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REVIEW

# Molecular Mechanisms of Homocysteine Toxicity

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**Abstract**—Hyperhomocysteinemia is a risk factor for a number of cardiovascular and neurodegenerative processes as well as a complicating factor in normal pregnancy. Toxic effects of homocysteine and the product of its spontaneous oxidation, homocysteic acid, are based on their ability to activate NMDA receptors, increasing intracellular levels of ionized calcium and reactive oxygen species. Even a short-term exposure of cells to homocysteic acid at concentrations characteristic of hyperhomocysteinemia induces their apoptotic transformation. The discovery of NMDA receptors both in neuronal tissue and in several other tissues and organs (including immunocompetent cells) makes them a target for toxic action of homocysteine. The neuropeptide carnosine was found to protect the organism from homocysteine toxicity. Treatment of pregnant rats with carnosine under conditions of alimentary hyperhomocysteinemia increases viability and functional activity of their progeny.

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The biological role of homocysteine (HC) has been investigated since 1932 when Vincent du Vigneaud described this compound as a product of methionine demethylation. Homocysteine is a sulfur-containing amino acid connecting metabolism of methionine and cysteine (as well as glutathione). Dietary methionine is transformed in the organism into S-adenosylhomocysteine and further into HC, which can be utilized in two different ways: methionine synthase transforms it to methionine and cystathionine-β-synthase – to cysteine.

Vitamin $B_{12}$ and 5-methyltetrahydrofolate (THF) (produced from folic acid by 5,10-methylene tetrahydrofolate reductase) are cofactors of methionine reductase, whose deficiency stimulates transformation of HC by cystathionine-β-synthase in the presence of vitamin $B_6$. During this transformation, HC is irreversibly transformed into cystathionine and into cysteine (this reaction is catalyzed by cystathionase). All these reactions utilizing HC support low levels of this compound in the bloodstream. Suppression of any of these reactions results in a pronounced increase in blood HC levels, which is aggravated by a low rate of HC filtration by the kidneys [1]. The general scheme of HC transformation is presented in Fig. 1.

## HOMOCYSTEINE AS A RISK FACTOR OF CARDIOVASCULAR AND NEURODEGENERATIVE PATHOLOGIES

Total HC in blood of healthy donors (termed "total homocysteine" includes both free HC and HC bound to cysteine and proteins or an oxidized form [2]) is 5-10 μmol/liter; it increases slightly with age. Before puberty, the HC levels in blood of boys and girls are similar and roughly equal to 5 μmol/liter. Later it increases to 6-7 μmol/liter, being somehow higher in boys than in girls. Adult patients usually have 10-12 μmol HC per liter (in men slightly higher than in women) [3]. Increase in HC levels with age is explained by defects of kidney function and higher HC levels in men by a feature of hormonal metabolism.

Homocysteine easily participates in redox reactions, and its spontaneous oxidation results in accumulation of homocysteic acid (HCA) [4]; in the bloodstream, it is found either in oxidized form or in a bound state with proteins or cysteine (70%).

*Abbreviations*: DCF, 2′,7′-dichlorodihydrofluorescein; fMLP, fMet-Leu-Phe; HC, homocysteine; HCA, homocysteic acid; ROS, reactive oxygen species.

