ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 4, pp. 467-472. © Pleiades Publishing, Ltd., 2011.
Published in Russian in Biokhimiya, 2011, Vol. 76, No. 4, pp. 573-580.

Effect of Homocysteine on Properties of Neutrophils Activated *in vivo*

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Received October 25, 2010
Revision received November 29, 2010

Abstract—We have found that neutrophils begin to express NMDA receptors on their membranes after *in vivo* activation. These receptors are the target for action of homocysteine (HC). After incubation of activated neutrophils with HC, the degranulation process is stimulated and generation of reactive oxygen species is increased. We conclude that expression of NMDA receptors on neutrophil membrane makes neutrophils sensitive to HC. Thus, hyperhomocysteinemia may induce additional stimulation of immune competent cells.

DOI: 10.1134/S0006297911040109

Key words: neutrophils, inflammation, NMDA receptors, homocysteine

Homocysteine (HC) is a metabolite related to transformation of methionine to glutathione. Under normal conditions, its stationary levels in blood does not exceed 15-20 μM, but when HC metabolism is disturbed hyperhomocysteinemia appears and blood HC levels may increase to 100-500 μM or higher. Such increase in stationary levels of HC in blood accompanies some neurodegenerative disorders, is considered as a risk factor of infarcts and stroke, and increases the development of atherosclerosis [1-4].

Toxic effect of HC is usually explained by is activating effect on glutamate receptors in the neural system, especially NMDA receptors, which take part in development of excitotoxicity [5, 6]. Recently, these receptors were discovered in red blood cells, cardiomyocytes, and lymphocytes. Moreover, it was found that toxic effect of HC is not limited by its action on neuronal cells [7, 8].

In this study, we demonstrate that neutrophils isolated from inflammatory centers express functionally active NMDA receptors whose interaction with HC activates degranulation and respiratory burst.

Abbreviations: HC, homocysteine; ROS, reactive oxygen species.

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MATERIALS AND METHODS

In the experiments white outbreed laboratory rats kept under standard living conditions were used. All experiments were done consistent with international rules for the use of laboratory animals [9]. Before experiments, the rats were treated with chloral hydrate (Reakhim, Russia) at 500 mg/kg body weight. From both jugular veins before arrest of the heartbeat a blood portion was taken using a syringe containing heparin (50 units/ml of blood) (Spofa, Czech Republic). Intact neutrophils were prepared by centrifuging blood samples using MonoPoly medium (ICN Biomedicals, USA). Cells suspended in Hanks solution were kept at 37°C no longer than 5 h. From flow cytometry analysis, it was noted that neutrophils are present as a homogenous population corresponding in size to 12 μm calibrated beads (BD Biosciences, Becton Dickinson and Co, USA) [10].

In the case of additional experimental treatment including incubation in plastic dishes, the cells were stirred in a vortex, returning adhesive cells into suspension. Flow cytometric measurement of the cells in final samples showed that their number was not decreased.

To prepare neutrophils activated under *in vivo* conditions, 500 μl of non-opsonized zymosan suspension

(4.5 mg/ml of Hanks solution) was administered to each animal intraperitoneally to induce local inflammation. Activated cells were isolated from peritoneal exudates by a procedure based on physiological features of neutrophils yielding a homogenous (95-98%) fraction of these cells [11]. To prevent intercellular adhesion during the preparative procedure, neutrophil suspension was diluted 1.5-fold with Hanks solution. Cells were then centrifuged for 7 min in 1 ml of Hanks solution at 600*g* and room temperature, and the pellet was resuspended in 1 ml of Hanks solution and kept during experiment (no longer then 3 h) at 37°C.

The number of cells in cell suspensions was counted using a Goryaev chamber. Because the number of cells in the samples prepared from several animals differed, in each experiment the volume of the suspension used was adjusted to provide final number of cells of 250,000 per sample.

Functional activity of neutrophils was estimated by production of reactive oxygen species (ROS) measured by the chemiluminescence method. Preliminary effect of HC on chemiluminescence of intact cells was tested during 5-30 min incubation, and it was found that it does not affect this parameter in this time interval. In further experiments for characterization of HC action, the cells were incubated with HC for 30 min at 37°C with constant stirring. Sample volume was 300 μl. Then the chemiluminescence probe luminol (1 μM) was added, whose oxidation to aminophthalic acid was measured at 425 nm [12]. In control samples, chemiluminescence of the cells with no ligand added was measured. Chemiluminescence signal was monitored using a Smartlum 5773 apparatus (St. Petersburg, Russia).

