* gene encoding the Hsc70 protein was significantly higher than in mononuclear cells. These variations in transcriptional activity of *HSPA* genes and intracellular HSP70 content in different populations of leukocytes may reflect specified requirements for the chaperone activity in the cells with a distinct functional role in the immune system.

Keywords Heat shock protein 70 · Intracellular HSP70 · *HSPA* expression · Leukocytes · Neutrophils · Cell stress

Introduction

As in other cell types, in leukocytes, highly conserved heat shock proteins of the 70 kDa family (HSP70) are essential for maintaining cellular homeostasis in normal physiological conditions and for cell viability in stress conditions. The HSP70 family has a broad spectrum of chaperone functions, by which they are involved in many intracellular processes. In humans, this family of proteins includes several constitutively expressed and inducible varieties with a molecular weight in the range of 66–78 kDa. These proteins are highly homologous but encoded by different *HSPA* genes. Their main function is to assist the correct functioning of other intracellular molecules, mainly proteins. The localization of intracellular HSP70 is diverse: they are found in the cytoplasm, in the nucleus, in the mitochondria, and in other cellular compartments where they can perform different functions (Ellis et al. 2000; Dagaard et al. 2007). Forming complexes with different regulatory proteins, they can modulate the activity of signaling pathways involved in cell differentiation, proliferation, and apoptosis (Guzhova et al. 1997; Helmbrecht et al. 2000; Maloyan and Horowitz 2002).

Some members of the HSP70 family are constitutively expressed in unstressed cells. They include an essential

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“house-keeping” protein Hsc70, the product of the *HSPA8* gene, and some other organelle-specific proteins. These proteins provide the correct folding and stabilization of newly synthesized polypeptide chains, degradation of misfolded proteins, and conformational remodeling of the proteins to facilitate their further import into organelles such as the mitochondria and endoplasmic reticulum. The Hsc70s stabilize lysosome membranes and participate in chaperone-mediated autophagy (Agarraberes and Dice 2001), and are implicated in nuclear targeting in stress conditions (Bański et al. 2010).

Various stress conditions stimulate the expression of inducible proteins of the HSP70 family that protect cells by promoting the disaggregation process, repair or elimination of damaged proteins, stabilizing the molecule conformation, and provide post-stress resistance to adverse external influence. Prevailing in this group, Hsp70 protein comprises two isoforms, HSPA1A and HSPA1B (or HSP70A and HSP70B) encoded by *HSPA1A* and *HSPA1B* genes, respectively. Differing only by two amino acids, these isoforms are thought to be interchangeable by their functions (Kampinga et al. 2009). Traditionally, these proteins are referred to as Hsp70. Another stress-induced protein is Hsp70B', a product of the *HSPA6* gene, that is either not detected or expressed at very low levels in intact cells, but strictly induced by stress, especially by hyperthermia, while being less sensitive to temperature as compared with Hsp70 (Noonan et al. 2007). In response to stress, a part of Hsp70s like Hsc70 accumulates in the cell nucleus where it is involved in the protection of the genetic material (Kotoglou et al. 2009), participating directly in the process of reconstitution of the mitotic centrosome (Hut et al. 2005). Also, Hsp70 induction in the cells inhibits the development of autophagy, an alternative, a more “radical” mechanism of the cellular response to stress (Dokladny et al. 2013). Despite a high similarity among the HSP70 family of protein members in gene sequences, increasing data indicate that different HSP70 proteins may vary in function depending on their sub-cellular localization, concentration, and availability of specific substrates (Ellis et al. 2000; Leppa et al. 2001; Arispe et al. 2002; Callahan et al. 2002; Mayer and Bukau 2005; Dugaard et al. 2007; Hageman et al. 2011).

Members of the HSP70 family may differ in their constitutive expression and in the range of stress-induced responses in different tissues of the organism. For example, the level of transcriptional activity of the *HSPA6* gene in white blood cells greatly exceeds the transcription levels in other tissues (kidney, liver, prostate, testicular tissue, uterus, brain) (Dugaard et al. 2007). The kinetic profile of HSP70 expression is highly dependent on the type and strength of the stress affecting the cells or the organism (Eid et al. 1987; Wang et al. 2003). At the same time, the detailed mechanism of different HSP70 protein expression and their functioning in various cell types, in particular white blood cells, is not fully understood.