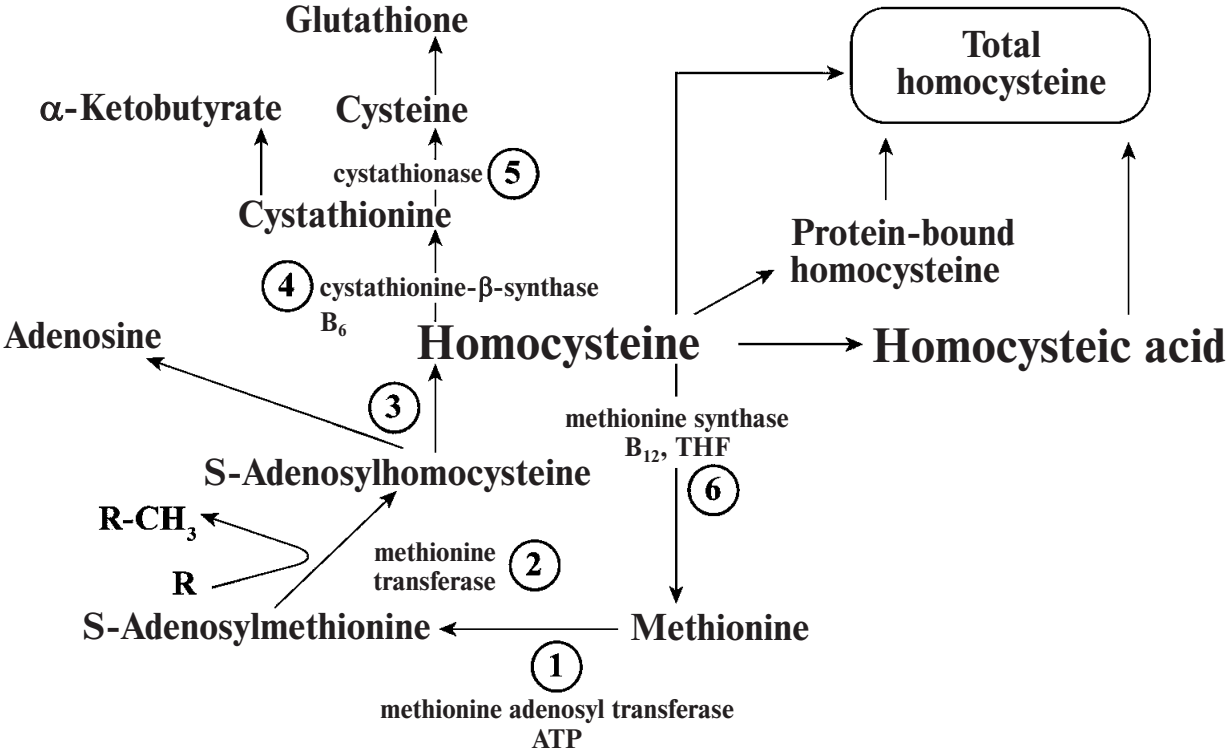


**Fig. 1.** Scheme of homocysteine metabolism in the body. HC is formed from methionine as a result of its transformation into S-adenosylmethionine (reaction 1) and S-adenosylhomocysteine (reaction 2). The latter supplies methyl groups for a diversity of metabolic processes, and S-adenosylhomocysteine accumulated is hydrolyzed to adenosine and homocysteine (reaction 3). Two main ways of HC utilization are represented by reactions 4 and 5 (transformation to cystathionine and then to cysteine) and by reaction 6 (transformation to methionine); these are under control of the enzymes whose activity depends on vitamins $B_6$ and $B_{12}$ and active form of folic acid (THF). Excess HC is accumulated in the blood as free molecule, as a complex with proteins or cysteine, or as a dimer (homocystine) as well as products of spontaneous oxidation of HC (preferably, homocysteic acid); all these forms are determined in clinical trials as "total HC".

Excess HC in the bloodstream is considered as risk factor for a number of pathologies. The term "hyperhomocysteinemia" is used when its levels exceed 15 μmol/liter. The concentration range of 15-30 μmol/liter indicates mild hyperhomocysteinemia, 30 to 100 μmol/liter – intermediate, and higher than 100 μmol/liter – severe hyperhomocysteinemia [4, 5]. Among the reasons for hyperhomocysteinemia are deficit of kidney function (either age-dependent or induced by pathologies) [1], deficit of vitamins taking part in HC metabolism [6, 7], or excess of dietary methionine. In some cases, decrease in blood HC can be achieved by diet normalization [8]. Caloric restriction is also supposed to be useful because one of the important consequences of energy limitation is decrease in availability of methionine [9].

Increased disposition to hyperhomocysteinemia is characteristic of smokers [10] and heavy coffee drinkers [11]. Small doses of alcohol suppress HC in blood, whereas large doses facilitate its accumulation.

