Effect of Homocysteine and Homocysteic Acid on Glutamate Receptors on Rat Lymphocytes

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Homocysteine and homocysteic acid increased the stationary level of reactive oxygen species in rat lymphocytes, homocysteic acid being more potent in this respect. The effect of this compound was realized via ionotropic NMDA receptors and group III metabotropic glutamate receptors. Incubation of lymphocytes with homocysteic acid increased intracellular Ca^{2+} concentration, activated of protein kinase C, and induced accumulation of reactive oxygen species, which reflected the involvement of homocysteic acid into cell signaling mechanisms.

Key Words: *glutamate receptors; reactive oxygen species; lymphocytes; homocysteic acid; homocysteine*

Various neurodegenerative and cardiovascular diseases are associated with increased blood concentrations of homocysteine (HC) and homocysteic acid (HCA). Blood HC concentration in healthy humans does not exceed 14 μM [13]. Disturbances in HC metabolism are followed by an increase in its concentration to 50-200 μM or higher [9]. Hyperhomocysteinemia increases the risk of atherosclerosis and myocardial infarction [11] and is a potent independent factor in the development of dementia and Alzheimer's disease [13].

Under conditions of hyperhomocysteinemia the cells are exposed to neurotoxic influences. Disturbed metabolism of excitatory neurotransmitters (*e.g.*, glutamate) causes the excitotoxic effects associated with increased intracellular level of reactive oxygen species (ROS) and oxidative damage to tissues. It was hypothesized that the excitotoxic effect of HC on neurons is realized via NMDA receptors [8]. Group I metabotropic receptor antagonist LY367385 and NMDA receptor antagonist MK-801 (trans-S-methyl-10,11-dihydro-5N-benzo[a,d]cyclopentene-5,10-imine maleate) partially prevent the toxic effect of HC. Combined treatment with these antagonists protects the neurons from HC [15]. Therefore, the neurotoxic effect of HC is realized via both ionotropic and metabotropic glutamate receptors.

HC can affect not only neurons, but also other cells expressing NMDA receptors. Incubation of cultured smooth muscle cells with HC was accompanied by an increase in the expression of interleukin-1β and other factors inducing cell proliferation. HC decreased the content of oxygen radicals in these cells. The effects of HC were blocked by NMDA receptor antagonist MK-801 [11].

At the same time, HC is a weak neurotoxin. The effects of this compound are observed only at a concentration >1 mM, *i.e.* the neurotoxic dose of HC 2-fold exceeds that of glutamate [8]. Various HC metabolites, including the oxidation product L-HCA, exhibit properties of endogenous exotoxins [15]. It should be emphasized that HCA produces a more potent toxic effect on neurons than HC [5].

NMDA receptors are expressed on immune cells. Long-term incubation of lymphocytes with NMDA and HCA increases ROS content in these cells and

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their death [1,2]. Published data show that lymphocytes express various subtypes of glutamate receptors [4,10]. It is important to compare the effects of HC and HCA on lymphocytes.

The present study shows that incubation of rat lymphocytes with HCA induces a more pronounced increase in intracellular ROS content compared to NMDA and HC. It should be noted that the effects of HCA are realized via glutamate receptors, are accompanied by an increase in intracellular Ca^{2+} concentration, and involve protein kinase C.

MATERIALS AND METHODS

Experiments were performed on outbred albino rats aging 9-12 days. The blood was taken from 5-6 animals and put in Hanks medium (1.26 mM $CaCl_2 \times 2H_2O$, 0.44 mM KH_2PO_4, 136.7 mM NaCl, 4.16 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, 0.2 mM $MgSO_4 \times 7H_2O$, 5.37 mM KCl, and 5.56 mM D-glucose) containing heparin (20 U/ml). The blood was layered on Lymphosep medium (ICN Biomedicals) and centrifuged at 400*g* and 18-20°C for 30 min. The lymphocyte ring was collected and used for further studies. The cells were counted in a Goryaev chamber. ROS content was measured using a fluorescent probe $DCFH_2$-DA (2',7-dichlorofluorescin diacetate, Sigma). This probe accumulating in cells is converted into DCF (2',7-dichlorofluorescin) interacting with H_2O_2 (amount of H_2O_2 is proportional to ROS content). $DCFH_2$-DA in a final concentration of 100 μM was added to the cell suspension. The mixture was incubated in darkness at 37°C for 45 min. The cell count was adjusted to a probe concentration of 20-25 nmol per million cells.