To estimate the involvement of NMDA receptors in activation of ROS production in the presence of HC, we used the specific antagonist of the receptors MK-801. Cells were incubated with 500 μM HC in the presence of 5 μM MK-801 for 30 min at 37°C (sample volume 300 μl), and ROS level was measured by the chemiluminescence method.

Another parameter used to characterize the functional activity of neutrophils was the degranulation process – release of antimicrobial cytotoxic molecules from granules formed by subsequent immune competent cells: granulocytes (neutrophils, basophils, eosinophils), mast cells, NK-cell, as well as other cells with similar functions [13].

In neutrophil cytoplasm two types of granules are present: specific and azurophilic ones containing a large amount of several proteins including myeloperoxidase. Because both types of the granules equally take part in immune response, for estimation of degranulation we determined total amount of protein released from the cells into supernatant after cell sedimentation using the Lowry method [14]. In addition, we performed electrophoretic analysis of supernatant proteins and identified the one corresponding to myeloperoxidase in terms of electrophoretic mobility.

We estimated the presence of NMDA receptors in neutrophils using two different methods. The first was immunocytochemical staining of the cells by antibodies toward the extracellular site of the NR2B subunit of the NMDA receptor, defining NMDA receptor molecules in the cell membrane. The second consisted of lysis of cells with subsequent co-immunoprecipitation with primary antibodies to the NR1 subunit of the NMDA receptor. This approach reveals all the receptor molecules accumulated in the cells.

Cytochemical treatment was made as follows. Samples containing 10^6 cells were incubated with primary antibodies (ab 28373; Abcam Inc., USA; titer 1 : 1000) for 30 min at 25°C. Then 500 μl of Hanks solution was added, and the samples were centrifuged for 5 min at 400*g*, and the pellet was resuspended in Hanks solution and incubated with secondary (FITC labeled) antibodies (F 0257; Sigma-Aldrich, USA; titer 1 : 200) for 30 min at 25°C in the dark. Samples containing only secondary antibodies served as control for nonspecific binding of secondary antibodies with the cell surface (a negative control). A suspension of rat cerebellum neurons, which contain NMDA receptors, was used as a positive control [16]. Fluorescence of FITC-labeled cells was measured using a FACStar flow cytometer (Becton Dickinson).

To prepare samples for immune precipitation, they were resuspended for 5 min in lysing buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF, 4% protease inhibitor (Protease Inhibition Cocktail, PIC) at 4°C. Then the samples were sonicated and centrifuged for 10 min at 13,000*g*. The supernatant was used for immunoprecipitation with antibodies to the NR1 subunit of the NMDA receptor immobilized on Protein A-Sepharose. The antibodies were immobilized using a commercial protocol: Protein A-Sepharose was incubated for 3 h with primary antibodies (ab 1880; Abcam Inc.; 1 : 300) in immune precipitating buffer (3 M NaCl, 1.5 M Gly, pH 8.9) at 4°C with constant stirring and then washed free from excess of antibodies. Immunoprecipitation was performed for 5 h at 4°C with constant stirring. The resulting complex was washed with lysing buffer, resuspended in medium containing 200 mM Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromphenol blue, 4% β-mercaptoethanol, pH 6.8, and heated to 90°C for 5 min, and then the Protein A-Sepharose was sedimented (30 sec at 200*g*). The supernatant containing the desired protein was subjected to Laemmli SDS-PAGE using 4.5% concentrating gel and 10% separating gel. After the electrophoretic procedure, the proteins were transferred to nitrocellulose membrane by Western-blotting while monitoring

protein transfer with preliminarily stained markers (Fermentas, Canada). The resulting membrane was washed with buffer containing 20 mM Tris-HCl, 137 mM NaCl, 1% Tween-20, pH 7.6. Nonspecific sites of antibody binding were blocked using 10% solution of dry milk (Sigma-Aldrich) for 10 min in the same buffer, and then the samples were incubated with primary antibodies to the NR1 subunit of the NMDA receptor (ab 1880; Abcam Inc.) in the presence of dry milk (5% solution) for 1 h. The samples were washed free from nonbound antibodies and incubated with secondary antibodies conjugated with horseradish peroxidase (Sigma-Aldrich) under the same conditions. Then the membrane was washed several times, and the NR1 subunit was identified by a sensitive chemiluminescence substrate system for Western blotting with horseradish peroxidase (Thermo Scientific, USA). The results were recorded using X-ray film (Sigma-Aldrich).