As early as in 1962, homocysteinuria syndrome was described and related to deficit of cystathionine-β-synthase [12]. This was followed by cognitive deficit, progressive cardiovascular diseases, and high frequency of thromboembolia. Later it was found that increased HC levels provoke atherosclerosis development and accelerate the atherogenic effect of cholesterol [12, 13]. Increased levels of HC in blood results in multiple damages to blood vessel walls and significantly elevated risk of thrombosis [14]. Thus, hyperhomocysteinemia is one of the pathogenic factors of atherosclerosis [15, 16]. It can also lead to heart attack [17] and brain stroke [18] as well as increase in diabetes complications [19] and stimulation of Alzheimer's disease [20]. Severe hyperhomocysteinemia results in convulsions and dementia [21, 22].

In the modern literature, hyperhomocysteinemia is considered as an important pathological factor [23-26], many authors suggest HC to be a risk factor of cardiovascular and neurodegenerative diseases [22, 27, 28], while the causes of its toxicity are not clear. The review is dedicated to analysis of molecular mechanisms of HC toxicity.

## NEUROTOXIC EFFECT OF HOMOCYSTEINE

Toxic effect of HC on cultured cerebellum cells was noted several years ago and has been addressed to activa-

**Table 1.** Effect of intracellular calcium buffer BAPTA on the levels of calcium ions and ROS in cerebellum neurons after their activation with NMDA, HC, or HCA (500 μM for 30 min)

| Parameter | Control | NMDA | HC | HCA |
|---|---|---|---|---|
| $Ca^{2+}$ level without additions | 41.5 ± 0.2 | 46.3 ± 0.1* | 58.2 ± 0.3* | 64.0 ± 0.3* |
| + 10 μM BAPTA | 41.0 ± 0.1 | 42.1 ± 0.1* | 47.2 ± 0.2* | 45.1 ± 0.3* |
| ROS level without additions | 48.0 ± 0.1 | 52.0 ± 0.1* | 52.3 ± 0.2* | 57.4 ± 0.2* |
| + 10 μM BAPTA | 48.0 ± 0.1 | 47.0 ± 0.2* | 48.1 ± 0.1* | 47.5 ± 0.3* |

Note: Data are expressed as arbitrary units (mean ± SE).
* Significant difference from control with $p < 0.05$.

tion of glutamate receptors [29-31]. The more expected target could be NMDA receptors, whose activation results in rise in calcium ion and reactive oxygen species (ROS) within neuronal cells [32]. ROS accumulation could be an immediate cause of toxic effect because incubation of neurons with superoxide dismutase or catalase efficiently suppressed the toxic effect of HC [29].

Homocysteine toxicity at short incubation times (1-3 h) and concentrations corresponding to evident hyperhomocysteinemia (200-300 μmol/liter) induced apoptosis as documented by externalization of phosphatidylserine on the outer space of the cell membrane, which is not accompanied by its disordering; this feature is considered as an early sign of the apoptotic process. Longer incubation time and two-to-three times increased HC concentration results in disordering and ruptures of neuronal membranes, which serves as a sign of necrotic cell death; such cells are labeled with propidium iodide, a marker for necrosis [32].

Use of specific inhibitors showed that the neurotoxic effect of HC is suppressed not only by MK-801, one of the antagonists of glutamate receptors of NMDA-class, but also by α-methylcarboxyphenyl glycine, an inhibitor of metabotropic glutamate receptors of group I, whose activation results in stimulation of phospholipase A2 and phosphatidylinositol-3-phosphate inducing calcium release from endoplasmic reticulum [33]. It was also found that another antagonist of metabotropic group I receptors, LY 367385, which can also partially induce toxic effect of HC when added simultaneously with MK-801, totally protected neurons from HC, whereas an agonist of metabotropic group I receptors, t-ADA, induced neuronal degeneration [34]. It was concluded that both ionotropic and metabotropic glutamate receptors are involved in neurotoxic effects of HC.