Circulating leukocytes actively participate in inflammatory reactions. HSP70 proteins expressed in leukocytes interact with units of the signaling pathways involved in immune responses and promote cell survival in sites of inflammation (Muralidharan and Mandrekar 2013). Cell-type specific variations in HSP70 expression in human leukocytes have been recently demonstrated (Oehler et al. 2001). Neutrophils, a subtype of granulocytes, constitute the predominant population of peripheral blood leukocytes in mammals. They are innate immune cells maintaining the non-specific first line of defense against bacterial pathogens. Neutrophils are actively involved in the inflammatory response and may exert damaging effects on the organism tissues. Although they are short-lived cells, neutrophils express a wide range of messenger RNA (mRNA) (including mRNA for HSP70) whose levels significantly increase upon stimulation (Subrahmanyam et al. 2001; Fessler et al. 2002; Kobayashi et al. 2002). Lymphocytes and monocytes are immune cells with an extended lifetime compared with neutrophils. They participate in various specific inflammatory and immune reactions and may have different requirements for HSP70 chaperone activity.

In a previous study, we demonstrated several significant age-dependent correlations between HSP70 levels and ROS production in human neutrophils (Kovalenko et al. 2014) that reflects an important but insufficiently studied role of HSP70 in the regulation and maintenance of functional activity of these cells. In this work, HSP70 expression was compared in cells isolated from healthy human polymorphonuclear leukocytes (granulocytes) and peripheral blood mononuclear cells containing lymphocytes and monocytes. The level of intracellular Hsc70 and Hsp70 was analyzed in relation to the transcriptional activity of the *HSPA* genes. Hyperthermia was used in the study as the classic stress model for the induction of HSP70 synthesis in cells.

Materials and methods

Participants

Healthy adults (18 persons (8 women), aged 20–65, median 27) were recruited for the study. Inclusion criteria for participation were the absence of active pathologies and treatment with corticosteroids or high doses of nonsteroidal anti-inflammatory drugs for all subjects. The study was approved by the local research ethics committee. All participants gave their informed consent prior to the study.

Granulocyte and mononuclear cell isolation

Cells were isolated from peripheral blood within 0.5 h after blood sampling by density gradient centrifugation at 500g for

30 min at RT using PolymorphPrep separation medium (Axis-Shield, Sweden). Fractions containing polymorphonuclear leukocytes (granulocytes) and mononuclear cells (lymphocytes and monocytes) were then collected. Cells were washed twice (400g, 15 min) in Dulbecco's phosphate buffer saline (DPBS), resuspended in RPMI-1640 media (Sigma-Aldrich, USA) supplemented with 2 mM L-glutamine, 15 mM HEPES, and 2 % fetal calf serum (HyClone, Thermo Scientific, USA) (referred to hereafter as assay media) at a concentration of 2×10^6 cells/ml and left for 30 min prior to use in assays. Granulocyte fraction purity assessed by flow cytometry analysis with CD66b as a marker for neutrophils was routinely ≥ 95 %. The population of monocytes determined as CD14-positive cells in mononuclear cell fractions amounted as a rule to not more than 10 %. Cell viability determined by trypan blue staining was no less than 97 %.

Heat treatment and HSP70 immunolabeling

Granulocytes and mononuclear cells in assay media were dispensed into polypropylene tubes (10^6 cells in 500 ml) and heated in a constant-temperature water bath at 40 °C for 1 h or at 41 °C for 1 h (heat shock, HS) with subsequent recovery period at 37 °C for different time intervals. Intracellular HSP70 levels were then determined by indirect immunofluorescent staining and flow cytometry analysis. For intracellular labeling the cells were fixed and permeabilized in DPBS containing 2 % paraformaldehyde (Riedel-de Haen, Germany), 0.05 % BSA and 0.05 % Triton X-100 (Sigma-Aldrich, USA) at 37 °C for 15 min. The permeabilized cells were incubated with primary HSP70-specific monoclonal antibody BRM22 (Sigma-Aldrich, USA) or Hsp70-specific antibody C92F3A-5 (Stressgen, Enzo Biochem, USA) for 30 min at RT and then stained with secondary sheep anti-mouse IgG Fab-fragments conjugated with PE (Sigma-Aldrich, USA) for 30 min at RT. Each stage of labeling was followed by two washes with DPBS containing 0.2 % BSA and 0.1 % Triton X-100. HSP70 intracellular levels were determined as means of fluorescence intensity (MFI) corrected for background fluorescence of the negative controls.