To check reproducibility of the data, all experiments were repeated 3-4 times using cells isolated from different animals. In the quantitative experiments three parallel samples were used for measurements.

Each set of experimental data was statistically analyzed using significance criterion $P > 0.1$, and the data from different series of experiments were compared using Student's *t*-test for independent experimental series with $P < 0.05$. Data from flow cytometry were analyzed using the WinMDI 2.9 program.

RESULTS

First we studied the effect of HC on the functional activity of *in vivo*-activated neutrophils by the oxygen-dependent mechanism [18]. To characterize degranulation we measured the amount of protein released from the cells into the incubation medium. We found that incubation of neutrophils with HC results in significant accumulation of protein in the supernatant – in control samples it did not exceed 80 ± 10 μg/ml, whereas in HC treated samples it reached 300 ± 18 μg/ml. This suggested additional activation of neutrophils by HC resulting in greater degranulation.

Figure 1 shows a cytogram of a population of *in vivo*-activated neutrophils in coordinates "forward scattering (FS) against side scattering (SS)", the latter characterizing the intracellular granulation [17], after 150 min incubation of the cells in the absence (control) (Fig. 1a) or in the presence (Fig. 1b) of 500 μM HC. It is seen that after incubation with HC the population of cells located in the upper right quadrant is increased, thus indicating accumulation of cytoplasmic granules in the HC treated neutrophils.

We have also shown that HC affects the efficiency of ROS generation by neutrophils – the process that is one of the basic cytotoxic functions of these cells [18]. Incubation of *in vivo*-activated neutrophils with 500 μM HC for 30 min results in considerable increase in the lev-

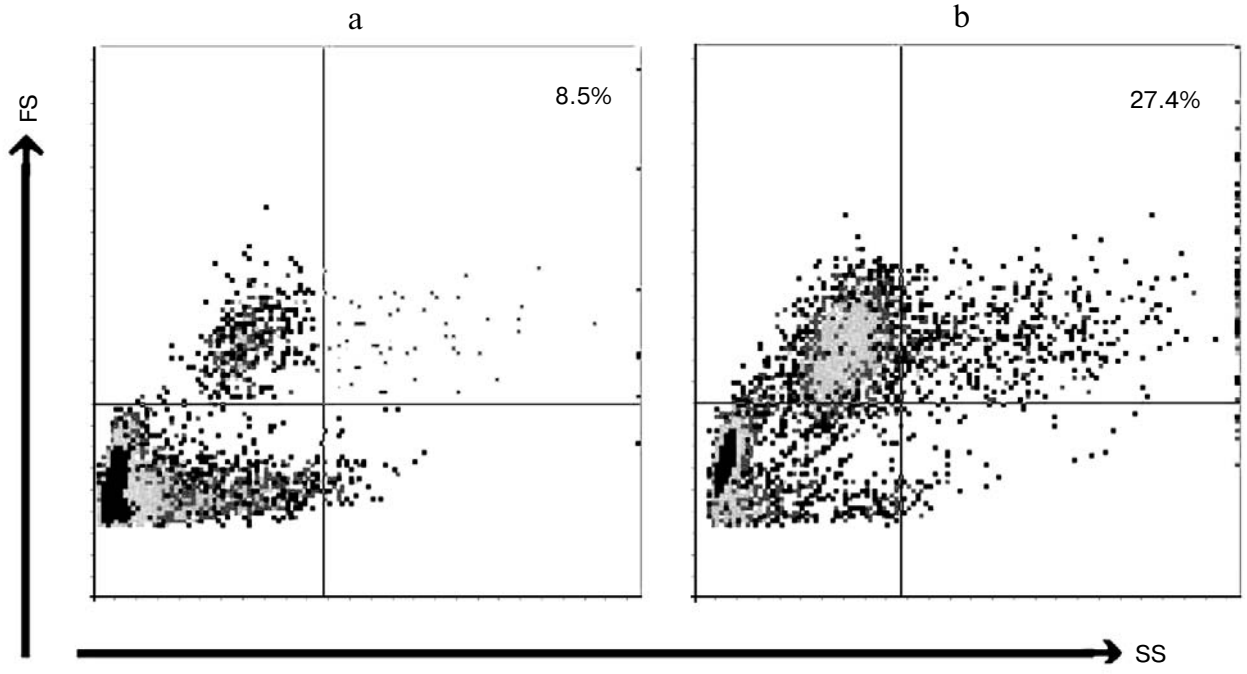

Fig. 1. Population of *in vivo*-activated rat neutrophils in FS vs. SS coordinates (in each case % of cells of increased granularity is noted). a) Control; b) after 150-min incubation with 500 μM HC; FS, forward scattering characterizing size of the cells; SS, side scattering characterizing intracellular granularity.