In spite of the described HC toxicity, it is suggested to be a relatively weak neurotoxin. Under *in vitro* conditions, its cytotoxic action results in necrotic death of the neurons only at concentrations above 1 mmol/liter [34]. This means that similar neurotoxic effect of HC appeared at 2-fold higher concentration than that of glutamate [29]. HCA is much more effective, and it was considered as an endogenous neurotoxin [34]; it was noted that HCA demonstrates more toxic effect on neurons than HC or glutamate [35, 36]. Neurotoxic concentrations of HCA are 0.3-0.5 mmol/liter, which is close to those in severe hyperhomocysteinemia.

Electrophysiological experiments also showed that sensitivity of neurons to HCA is sufficiently higher than to HC, thus HCA activates NMDA receptors at 50-100 μmol/liter concentrations (corresponding to modest hyperhomocysteinemia) [37].

Similarly to NMDA or HC, HCA induced calcium influx in neuronal cytoplasm and ROS accumulation, the latter being suppressed either by N-acetylcysteine (intracellular antioxidant) or by intracellular Ca-chelator BAPTA (Table 1). This shows that increase in intracellular calcium precedes the accumulation of ROS in the neurons. It is important to note that free radical signal induces rather quick (after 1-3 h) externalization of phosphatidylserine on the neuronal membrane [33, 37], which corresponds to initiation of apoptosis of neuronal cells by HCA.

It was demonstrated that intracisternal administration of either NMDA or HCA to the brain of young (2-week-old) rats induces long lasting convulsions accompanied by massive apoptotic processes in adjoining regions of the brain; epileptic attack induced by HCA is prevented by antagonists of NMDA receptors [38].

## DISCOVERY OF NMDA RECEPTORS IN NON-NEURONAL CELLS

It was demonstrated recently that glutamate receptors are found not exclusively in neuronal tissue. Metabotropic receptors that are associated with G-proteins are found in a large variety of tissues [39], and ionotropic receptors are not completely neurospecific, as thought earlier. Several years ago, NMDA receptors were described in rodent and human lymphocytes [40, 41]; it was found that their activation induces accumulation of calcium ion [42] and ROS [43]. These data obtained simultaneously in several laboratories showed both the

presence of mRNA for channel subunit of NMDA receptors (NR1) using the RT-PCR technique [41] and the presence of NMDA receptors on lymphocyte membranes using fluorescent antibodies [43] as well as functional activity of these receptors using pharmacological analysis (agonist/antagonist effects) [42, 43].

Diverse evidences related to the presence of NMDA receptors in immune competent cells raise the question of their functional role. NMDA was found to stimulate (similar to staurosporine A) caspase-3 activity, which is known to participate in lymphocyte activation (Fig. 2a). It is important to stress that expression of these receptors is changeable and depends on the functional state of the cells. Activation of lymphocytes *in vitro* by addition of phytohemagglutinin increases the proportion of cells expressing NMDA receptors, and this effect is time-dependent [44]. Under *in vivo* conditions, it was also shown that exposure of lymphocytes in the inflammation area results in expression of these receptors on their membranes [45].

Simultaneous presence on the membranes of activated lymphocytes of both ionotropic and metabotropic receptors makes these cells similar (to some extent) to neurons, which use the interaction between these receptors to regulate intracellular levels of secondary messengers [46].

It is possible that in immune competent cells these receptors serve a similar role in regulation of cytokine synthesis. Interesting data were found when synthesis of interferon-γ was measured in a suspension of activated lymphocytes expressing ionotropic receptors of AMPA- and NMDA-classes as well as metabotropic glutamate receptors of Group I [41]. It was shown that neither NMDA nor glutamate affected the interferon-γ synthesis by intact NK- or T-cells. When the cells were then activated with interleukin-2 and started to synthesize interferon-γ, both NMDA and glutamate demonstrated regulatory action: the former suppressed and the latter stimulated accumulation of the cytokine (Fig. 2b). Based on these data, the authors ascribed to the glutamate receptors an ability to regulate production of cytokines, suggesting that ionotropic receptors suppress and metabotropic receptors accelerate this process [43]. Thus, glutamate can serve in mammals not only as a neuromediator, but also as immunomediator [39].