Flow cytometry

Flow cytometry analysis was carried out on a FACSCalibur flow cytometer (BD Biosciences, USA) equipped with 488- and 640-nm lasers and an appropriate set of detectors and filters. Cytofluorimetric analysis was performed using separated granulocyte and mononuclear cell fractions. In the case of mononuclear cell population, lymphocyte and monocyte subsets were discriminated by gating the cells using forward and side scatter cytogram (Fig. 1a). A minimum of 10,000 gated events were collected for each sample. Data were

analyzed using CellQuest ver. 3.4 (BD Biosciences) and FlowJo version 7.6.5 flow cytometry analysis programs.

Western blot analysis

Granulocytes and mononuclear cells (6×10^6 cells per sample) were harvested by centrifugation, washed one time with cold DPBS and treated with lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA , 1 % Triton X-100, protease inhibitors cocktail (Thermo Scientific, USA)) in 200 μl for 5 min on ice. After the lysed cell sample centrifugation (10,000g, 10 min) the supernatants were collected. The protein samples obtained from equal numbers of cells unless otherwise indicated were loaded into sodium dodecyl sulfate-polyacrylamide gels (10 %), separated by electrophoresis and blotted to nitrocellulose membranes for the HSP70 Western blot analysis according to the standard protocol (<http://www.cellsignal.com/contents/resources-protocols/western-blotting-protocol/western>). In experiments on intracellular Hsp70 dynamics, the protein in samples was equalized using a colorimetric assay with Bradford reagent. After the sample transference nitrocellulose membranes were stained with primary antibody BRM22 or C92F3A, anti- β -actin antibody AC-15 (Sigma-Aldrich, USA) and secondary HRP-conjugated antibody (Sigma-Aldrich, USA). The results were visualized using enhanced chemiluminescence Western blotting reagents (Immun-Star HRP, Biorad, USA). The data were analyzed using ImageJ software (ver.1.38x Wayne Rasband, NIH, USA).

Real-time PCR procedure

Total RNA for each time point was extracted from 6×10^6 cells of granulocytes or mononuclear cells using TRIzol reagent (MRC, UK) according to the manufacturer's instructions. All RNA samples were treated with DNaseI (Thermo Scientific, USA) to remove residual DNA. Total RNA was quantified with NanoVue Plus spectrophotometer at A260/A280 (GE Healthcare Life Science, UK). Complementary DNA (cDNA) synthesis was performed using random hexamer primers with the addition of MINT Reverse Transcriptase (Evrogen, Russia) following the manufacturer's instructions. Real-time PCR primers (5'-3') designed with Primer Blast software (www.ncbi.nlm.nih.gov/tools/primer-blast) and controlled with OligoAnalyzer ver. 3.1 program are listed below (gene: forward primer, reverse primer).

HSPA1A/B: AGGTGCAGGTGAGCTACAAG,
CTCGGCGATCTCCTTCATC
HSPA8: TGCTGCTCTTGATGTCACT, AAGGTCTG
TGTCTGCTTGGT

Fig. 1 Flow cytometric analysis of granulocytes and mononuclear leukocytes. **a** Typical FS-SS dot-blots of granulocyte and mononuclear cell fractions. **b** Basal overall intracellular HSP70 (Hsc70 + Hsp70) levels in granulocytes, lymphocytes, and monocytes measured by flow cytometry using BRM-22 antibody recognizing both inducible Hsp70 and constitutive Hsc70 proteins of HSP70 family. **c** Hsp70 levels measured using C92F3A-5 antibody specific for Hsp70. The results are presented in **b** and **c** as an average of means of fluorescence intensity (MFI) of n independent experiments $\pm$ SD, where for **b** $n \geq 6$, and for **c** $n \geq 3$. Hereinafter, significant differences between the groups are indicated by asterisks (***) $p < 0.005$; ** $p < 0.01$; * $p < 0.05$)