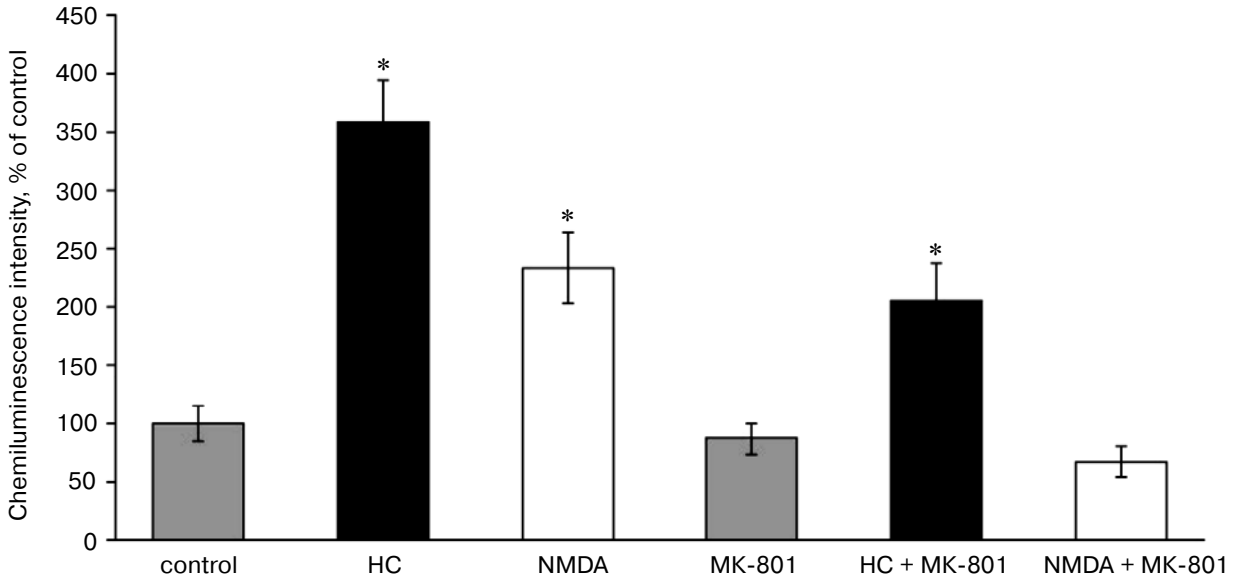

Fig. 2. Effect of HC and NMDA on ROS accumulation by *in vivo*-activated rat neutrophils. Results of three independent measurements are presented. Sign (*) corresponds to statistically significant difference from control ($P < 0.05$).