Recently, similar behavior of NMDA receptors was described in phagocytic immune competent cells, neutrophils. These cells, when they were purified from peripheral blood of intact animals, did not possess NMDA receptors, whereas their membrane presented practically all classes of adenosine receptors involved in regulation of immune response, namely, A1, A2, and A3 receptors. Activation of A1 adenosine receptors results in inhibition of adenylate cyclase, activation of K-channels and inhibition of Ca-channels, as well as activation of phospholipase C; similar processes were noted after activation of A3 adenosine receptors. Nevertheless, activation of A1 results in stimulation of chemotaxis, phagocytosis, and adhesion of neutrophils, whereas activation of A3 only suppresses degranulation [47]. Activation of A2a

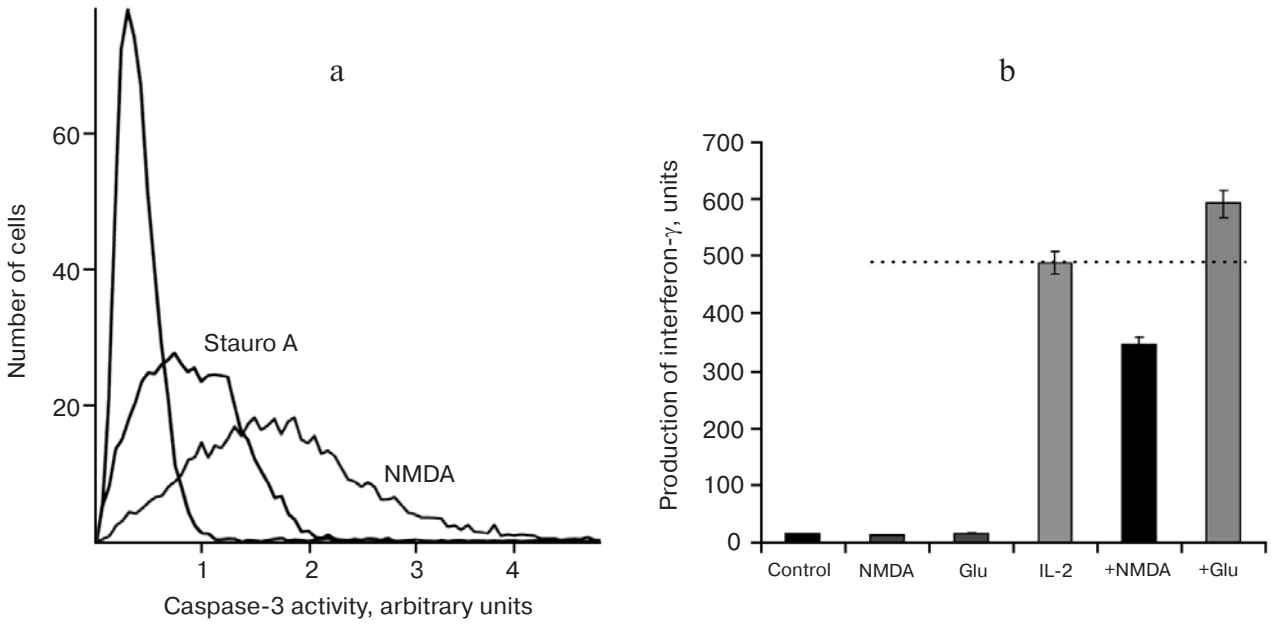


**Fig. 2.** Participation of NMDA receptors in activation of caspase-3 (a) and production of interferon-γ (b) by lymphocytes: a) NMDA (250 μM, 6 h incubation at 37°C) activates caspase-3 in lymphocytes like staurosporine A (Stauro A, 10 μM); b) NMDA (0.5 mM) and glutamate (Glu, 0.5 mM) do not affect interferon-γ production by intact lymphocytes, whereas the former inhibits and the latter activates this process in lymphocytes activated by interleukin 2 (IL-2) (from [42] and [80] with modifications).

and A2b receptors results in increase in intracellular concentration of cAMP. Adenosine effect on A2a suppresses both adhesion and activation of neutrophils induced by fMLP (fMet-Leu-Phe), this effect being suppressed by inhibitors of protein kinase A. A suggestion has been made that occupation of A2 receptors on neutrophils facilitates dissociation of chemo-attractant molecules from membrane receptors and suppresses activation of neutrophils without participation of cAMP [48]. Thus, activation of A2a may affect the anti-inflammatory mechanisms.