Cytoplasmic Ca^{2+} concentration was measured using an intracellular fluorescent probe Fluo-3 AM [6]. The cells were loaded with Fluo-3 AM under similar conditions. The final concentration of Fluo-3 AM was 20 μM (4-5 nmol Fluo-3 AM per million cells). Propidium iodide (Sigma) in a final concentration of 10 μM was added the cell suspension to estimate the ratio of dead cells. The measurements were performed on a FacStar flow cytometer (Becton Dikinson). The sample volume was 0.6 ml (~50,000 cells per sample). The measurements were performed over 1.5-2.0 h (cells labeled with propidium iodide did not appear in the suspension within this period). Lymphocytes were preincubated with glutamate receptors agonists at 37°C.

We used HCA (100-1000 μM, Sigma), ionotropic NMDA receptor antagonist MK-801 (10 μM, RBI), and group III metabotropic glutamate receptor antagonist MSOP (25 μM, Tocris). The concentrations of antagonists were chosen on the basis of their K_D. The above antagonists in specified concentrations had no effect on the stationary level of probe fluorescence in lymphocytes. We also use A_{23187} (10 μM, Sigma), chelerythrine (1 μM, Alomon Labs), NMDA (100-1000 μM, Sigma), and HC (100-1000 μM, Sigma). Control samples were incubated over the same period of time. The measurements with each sample were performed in at least 3 repetitions. The results were analyzed by means of WINMDI software and processed statistically using Biostatistika software.

RESULTS

Incubation of lymphocytes with HCA was followed by an increase in fluorescence of DCF, which serves as an intracellular marker of free radicals [6]. Figure 1 illustrates results of a representative experiment. Incubation of cells with 250 μM HCA for 15 min led to a shift in DCF fluorescence, which reflects the increase in intracellular ROS level. When comparing the concentration dependencies of the effect of HC, HCA, and NMDA on stationary ROS content (Table 1) we found that HC and NMDA produce similar effects, while HCA was most potent in inducing ROS accumulation in lymphocytes.

Activation of glutamate receptors in neurons promotes ROS accumulation due to a transient increase in cytoplasmic Ca^{2+} concentration [14]. Similar changes were observed during incubation of lymphocytes with HCA (fluorescence of DCF and Fluo-3 AM was recorded in parallel samples under similar conditions). Incubation of lymphocytes with HCA increased in intracellular Ca^{2+} concentration (peaked after 5-10 min, Fig. 2, *a*) and then ROS content (similarly to neuronal cells).

DCF fluorescence increased most significantly after 15 min (Fig. 2, *b*).

For identification of receptors mediating the effect of HCA we evaluated the sensitivity of lymphocytes to this ligand in the presence of antagonists of various types of these receptors. Previous studies showed that the lymphocyte membrane in rodents expresses ionotropic NMDA receptors [3] and group III metabotropic receptors [3,11]. We used NMDA receptor antagonist MK-801 (10 μM) and group III metabotropic receptor antagonist MSOP (25 μM). After 15-min incubation of cells with HCA (0.25 mM), HCA+MSOP, and HCA+MK-801, DCF fluorescence was 152±8, 116±6, and 114±7% of the control, respectively. DCF fluorescence induced by incubation of lymphocytes with HCA was suppressed by both antagonists, *i.e.* the effects of HCA are mediated by both classes of glutamate receptors.

