BIOPHYSICS AND BIOCHEMISTRY

Homocysteinic Acid Causes Oxidative Stress in Lymphocytes by Potentiating Toxic Effect of NMDA

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Short-term incubation of lymphocytes with homocysteine or its oxidation product homocysteinic acid increased the formation of reactive oxygen species and cell necrosis (in case of homocysteinic acid). Effective concentration of homocysteine and homocysteinic acid (500 μM) significantly surpassed the level observed during hyperhomocysteinemia. The addition of homocysteinic acid in a nontoxic concentration of 100 μM potentiated the toxic effect of NMDA and led to massive cell death. During hyperhomocysteinemia the amount of these metabolites in the blood was much higher than in the brain. Oxidative stress produced by these substances can result from activation of NMDA glutamate receptors that were recently detected on lymphocytes.

Key Words: *lymphocytes; oxidative stress; homocysteinic acid; glutamate receptors; NMDA*

Hyperhomocysteinemia often accompanies vascular disorders and serves as a risk factor for cardiac, vascular, and renal pathologies. The upper boundary of normal for plasma homocysteine (HC) in humans is below 10 μM. However, metabolic disturbances are accompanied by the increase in plasma HC concentration more than by 5-10 times [11]. HC produces toxic effect on metabolism under conditions of moderate homocysteinemia. Cognitive disorders are revealed at a plasma HC concentration of 15-50 μM, while in a concentration of 200 μM or higher HC leads to neuronal dysfunction and psychosis [7].

HC is synthesized from S-adenosyl-cysteine, which in turn is formed from S-adenosyl-methionine, the source of methyl groups for methylation of nucleic acids, histones, phosphatidylcholine, and neuromediators. S-Adenosyl-homocysteine can suppress consumption of S-adenosyl-methionine during methylation [13]. Deficiency of cystathionine-β synthase responsible for conversion of HC into cystathionine is the major cause of HC accumulation in tissues. This disorder has a variety of complications, including renal failure [11].

Transformation of HC into homocysteinic acid (HCA) is accompanied by the formation of reactive oxygen species (ROS). It is associated with the ability of HC to cause oxidative stress that impairs function of brain neurons [10]. Intracerebroventricular administration of HC potentiates the excitotoxic effect of N-methyl-D-aspartate (NMDA). This antagonist of glutamate receptors is involved in the formation of long-term memory. These changes are followed by oxidative damage and death of neurons [15]. However, the toxic effect is observed only upon exposure to HC in high concentrations ($K_{0/5}$=17.4 mM). This high level of HC cannot be achieved in the brain even during hyperhomocysteinemia. HC in high concentrations (≥1 mM) is toxic for splenocytes. HC in this concentration increases ROS concentration and stimulates proliferation of splenocytes, which is mediated by protein kinase C, p38 MAP kinase, and other ROS-dependent

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compounds [14]. These changes reflect toxicity of HC, but do not explain its toxic effect on the organism.

Recent studies revealed glutamate receptors on lymphocyte membrane [1,5]. Activation of these receptors with NMDA increased Ca^{2+} concentration, stimulated generation of free radicals, and activates caspase-3. Therefore, these receptors play a role in the regulation of cell function. In the present work we showed that activation of lymphocytes with NMDA in the presence of HC and particularly HCA provides conditions for oxidative stress and causes cell death. These changes are observed upon treatment with ligands in physiological concentrations.

MATERIALS AND METHODS

Lymphocytes were routinely isolated from the blood of 10-15-day-old ICR mice [5,9]. The cells were incubated in Tyrode medium (Sigma) at 37°C. Before the experiment lymphocytes were loaded with CDCFDA (Molec. Probes) for 45 min. This dye serves as a source of CDCF (fluorescent trap of free radicals) [5]. The cells were incubated with NMDA, HC, or HCA (in combination or individually) for 30-120 min. Propidium iodide (PI, Sigma) in a concentration of 10 μM was added 5 min before recording to estimate the count of necrotic cells. NMDA, HC, and HCA were obtained from Sigma. Fluorescence of CDCF and PI was measured on a flow cytometer (Beckman Coulter, Epics Altra). Each series was performed at least in 3 repetitions. Experiments were conducted on animals from various families. Each measurement was performed 3 times. The results were statistically analyzed by means of Biostatistika software.