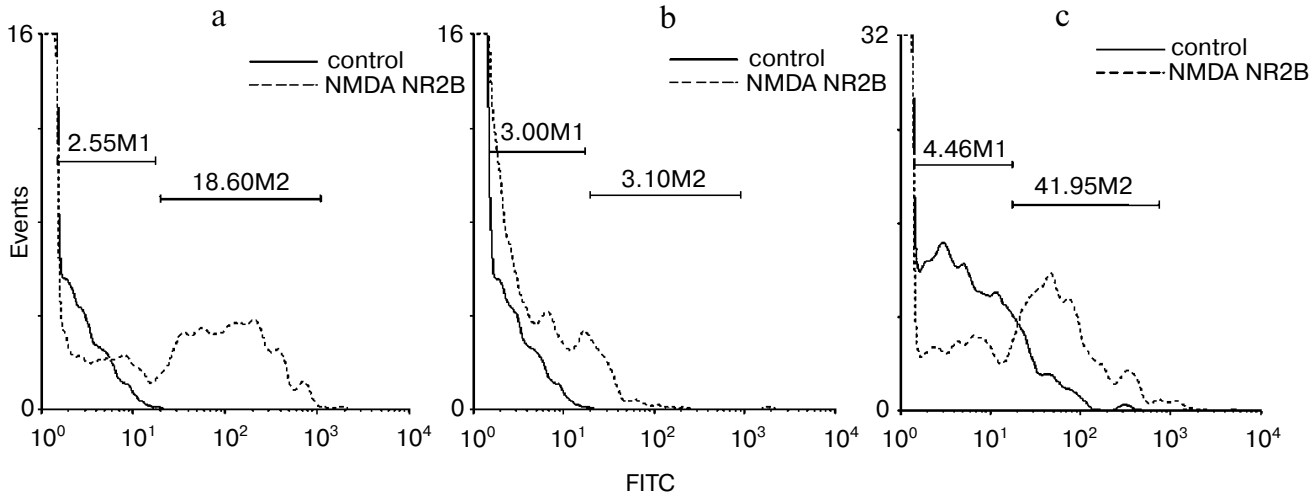

Fig. 3. Immunocytochemical staining of *in vivo*-activated (a) and intact (b) neutrophils by FITC-labeled antibodies against NR2B subunit of NMDA receptor; c) positive control (staining of neuronal cells by the same FITC-labeled antibodies). Y-axes, intensity of fluorescence of FITC-labeled antibodies bound to receptors. Numbers designate mean fluorescence intensity in the areas marked with markers M1 (fluorescence of control samples) and M2 (fluorescence of samples stained with FITC-labeled antibodies).

els of ROS generated (Fig. 2). At the same time, intact cells isolated from peripheral blood are not affected by HC.

In the presence of 500 μM NMDA, *in vivo*-activated neutrophils enhance ROS production, and this effect is prevented in the presence of the specific antagonist of NMDA receptors MK-801. Figure 2 shows that the effect of HC also depends on the presence of MK-801, which diminishes ROS generation by neutrophils preincubated with HC, whereas ROS levels in the samples preincubated with MK-801 alone do not differ from control.

These results suggest the expression of NMDA receptors on the neutrophil membrane, which had not been published previously. To check this suggestion, we attempted to establish the presence of these receptors in neutrophils. For identification of NMDA receptors we incubated intact and *in vivo*-activated cells with antibod-

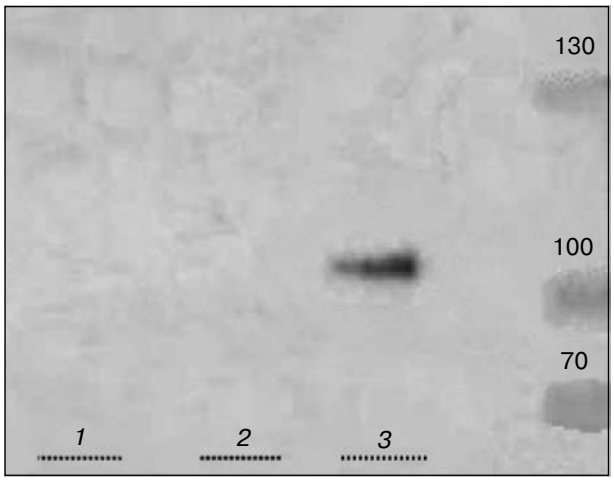

Fig. 4. Identification of NMDA receptors in *in vivo*-activated rat neutrophils: *1*) cell lysate; *2*) control for nonspecific binding of Protein A-Sepharose with lysate components; *3*) immune precipitation of lysate components with antibodies against NR1 subunit of NMDA receptor. Right lane, markers with known molecular mass.

ies against the extracellular site of the NR2B subunit of the NMDA receptor with following immune precipitation as described in "Materials and Methods". A suspension of cerebellum granule cells was used as a positive control.

Figure 3 shows that when cerebellum neurons were treated with antibodies against the NR2B subunit of the NMDA receptor, the FITC-labeled population of cells on the histogram shifts to the right compared to the control, thus demonstrating that these cells possess a target for antibodies against NMDA receptors (positive control). A similar shift is seen when *in vivo*-activated neutrophils (not intact) underwent such treatment. In other words, activation of neutrophils *in vivo* is coupled with appearance of cells expressing NMDA receptors.