HSPA6: ACCCAGGTGTATGAGGGTGA, TCTATCTG GGGGACTCCACG

β -actin: CACCACACCTTCTACAATGAG, GTCTCAACATGATCTGGGTC

Each real-time PCR mixture (final volume 25 μ l) contained 5 μ l of qPCRmix-HS SYBR mixture (Evrogen, Russia), 1 μ l of 3 mM forward and reverse primers, 0.5 μ l of cDNA template, and 17.5 μ l of nuclease-free water. The reactions were carried out using a LightCycler 480 real-time PCR detection system (Roche Diagnostics, Deutschland GmbH) as follows: pre-incubation at 95 $^{\circ}$ C (5'), and then 40 cycles of 95 $^{\circ}$ C for 10 s, 60 $^{\circ}$ C for 10 s, and 72 $^{\circ}$ C for 10 s. At the end of the amplification, a dissociation curve was plotted to confirm the specificity of the product. All real-time experiments were repeated in triplicates. The results were normalized against house-keeping gene *β -actin* to correct the sample-to-sample variation. The fold increase gene expression ratio was estimated as a ratio of normalized mRNA level from cell samples treated by HS 40 $^{\circ}$ C 1 h or 41 $^{\circ}$ C 1 h to that of the intact samples.

Statistical analysis

Statistical analysis was performed using Microsoft office Excel 2007 and SigmaPlot (ver. 11.0, Systat Software Inc.). Data are expressed as mean $\pm$ standard deviation. For data that followed a normal distribution, t tests were performed; for

non-normally distributed data, a Mann-Whitney U test was used. Results were considered statistically significant as *** $p < 0.005$; ** $p < 0.01$; * $p < 0.05$.

Results

Analysis of intracellular HSP70 in intact peripheral blood granulocytes and mononuclear cells

In this work, we estimated the HSP70 content in the granulocytes or mononuclear cells of healthy volunteers. Two cell fractions were isolated by specialized gradient cell separation from a single sample of peripheral blood. In the granulocyte fractions, most cells were neutrophils (over 95 % CD66b-positive cells). The mononuclear cell fractions contained lymphocytes and monocytes. The procedure used for cell separation allows for isolating the cell with minimal side effects, such as induction of reactive oxygen species (ROS) generation in neutrophils. Overall intracellular content of HSP70 (constitutive Hsc70 and inducible Hsp70) was measured by flow cytometry with BRM-22 antibody (Sigma-Aldrich, USA) recognizing a conservative epitope of HSP70 molecules. Inducible Hsp70 was detected by C92F3A-5 antibody (Stressgen, USA) specific for this protein. Cytofluorimetric analysis was performed using the separated granulocyte and mononuclear cell fractions; in the case of the mononuclear cell population, lymphocyte and monocyte

subsets were discriminated by gating the cells using forward and side scatter cytogram (Fig. 1a).

The results demonstrated that the total level of intracellular HSP70 detected in granulocytes by using BRM-22 antibody was approximately three times lower than in mononuclear cells; there was no significant difference in the HSP70 content between lymphocytes and monocytes (Fig. 1b). In contrast, the levels of stress-inducible Hsp70 detected with C92F3A-5 antibody were considerably higher in granulocytes compared to lymphocytes but did not differ from Hsp70 levels in monocytes (Fig. 1c).

Taking into consideration that intracellular HSP70 proteins being chaperones are often in a substrate-associated or in autoaggregate states (Angelidis et al. 1999), that may restrain their epitope accessibility for binding with a specific antibody used for a cytofluorimetric method of protein detection (Boyko et al. 2014), we applied Western blot along with flow cytometry for a comparative analysis of intracellular HSP70 in polymorphonuclear and mononuclear leukocyte fractions. The data obtained demonstrated that with an equal number of cells per sample, the amounts of β -actin in mononuclear cells were higher than in granulocytes (Fig. 2a), that was in accordance with the data about low biosynthetic activity of neutrophils (Murphy 1976). We could not find a considerable difference in total HSP70 content normalized for β -actin between granulocytes and mononuclear cells. Intensities of HSP70 band density analyzed by ImageJ program were 0.62 and 0.51 relative units for granulocytes and mononuclear cells, respectively. At the same time, the amounts of Hsp70, both original and normalized for β -actin, were for the most part higher in granulocytes than in mononuclear cells that is corresponded to flow cytometric data (Fig. 2b). Thus, intact human neutrophils, despite the low total content of HSP70, express more stress-inducible Hsp70s compared with lymphocytes and monocytes.