RESULTS

Incubation of lymphocytes with 0.5 mM NMDA, HC, or HCA for 30 min increased fluorescence of CDCF (intracellular marker of ROS) [5]. NMDA and HC were equally potent in this respect. It should be emphasized that HC concentration surpassed the upper limit of its plasma level during hyperhomocysteinemia. Measuring of PI fluorescence showed that NMDA (Fig. 1, *b*) and HC (Fig. 1, *c*) do not cause cell death.

Treatment with HCA in the same concentration more significantly increased CDCF fluorescence and was followed by the appearance of PI-labeled cells (Fig. 1, *d*). PI is a marker of nucleic acids, which cannot cross intact cell membrane. Hence, the presence of cell stained with this agent reflects serious and fatal damage to the membrane. Therefore, the zone of high PI fluorescence included necrotic cells (Fig. 1, *d*).

HC and HCA produced a dose-dependent effect on intracellular ROS concentration (Table 1) and ratio of PI-labeled necrotic cells (Table 2). HC and HCA in concentrations of 500 and 250 μM, respectively, in-

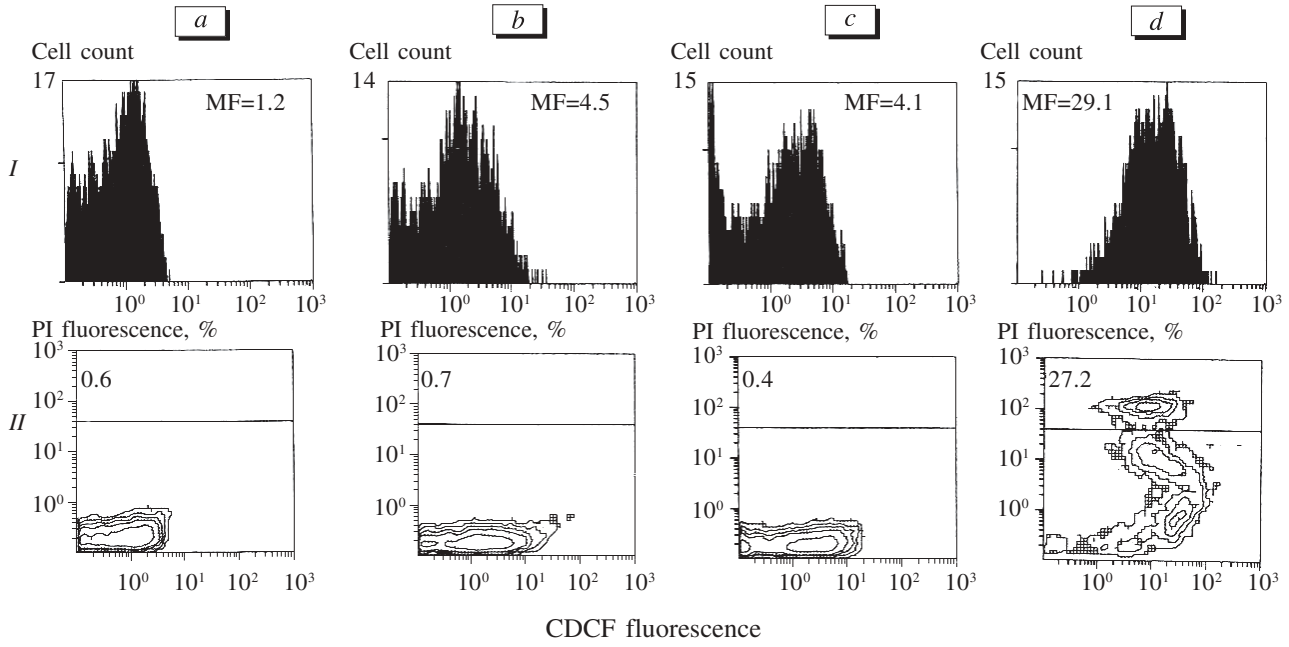

Fig. 1. Activity of mouse lymphocytes after incubation with NMDA, homocysteine (HC), or homocysteinic acid (HCA). Ligand concentration, 0.5 mM. Incubation at 37°C for 30 min. Logarithmic coordinates of CDCF concentration and cell count (*I*); distribution of cells by the ability to interact with PI (ordinate) or CDCF (abscissa, *II*). Control (*a*), NMDA (*b*), HC (*c*), and HCA (*d*). Shift of the curve along the abscissa corresponds to the increase in intracellular ROS concentration (*I*; MF, mean value of CDCF fluorescence, arb. units). Displacement of the cell population along the ordinate reflects the appearance of PI-labeled necrotic cells (*II*, percentage of dead cells in the left upper corner of quadrant).

TABLE 1. Concentration Dependence for the Effects of HC and HCA on Intracellular ROS Concentration

Concentration, mM	CDCF fluorescence, arb. units	
	HC	HCA
0.05	5	4