In the literature, interaction of A1 and A2 adenosine receptors with Fcγ-receptor has been noted [49]. Using selective agonists, it was demonstrated that activation of A1 adenosine receptor stimulates Fcγ-receptor mediated phagocytosis and generation of superoxide anion, whereas activation of A2 receptors suppresses both reactions. Thus, the final result of activation of Fcγ-receptors in neutrophils will depend on the presence of adenosine, which at low concentration will affect A1 receptors and stimulate Fcγ-receptor and at high concentrations will affect A2 receptors and suppress Fcγ-receptor [50].

In neuronal cells, the possible interaction between A1 adenosine receptors and NMDA receptors has been described. Activation of A1 receptors on postsynaptic membrane has been found to suppress ion fluxes via NMDA receptors [51]. At the same time, on presynaptic membrane adenosine suppresses glutamate release. On the other hand, activation of NMDA receptors by glutamate facilitates adenosine release in the synaptic cleft [52]. Because NMDA receptors were recently found in lymphocytes [41], it is possible to speculate that similar interaction between adenosine receptors and NMDA receptors takes place not only in brain but also in the bloodstream.

It was found that neutrophils isolated from an area of inflammation after treatment of rats with opsonized zymosan demonstrate on their membranes NMDA receptors, in contrast with those cells isolated from intact animals (Fig. 3). The functional meaning of this fact is still obscure, but the possibility exists that they can take part in regulation of respiratory burst and ROS production [53].

Recently, NMDA receptors were found in precursors of platelets, megakaryocytes [54, 55], and in cardiomyocytes where they may take part in regulation of Ca fluxes [56]. Thus, we can conclude that NMDA receptors are broadly expressed in diverse tissues, where they are involved in intracellular signaling mechanisms.

## EFFECT OF HOMOCYSTEINE ON BLOOD CELLS

The ability of immune competent cells to express NMDA receptors suggests that they can be affected by