Fig. 2 Intracellular HSP70 contents in granulocytes and mononuclear cells analyzed by immunoblotting. **a** Overall Hsp70 + Hsc70 content measured using BRM-22 antibody. β -actin intracellular content was analyzed as internal control of biosynthetic activity of cell fractions. **b** Hsp70 content determined by C92F3A-5 antibody

Transcriptional activity of HSP70 genes in intact granulocytes and mononuclear cells

The intracellular level of Hsp70 is dependent on the accumulation and stability of mRNA of *HSPA* genes (Balakrishnan and De Maio 2006). In the next step of the investigation, we analyzed the basal transcriptional activity of *HSPA8*, *HSPA1A/B*, and *HSPA6* encoding constitutive protein Hsc70 and stress-inducible proteins Hsp70 and Hsp70B', respectively. The basal *HSPA8* expression that was found using qRT-PCR was higher in mononuclear cells than in granulocytes (Fig. 3). In contrast, *HSPA1A/B* expression was higher in granulocytes compared with mononuclear cells. Similar to *HSPA1A/B*, transcriptional activity of *HSPA6*, another stress-inducible gene, was higher, although insignificantly, in granulocytes than in mononuclear cells (Fig. 3). Within the mononuclear cell fraction, *HSPA8* expression was considerably more intensive than *HSPA1A/B* or *HSPA6* expression whereas comparable expression levels of *HSPA8*, *HSPA6* and *HSPA1A/B* were observed in intact granulocyte fractions.

These results conform to the data described above on the intracellular content of Hsc70 and Hsp70 in granulocytes and mononuclear cells, showing a prevalence of Hsc70 expression in mononuclear cells and increased expression of stress-inducible proteins in granulocytes. Collectively, these data indicate significant differences in HSP70 expression between different leukocyte subsets in healthy humans.

Heat shock-induced changes in HSPA transcriptional activity in granulocytes and mononuclear cells

The efficiency of the HSP70 protective system is mediated by the proteins constitutively expressed in cells and induced during cell stress. To analyze the relationship between the transcriptional activity of *HSPA* genes (*HSPA1A/B*, *HSPA6*,

Fig. 3 Transcriptional activity of *HSPA* genes in granulocytes and mononuclear cells, analyzed by qRT-PCR. Data of mRNA levels normalized by β -actin were averaged for granulocyte and mononuclear cell fractions isolated from three different donors aged 22–35 years

HSPA8) and the protein content in granulocytes and mononuclear cells in stress conditions, we used the cell samples subjected to hyperthermia *ex vivo*. Two types of heat shock (HS) were applied to the cells: (1) mild HS (40 °C, 1 h) that might occur in vivo during inflammation and fever and (2) physiologically sublethal severe HS (41 °C, 1 h).

Mild HS did not induce mRNA synthesis in granulocytes for any of the stress-inducible genes (*HSPA1A/B* and *HSPA6*) but led to the induction of *HSPA8* transcription (Fig. 4a). At the same time, intensive generation of mRNA for stress-inducible Hsp70 proteins in mononuclear cells was registered (2 h after the beginning of treatment, or 1 h after HS completion). *HSPA6* transcription in these cells was also induced by mild HS but to a lesser extent than *HSPA1A/B*. Induction by mild HS of *HSPA8* transcription was much lower in mononuclear cells than in granulocytes. The dynamics of *HSPA* mRNA synthesis was similar in both cell fractions: after a short increase, the mRNA accumulation rate measured 4 h after the beginning of HS declined and, in some experiments, was closer to the initial level (Fig. 4a).

Exposure of cells to severe HS (when the temperature was increased to 41 °C) led to a much more significant increase in mRNA levels for all genes tested in both mononuclear cells and granulocytes compared to mild HS. The increase was less pronounced for *HSPA8* expression, compared to *HSPA1A/B* and *HSPA6* (Fig. 4b). Again, HS-mediated induction of

HSPA1A/B genes was higher in mononuclear cells than in granulocytes, and *HSPA8* induction was higher in granulocytes compared with mononuclear cells. The maximal mRNA accumulation rate was observed for the *HSPA1A/B* gene in mononuclear cells an hour after HS completion (more than 600-fold increase). The dynamics of *HSPA* gene mRNA synthesis induced by severe HS were mostly similar to the dynamics observed after mild HS. A maximal increase in mRNA accumulation rate was observed 2 h after beginning HS. For all tested genes, the mRNA synthesis decreased (or at least did not increase) at 4 h compared with the 2-h point of measurement. No decrease in mRNA accumulation rate for *HSPA1A/B* was observed in granulocytes after 4 h; a minimal reduction of *HSPA8* mRNA synthesis was observed at this time point for both cell fractions.