0.10	4	3
0.25	7	39
0.50	41	103
1.00	109	88
2.5	122	119

TABLE 2. Effect of HC and HCA in Various Concentrations on the Ratio of PI-Labeled Necrotic Cells (%)

Concentration, mM	HC	HCA
Without additive	1	3
0.75	3	8
1	4	23

creased ROS concentration in lymphocytes. The maximum increase in ROS concentration was similar for these compounds, but HC did not cause cell death, while HCA in concentrations >250 μM significantly increased the count of necrotic cells (Table 2). Therefore, the increase in ROS concentration is not sufficient for inducing cell death. This process occurs in the presence of HCA.

The concentration of free radicals and count of dead cells were estimated in the presence of NMDA and HCA. HCA in a nontoxic concentration of 100 μM potentiated the increase in ROS concentration observed upon treatment with NMDA in low dose (Fig. 2, *c*). The histogram included cells with high (right peak, Fig. 2, *d*) or low sensitivity to HCA (left peak, Fig. 2, *d*). Individual or combined treatment with these ligands did not cause cell death. Selectivity of HCA reflects the specific effect mediated by structures not present in HCA-insensitive cells (left peak, Fig. 2, *d*).

NMDA and HCA in high concentrations more significantly increased intracellular ROS concentration (Fig. 3). These changes were accompanied by massive necrosis. More than two thirds cells died after 30-min incubation. We analyzed the distribution of cells by size in coordinates cell count—forward scattering (FS). HCA split the population of cells by size. The left peak corresponded to relatively small cells (Fig. 3, *d*, II). The right peak corresponded to large and swollen cells [8]. NMDA and HC were not capable of producing these changes in the size of cells. HC in a concentration below 1 mM did not increase the number of dead cells (even in the presence of NMDA in equimolar concentration).

HC-produced oxidative stress was accompanied by cell damage. Incubation of lymphocytes with HC