To confirm this conclusion, we identified NMDA receptors in activated neutrophils using the immune precipitating procedure. Figure 4 shows the result of interaction of cell lysate with specific antibodies against the NR1 subunit of NMDA receptors with subsequent identification of the resulting spot. After staining of cell lysate samples using antibodies against NR1 subunit of NMDA receptor, a protein is revealed (Fig. 4) with molecular mass within the range (100-110 kDa) corresponding to the molecular mass of NR1 known from the literature [19]. Samples not subjected to preliminary immunoprecipitation (Fig. 4, lane *1*) did not demonstrate such staining.

Thus, we conclude that *in vivo* activation of rat neutrophils is coupled with expression of NMDA receptors, and they possess both subunits important for rigorous functioning of NMDA receptors [20].

DISCUSSION

Increase in blood levels of HC is known to correlate with various cardiovascular and neurodegenerative diseases [1, 2, 21]. Considerable neurotoxic effect of HC is also described [22]. Moreover, an ability of HC to affect immune competent system is also known: HC enhances zymosan-induced activation of polymorphonuclear cells, inhibits chemotaxis, suppresses phospholipid methylation and protein carboxylation in neutrophils after fMLP activation, and stimulates assembling and activation of NADPH oxidase in neutrophils and monocytes [23, 24]. In our work, we have shown that HC does not affect the function of intact cells but stimulates ROS generation (Fig. 2) and degranulation in *in vivo*-activated neutrophils isolated from inflammatory area (Fig. 1). These facts indicate that HC is able to modify neutrophil function by stimulating their cytotoxicity.

It is known from the literature that the action of HC on neurons and lymphocytes may be mediated both by metabotropic and ionotropic glutamate receptors [27-29], the ionotropic receptors of the NMDA class immediately participating in regulation of cytokine production [28].

In our work we have shown that HC-induced stimulation of ROS production is suppressed in the presence of antagonist of NMDA receptors MK-801. In terms of these data and evidence that HC may activate NMDA receptors in neurons and lymphocytes [8, 27, 29], we suggested that NMDA receptors can be expressed on the *in vivo*-activated neutrophil membrane.

After immune precipitation of cell lysates from activated neutrophils with antibodies against NR1 subunits of NMDA receptors, we identified a protein whose molecular mass corresponds to that of NR1 protein. Moreover, we performed immunocytochemical staining of neutrophils with antibodies against extracellular site of the NR2B subunit, thus demonstrating its presence in the studied cells. The presence in membrane of neutrophils of both subunits of the receptors, which are necessary for activity of NMDA receptor-operated ionic channel, along with the data on suppression of ROS generation by NMDA receptor antagonist MK-801, indicates that in these cells functionally active NMDA receptors are expressed. Staining of activated neutrophils by fluorescent antibodies against both subunits of the receptor suggest that they are expressed on neutrophil membrane after cell activation. Moreover, positive reaction of cells to antibodies against extracellular site of the regulatory subunit shows that these receptors are not only synthesized *de novo* but also inserted into the cell membrane. Sensitivity of HC effect on ROS generation to MK-801 demonstrates that these receptors are functionally active.

Thus, one of the consequences of *in vivo* activation of neutrophils is expression of NMDA receptors, which may be the target for natural regulatory ligands, and HC

is one of these. It is necessary to note that several signaling cascades function in cells either sequentially or in parallel to each other depending on the type of receptors involved as well as on the functional state of the cells.

It is well known that activation receptors of the complement system and/or Fc receptors of neutrophils results in activation of phospholipase A2, whose operation is mediated by arachidonic acid [30]. For activation phospholipase A2 has to be phosphorylated at Ser505 by MAP kinases, including Erk1/2 – an enzyme that plays a key role in assembly of NADPH oxidase complex involved in respiratory burst [30, 31]. Activation of enzymes providing respiratory burst critically depends on intracellular Ca^{2+}. Degranulation is regulated by adenosine receptors of neutrophils. Their activation directly depends on the MAPK cascade, some of them being related to increase in intracellular cAMP concentration [32].

Expression of another class of receptors (NMDA receptors) regulating neutrophil activity after their *in vivo* activation reveals cytotoxic effect of HC, which results in ROS accumulation because of possible stable activation of these receptors controlling this process. Recently, the ability of HC to stimulate ROS accumulation by peripheral blood lymphocytes was found [27, 28]. All these facts may explain the extra activation of immune competent cells under hyperhomocysteinemia.

The study was supported by RFBR grants (Nos. 09-04-00507 and 10-04-01461).

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