Thus, *HSPA* gene transcription differs significantly depending on hyperthermia conditions. Translation efficacy of the genes might also change with temperature increase (Finka et al. 2015). To trace the hyperthermia effects, intracellular Hsp70 levels were assessed in granulocytes and mononuclear cells after both mild and severe HS. Despite the multifold induction of *HSPA1A/B* transcription, only a small increase in intracellular Hsp70 content was shown in mononuclear cells by Western blot analysis under mild HS. The increase was detected an hour after HS completion but no further Hsp70 elevation with time was observed (Fig. 5a). Severe

Fig. 4 Transcriptional activity of *HSPA* genes in granulocytes and mononuclear cells in conditions of hyperthermia. Transcription levels were determined by qPCR using β -actin as a reference. *HSPA1A/B*, *HSPA8*, and *HSPA6* mRNA synthesis induced by mild HS (40 °C, 1 h) (a) and severe HS (41 °C, 1 h) (b) was calculated in dynamics (1, 2, 3, and 4 h after HS). Representative data were obtained in cell fractions isolated from three different donors aged 22–35 years in independent experiments

Fig. 5 Western blot analysis of changes in intracellular Hsp70 (inducible protein) contents in granulocytes and mononuclear cells induced by mild (a) and severe (b) HS. Monoclonal antibody C92F3A-5 was used for

Hsp70 detection. Within each experiment the samples were equilibrated by protein measuring method. Time intervals were counted from the start of the procedure. C basal level of Hsp70 without hyperthermia

HS led to a more pronounced accumulation of Hsp70 in mononuclear cells compared with mild HS. However, in granulocytes *HSPA1A/B* transcription induced by HS (Fig. 4b) was not accompanied by an increase in Hsp70 intracellular content (Fig. 5b).

The dynamics of intracellular Hsp70 content in response to hyperthermia in granulocytes and mononuclear cells was also analyzed by flow cytometry (Fig. 6) that allowed us to discriminate Hsp70-containing lymphocytes and monocytes in mononuclear cell fraction using a gating strategy (Fig. 1a). Mild HS induced a significant increase of Hsp70 in monocytes but did not cause Hsp70 accumulation in lymphocytes (Fig. 6a). We can suggest that the small increase in Hsp70 content registered by Western blot in mononuclear cells fraction (Fig. 5a) was associated mainly with monocytes, although the portion of these cells in the fraction was usually less than 10 %. An increase in temperature (severe HS) led to a further accumulation of Hsp70 in monocytes and to a moderate increase in intracellular Hsp70 in lymphocytes (Fig. 6b). The induction of Hsp70 expression in neutrophils treated at 41°C for 1 h was negligible there being a more than 150-fold increase in the induction of *HSPA1A/B* gene transcription (Fig. 4b).

Fig. 6 HS-induced dynamics of intracellular Hsp70 (inducible protein) in granulocytes and mononuclear cells analyzed by flow cytometry. a Hsp70 accumulation in monocytes and lymphocytes induced by mild HS. b Hsp70 accumulation in monocytes, lymphocytes, and neutrophils, induced by severe HS. Data of a typical experiment are presented

Thus, different populations of leukocytes, namely granulocytes and mononuclear cells differ significantly by both basal HSP70 expression and the level of HSP70 induction in unfavorable conditions such as hyperthermia. These differences are observed at the levels of *HSPA* gene transcription and HSP70 protein synthesis.

Discussion

Despite the high homology of proteins in the multigene HSP70 family and the similar manner of their regulation by stress-inducible systems of transcription factors, the levels and ratio of expression of members of this family can differ in different types of cells in the organism. These cell-type-specific variations in HSP70 expression seem to reflect the association of the protein activity with cell functions (Finka and Goloubinoff 2013). In agreement with this idea, we found in this study considerable differences in the transcriptional activity of *HSPA* genes and the intracellular contents of HSP70 proteins between different populations of human leukocytes (granulocytes and mononuclear cells) closely related by their hematopoietic origin.