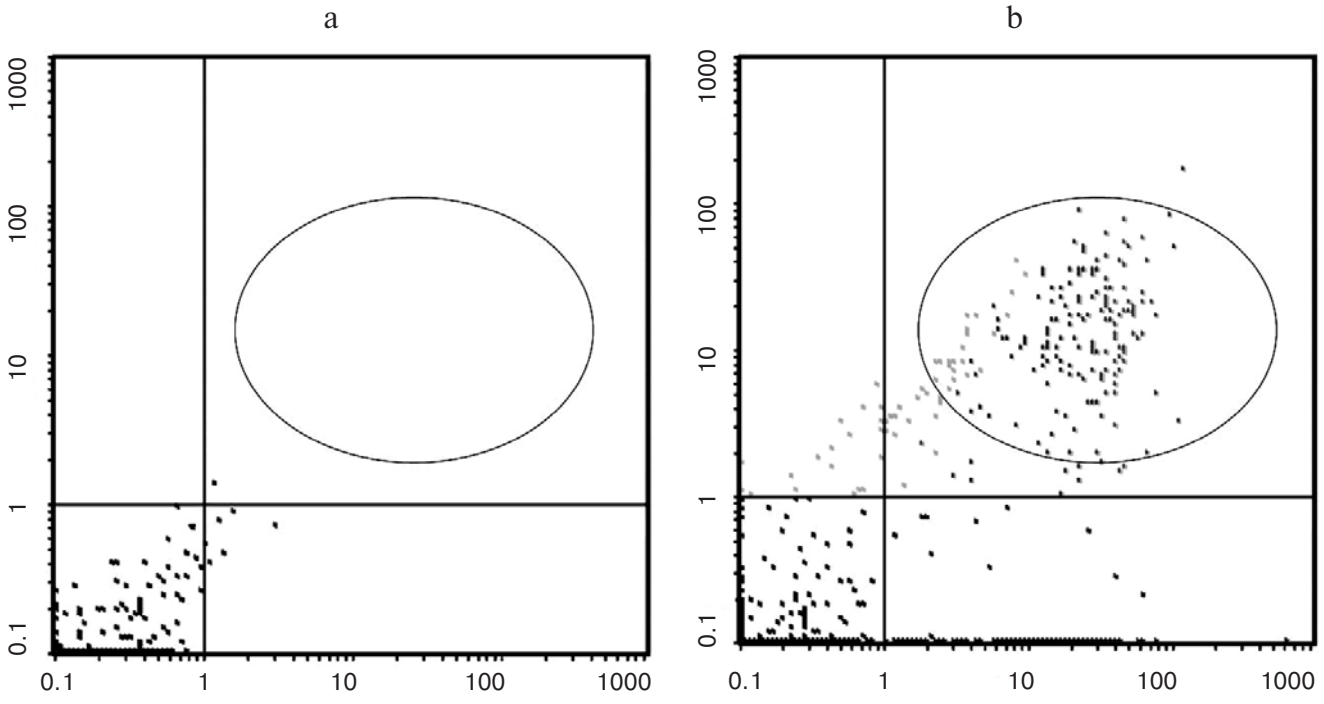


**Fig. 3.** Disclosure of NMDA receptors in neutrophils isolated from an area of inflammation. Results of immunocytochemical staining of intact (a) and *in vivo* activated (b) neutrophils with FITS labeled antibodies toward NMDA receptors; the circled area contains cells expressing NMDA receptors. Ordinate, fluorescence of FITS-labeled antibodies to NMDA receptors; abscissa, fluorescence of phycoerythrin-labeled marker of specific neutrophil protein (from [53] with modification).

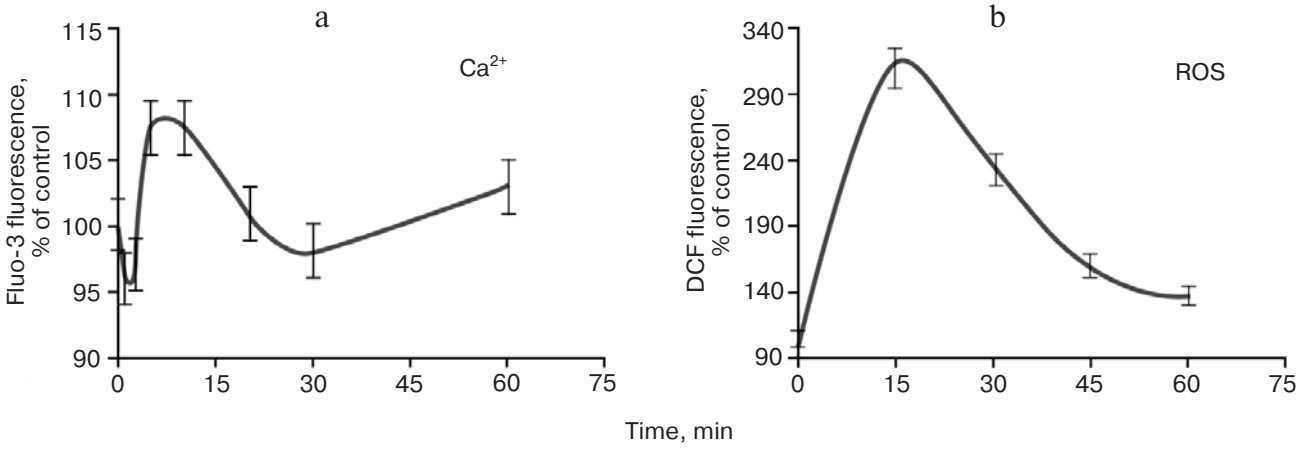


**Fig. 4.** Effect of HCA on calcium and ROS levels in rat lymphocytes. Time dependence of fluorescence of Fluo-3 (intracellular marker for calcium ions) (a) and DCF (intracellular marker for ROS) (b) was measured after incubation of rat lymphocytes with HCA (500 μM at 37°C). For experimental conditions, see [57].

homocysteine. In our laboratory, the effect of HC and HCA both on the cells producing cytokines (lymphocytes) and on the phagocytic cells (neutrophils) has been studied.