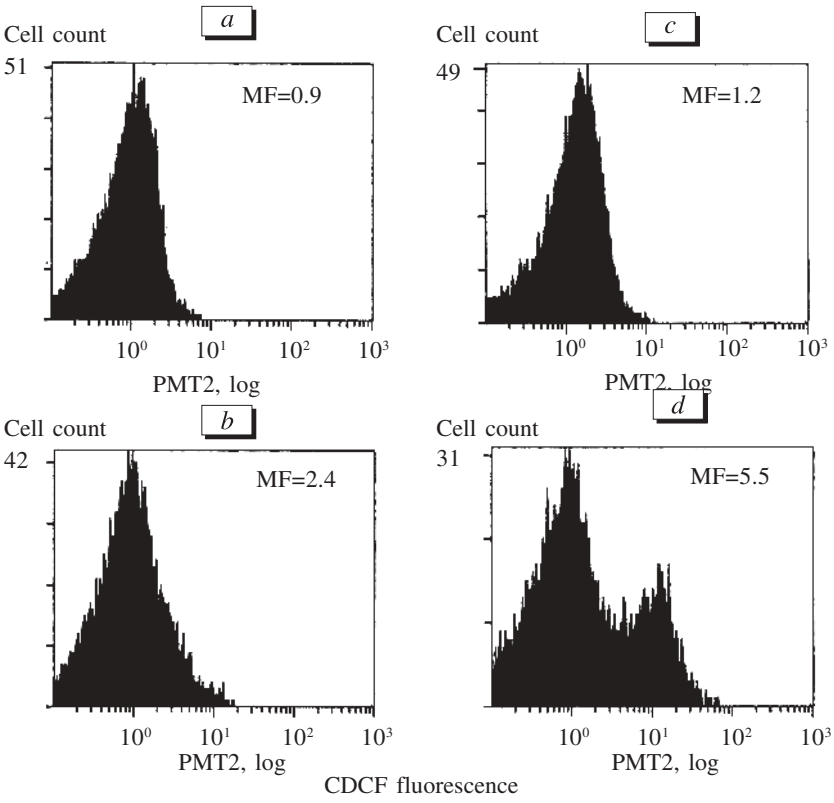

Fig. 2. Distribution of cells in coordinates cell count — CDCF fluorescence: control (*a*); 30-min incubation of lymphocytes with 0.25 mM NMDA (*b*), 0.1 mM HCA (*c*), or both compounds (*c*). The mean value of CDCF fluorescence is shown in each quadrant (MF, arb. units).