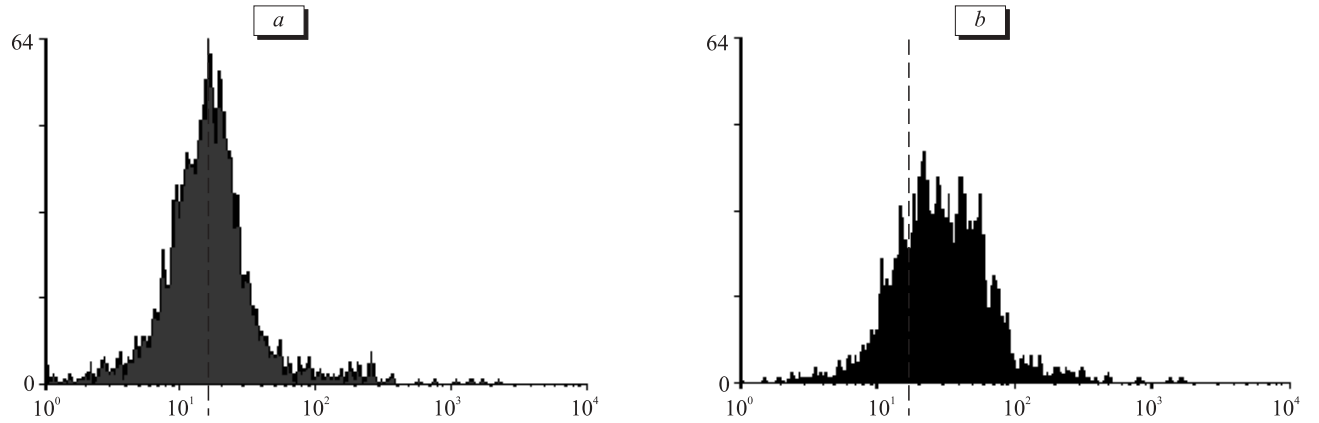

Fig. 1. DCF fluorescence in lymphocytes from 12-day-old rats under control conditions (*a*) and after 15-min incubation with 0.25 mM homocysteic acid (HCA, *b*). Mean fluorescence: 18 (*a*) and 30.5 rel. units (*b*).

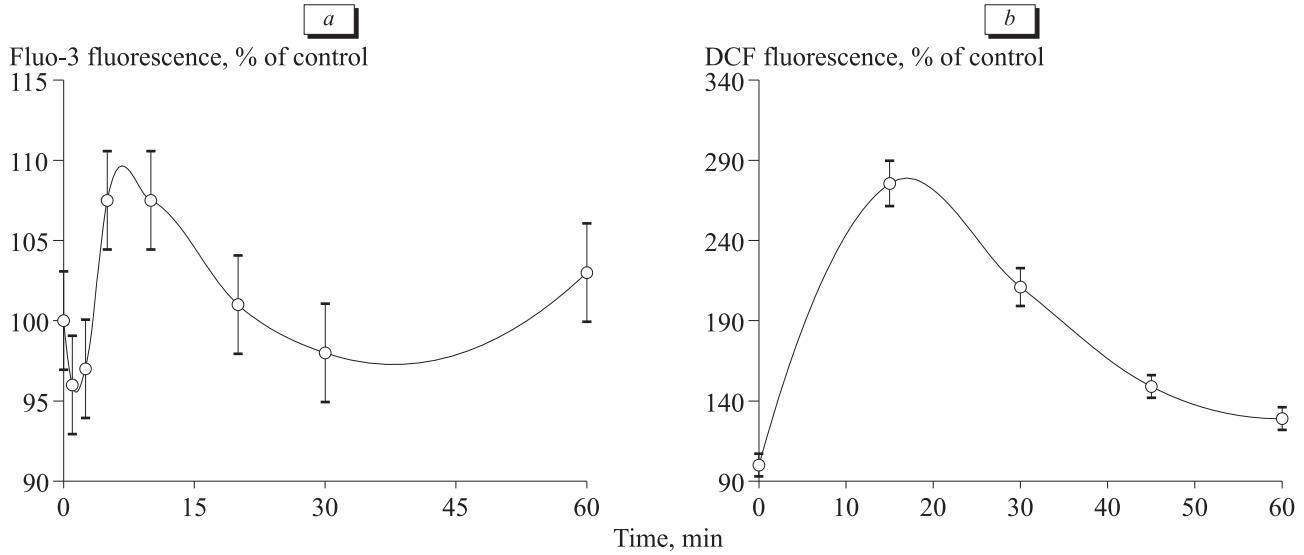

Fig. 2. Time dependence for the effect of HCA in a concentration of 0.25 mM on stationary level of Ca^{2+} (*a*) and ROS in rat lymphocytes (*b*). Incubation at 37°C.