Although neutrophils are considered as terminally differentiated cells, they demonstrate significant transcriptional and translational activity (Luerman et al. 2010). Nevertheless, we have shown by flow cytometry and Western blot analysis that a granulocyte fraction consisting mainly of neutrophils contains fewer HSP70 proteins than a mononuclear cell fraction. At the same time, we could not find any significant differences between granulocytes and mononuclear cells in the intracellular level of HSP70 (including both Hsc70 and Hsp70) normalized to β -actin that is often used as the reference protein because of its stable constitutive expression in the cell. Low content of both β -actin and HSP70 in neutrophils can be explained by low biosynthetic activity of these cells characterized by small number of ribosomes and condensed state of chromatin (Murphy 1976). Possibly, the low intensity of protein synthesis in a cell determines the moderate level of intracellular of HSP70 that is required for assisting correct folding, stabilization, and targeted transportation of newly synthesized proteins. However, intracellular levels of stress-inducible

protein Hsp70 in intact granulocytes exceed the Hsp70 levels in a mononuclear cell fraction in which, in contrast to granulocytes, constitutive protein Hsc70 prevailed. Predominant expression of Hsc70 within the HSP70 family was shown previously for many non-tumor and non-stressed cells (Finka and Goloubinoff 2013). Higher levels of Hsp70 in intact granulocytes compared to lymphocytes and monocytes were shown by flow cytometry in an earlier work (Oehler et al. 2001). The main pattern of Hsp70 and Hsc70 protein intracellular levels in granulocytes and mononuclear cells corresponded to the higher transcriptional activity of *HSPA1A/B* and lower *HSPA8* expression in intact granulocytes compared with mononuclear cells demonstrated in this work. The considerable transcriptional activity of *HSPA6* was registered in intact granulocytes. In earlier works, the *HSPA6* was suggested to be expressed in a number of cell types predominantly only under stress conditions (Leung et al. 1990; Noonan et al. 2007).

Differences in intracellular content of constitutive and inducible forms of HSP70 may be associated with primary functional activity of these proteins in different subsets of leukocytes. Although inducible Hsp70 and constitutive Hsc70 are considered to possess mainly similar functions in the cells, one of these proteins can be more efficient in some processes than another protein. An example of these differences is the better ability of Hsp70, compared with Hsc70, to interact in stress conditions with peptides to facilitate their presentation in a complex with MHC class I molecules (Callahan et al. 2002). Increased Hsp70 levels in intact neutrophils may be associated with high reactivity of these cells. Hsp70 was shown to interact with many components of signal transduction pathways involved in cell activation including MAP kinases (MAPKs) and Src family kinases (Helmbrecht et al. 2000). We can speculate about participation of this chaperone in the control of neutrophil responses to environmental stimuli, such as their rapid migration to the sites of inflammation by gradient of chemokines whose receptor-mediated signaling involves Src-kinases, or due to sensing IL-1, IL-18, and various pathogen-associated molecular patterns (PAMPs) leading to activation of MAPKs. Hsp70, as suggested, is involved in LPS-triggered pro-inflammatory signaling by binding Toll-like receptor 4 (TLR-4) within lipid rafts and by facilitating intracellular trafficking of the ligand-receptor complex (Triantafilou and Triantafilou 2004). Besides, multi-pointed involvement of Hsp70 in the regulation of apoptosis has been shown (Beere 2005; Mosser et al. 2000; Park et al. 2002). Programmed cell death is a tightly regulated process in neutrophils because it restricts destruction of tissues by released products of lytic granules. However, the mechanisms of the relationship between the cell activity and intracellular content of Hsp70 remain elusive. Uncovering these mechanisms is an important task for further studies.

Constitutive Hsc70 was shown in this work to be more expressed in mononuclear cells compared with granulocytes. Since the life span of the majority of lymphocytes and monocytes are significantly longer than neutrophils, we can assume that increased content of Hsc70 in mononuclear cells, compared to neutrophils, as well as their stronger response to cellular stress, is associated with long-term adaptive immune response. Hsc70 participates also in chaperone-mediated autophagy (CMA) extensively used by T and B lymphocytes to regulate cell homeostasis as well as immune functions (McLeod et al. 2012).