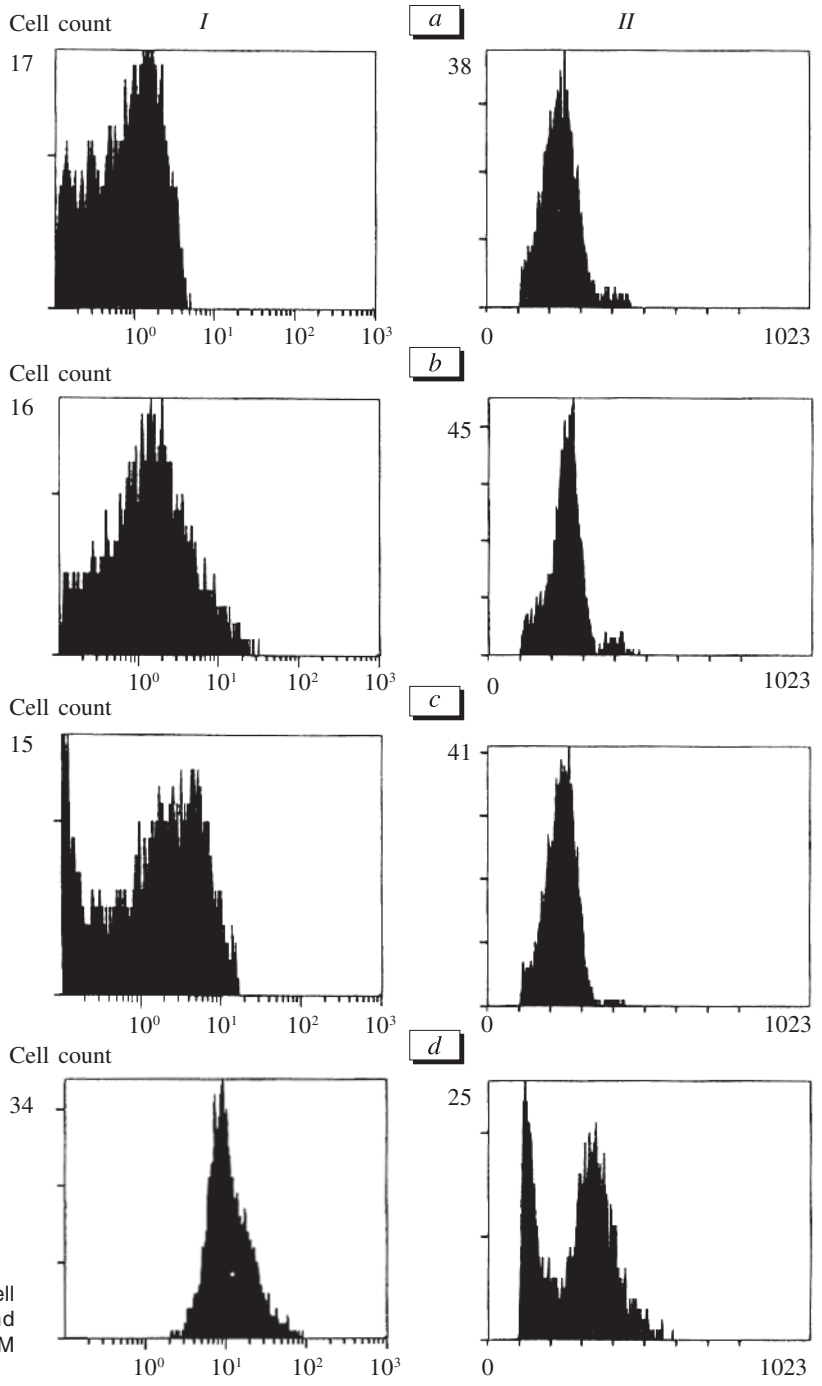

Fig. 3. Distribution of lymphocytes in coordinates cell count — CDCF fluorescence (*I*) or cell count and forward scattering (*II*): control (*a*); presence of 0.25 mM NMDA (*b*), 0.1 mM HCA (*c*), or both compounds (*c*).

in concentrations of 50-400 μM for 24-72 h was followed by progressive accumulation of genetic defects and cell necrosis [12]. This delayed effect is probably associated with HC metabolism and formation of secondary products. HC does not cause cell death. Its metabolite (HCA) exhibits higher toxicity and causes not only ROS generation, but also cell death. Similar results were obtained in studies of spontaneous electric activity of cultured nerve cells [7,11].

The mechanism of HCA toxicity remains unclear. In most experiments the concentrations of HC and HCA surpassed tissue concentrations of these substances during hyperhomocysteinemia [14]. Experiments on mice with a genetic defect in cystathionine-β synthase showed that the increase in the concentration of HC in the plasma and brain results in activation of stress-activated protein kinase and Jun-activated N-terminal kinase (SAPK/JANK) and stimulation of the JNK-c-Jun cascade. These changes are accompanied by impairment of calcium homeostasis and increase in ROS concentration [13]. Toxicity of HC increases with lengthening of the incubation per-

iod. These data indicate that HC metabolites exhibit higher toxicity. This conclusion is supported by pub lished data [7] and comparison of the effects produced by HC and HCA (Fig. 1).

Toxicity of HCA is related not only to ROS generation. Our experiments showed that HCA causes cell death, while HC does not produce this effect (even at the same level of free radicals, Tables 1 and 2). It is necessary to explain the differences in toxicity of HC and HCA. Otherwise, an attempt to decrease the severity of complications during hyperhomocysteinemia will fail.

Recent experiments revealed expression of glutamate receptors on lymphocyte membrane. Mouse lymphocytes express ionotropic NMDA receptors and group III metabotropic receptors (mGluRIII) [5]. mGluRIII can increase toxicity of NMDA receptors [3,5,10]. Moreover, HAC serves as an agonist of mGluRIII [6]. It can be hypothesized that this effect contributes to the increased toxicity of HCA for cells.

HCA is a normal metabolite formed in nervous tissue. Published data show that glutamate induces the release of HCA from astrocytes. This compound is involved in intercellular signal transduction [2]. The mechanism for this process includes a specific increase in ROS concentration that serve as secondary messenger in neurons [4]. Toxicity of HCA for neurons manifested after local changes in the interaction between ionotropic (NMDA-activated) and metabotropic receptors on neuronal cells.

We showed that circulating lymphocytes are involved in the realization of HCA toxicity by the same mechanism. They increase toxicity of NMDA receptors due to their activation with mGluRIII. It is observed in the presence of HCA. Taking into account these data, it is necessary to evaluate the ability of mGluRIII antagonists to decrease the risk of hyperhomocysteinemia-associated diseases.

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