NADPH oxidase is the major source of ROS in immunocompetent cells. Activation of NADPH oxidase is realized via protein kinase C [7]. We studied the role of this enzyme in processes occurring during activation of lymphocytes with HCA. Comparative study was performed to evaluate the effects of HCA and phorbol myristate acetate (PMA) on ROS production by lymphocytes. We also studied the influence of chelerythrine on this process. PMA increased the stationary level of ROS in lymphocytes during 5-15-min incubation. Further incubation was accompanied by a decrease in ROS level. The same time dependence was found during incubation of lymphocytes with HCA (Fig. 2, *b*). PMA in a concentration of 10^{-7} M most significantly modulated free radical production by lymphocytes. Chelerythrine abolished the increase in DCF fluorescence induced by PMA or HCA in optimal concentrations. These data show that protein kinase C plays a role in the effect of HCA (Fig. 3).

TABLE 1. Concentration Dependence for the Effects of NMDA, HC, and HCA on ROS Level Estimated by DCF Fluorescence in Rat Lymphocytes

Substance concentration, mM	NMDA	HC	HCA
0	100	100	100
0.1	9.1	100.7	170
0.25	103.6	118.9	201.7
0.5	120	123	158.9
0.75	123.8	118.9	153.6
1.0	122.7	120	138.8

Note. Incubation at 37°C for 30 min.

Our results indicate that HC and HCA produce a strong effect on lymphocytes and increase intra-

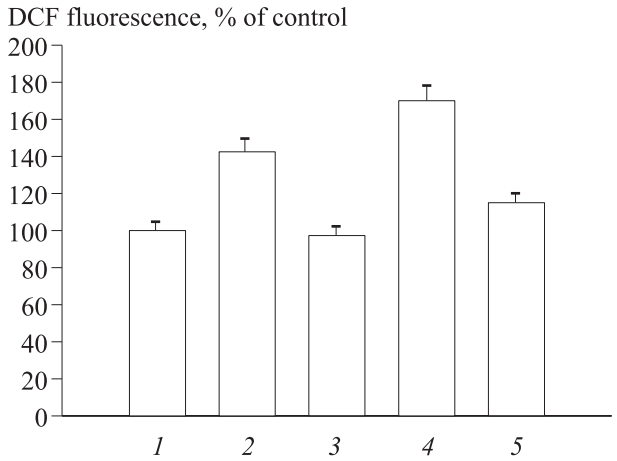

Fig. 3. Effects of HCA and protein kinase C activator phorbol myristate acetate (PMA) on DCF fluorescence in the presence or absence of chelerythrine. HCA, 0.25 mM; PMA, 10^{-7} M; chelerythrine, 1 μM. Incubation at 37°C for 15 min.

cellular Ca^{2+} concentration and ROS level. Similar results were obtained in experiments with neurons. The effect of the test ligands is realized via ionotropic NMDA receptor and group III metabotropic glutamate receptors. ROS level significantly increases even after short-term incubation of lymphocytes with 250 μM HCA (Table 1). This dose of HCA corresponds to the concentration of HC in the blood of patients with severe hyperhomocysteinemia. The increase in free radical concentration in lymphocytes serves as a signal mechanism for activation of proliferation and cytokine production [3]. It can be hypothesized that permanent increase in the concentrations of HC and its oxidation product (HCA) provides conditions for oxidative stress and stimulation of immune cells. Our previous studies showed that long-term incubation of lymphocytes with HCA *in vitro* causes massive cell death [1]. We conclude that glutamate receptors on immune cells serve as the target for the effect of HC and HCA during hyperhomocysteinemia.

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