Hyperthermia provokes an accumulation of damaged proteins, as well as their aggregation and inhibition of cellular metabolic processes and signal pathways. On the other hand, heat shock causes an induction of a massive synthesis of HSPs to protect cells against the stress (Trotter et al. 2001; Shalgi et al. 2013; Finka et al. 2015). Both regimes of hyperthermia used in this work induced immediate transcription of *HSPA* genes in granulocytes and mononuclear cells that peaked within the first hour after exposure and then greatly declined (Fig. 4a, b). This decline in *HSPA* mRNA levels may be associated with increased mRNA degradation. In fact, a similar mechanism of self-limiting control of intracellular Hsp70 levels was described in an earlier work (Balakrishnan and De Maio 2006). Another mechanism, controlling the expression of Hsp70 and Hsc70 in cells, is mediated by a complex formation of over-expressed proteins with transcriptional factor heat shock factor 1 (HSF1), one of the main factors responsible for *HSPA* expression (Abravaya et al. 1992; Nunes and Calderwood 1995). The expression of all tested genes was more pronounced after severe HS (41°C for 1 h), with a higher fold induction for stress-inducible genes. However, the considerable observed difference between HS-induced expression of *HSPA1A/B* and *HSPA6* was mediated possibly by differences in the promoter regions of these genes (Leung et al. 1990).

For many years, it was considered that expression of *HSPA8* gene (as well as synthesis of constitutive Hsc70) did not change much under the influence of hyperthermia (Ingolia and Craig 1982). However, the hyperthermia-induced accumulation of Hsc70 was subsequently described for several cell types (Finka et al. 2015). The authors noted that despite the lower *HSPA8* extent of expression in comparison with *HSPA1A/B*, a copy number of newly synthesized Hsc70 molecules may greatly exceed the number of Hsp70 molecules due to an initially greater intracellular Hsc70 content that can be important for the further successful functioning of the cell. In the present work, we observed a HS-dependent increase in *HSPA8* mRNA synthesis in different leukocyte populations (Fig. 4). The more significant HS-induced *HSPA8* expression in neutrophils compared to that in mononuclear cells (Fig. 4a, b) was associated with the low intracellular Hsc70 level observed in intact granulocytes (Fig. 1b and

Fig. 2a). The association between the initially low intracellular content of proteins of the HSP70 family and the more intensive synthesis of their mRNA under stress conditions had been previously described (Balakrishnan and De Maio 2006).

Neutrophils, both resting and activated, differ significantly from other circulating leukocytes by a gene expression pattern that determines their unique functional activity (Subrahmanyam et al. 2001). Our results emphasize that the responses to HS of these cells are also different from the response of other leukocytes. The transcriptional activity of *HSPA* genes, both in the absence of stress and in various stress-induced conditions, can be modulated by multiple intracellular molecules including TATA-binding and GAGA proteins, STAT1, STAT3, AP1, NF-IL6, etc. (Kurshakova et al. 2006; Wigmore et al. 2007; Stephanou and Latchman 2011). These factors bind themselves to the promoter regions of the genes or influence HSF binding to the gene promoters mediating diverse patterns of *HSPA* gene expression. The variation of activities of such factors appears to contribute to the different basal and temperature-dependent *HSPA* transcription found in this work for granulocytes and mononuclear cells. The relatively poor response of neutrophils to HS may be associated with their higher resistance to this type of stress, since in vivo neutrophils migrate to sites of inflammation, where they are extensively exposed to hyperthermia.

In our experiments, the highest HS-induced increase in intracellular Hsp70 was detected in monocytes and to a lesser extent in lymphocytes, that corresponds to data reported in earlier works (Polla et al. 1995; Njemini et al. 2002). At the same time, here and in a previous work (Boyko et al. 2014), we could not detect any significant augmentation of intracellular HSP70 content in neutrophils subjected to hyperthermia despite a considerable increase in *HSPA* mRNA synthesis. This may be partially explained by the relatively low levels of *HSPA* expression of all three gene types in intact granulocytes (compared to high constitutive *HSPA8* expression in mononuclear cells). On the other hand, the poor concordance between mRNA transcription and protein expression changes in neutrophils has been demonstrated by other authors (Fessler et al. 2002). An additional cause of the negligible HS-induced augmentation of HSP70 in neutrophils registered in our experiments might be connected with the release of a part of the intracellular pool of these proteins to extracellular space. Exocytosis of HSP70 in different populations of leukocytes was described earlier (Hunter-Lavin et al. 2004; Asea 2007). Less effective work of translational and transcriptional systems in these terminally differentiated cells may be the reason for this weak response to the HS.

Acknowledgments This work was supported by the Russian Science Foundation, grant no. 16-15-10404.

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