It was found that in lymphocytes, not only properties of NMDA receptors are similar to those in neurons [33, 45], but HC and HCA demonstrate similar effect on them [57]. The effective concentrations of HCA (which is more toxic than HC) are comparable with those found under hyperhomocysteinemia (100-500 μmol/liter), and the effect appears very quickly, i.e. 30 min incubation of lymphocytes with these ligands is enough to activate the cells (Fig. 4).

As noted earlier, neutrophils isolated from peripheral blood of intact animals do not express functionally active NMDA receptors, and HC does not affect the stationary ROS level of these cells. It is able, however, to increase the ROS levels in the presence of fMLP, which induces so-called "respiratory burst" in neutrophils. Because this ligand is known to activate a specific receptor on the neutrophil membrane [58] and HC cannot interact with this receptor directly (when HC is added to neutrophil suspension they are not activated), its modulating effect is supposed to be realized *via* interaction of HC with another membrane receptor.

In neutrophils, adenosine receptors act as modulators of respiratory burst [59]. Thus, one can suggest that in the intact neutrophils activated by fMLP, HC affects the function of these receptors [60, 61]. This hypothesis was tested using inhibitors of several adenosine receptors, showing that the activating effect of HC on ROS production is directed to A2 receptors because its activation was eliminated by ZM24385 (inhibitor of A2 receptors) but not by DCPCX or MRS 1220 (inhibitors of A1 and A3 adenosine receptors, respectively). It is interesting that HCA reveals a weaker effect on neutrophils than HC, suggesting that adenosine receptors possess higher affinity to HC whereas NMDA receptors demonstrate higher affinity to HCA [60].

The fact that neutrophils isolated from an inflammatory area express NMDA receptors on their membrane suggests their participation in accumulation of ROS by these cells. In agreement with this, HC induces respiratory burst in neutrophils activated with opsonized zymosan, and this effect is suppressed by an antagonist of NMDA receptors, MK-801 [53].

Thus, HC can induce the hyperactivation of NMDA receptors of both neuronal cells and immune competent cells that express NMDA receptors. This means that HC accumulation can result in exhaustion of both neuronal and immune systems of the body, which is a known feature of hyperhomocysteinemia.

Recently, an ability of HCA to stimulate red blood cells hemolysis was described that is induced by several unfavorable factors [62]. Preincubation of red blood cells with HCA accelerated acidic hemolysis and decreased the lag-period prior to disordering of erythrocyte membranes. Such effect is quite predictable in terms of the discovery on the erythrocyte membrane of a protein reacting with antibodies to NMDA receptors and inducing calcium flux into red blood cells after their incubation with N-methyl-D-aspartate [54].

Toxic effect of HC on platelets is also important. High levels of HC in the blood stimulates platelet aggregation [63], which possibly relates to damaging effect of HC on vessel endothelial cells, inhibition of NO-synthase, activation of factor V, decrease in activating action of C-reactive protein, misbalance of thrombomodulin expression, and inhibition of binding of tissue plasminogen activator by endothelial cells [64]. All these effects (or at least some of them) may result from the effect of HC or its metabolites on NMDA receptors recently described in

megakaryocytes [54, 55], a fact whose importance was not properly evaluated till recently.

## POSSIBLE STRATEGY OF PROTECTION FROM TOXIC EFFECTS OF HOMOCYSTEINE

As one can see from the above data, disordering of HC metabolism results in its accumulation in the bloodstream and induces or aggravates several pathologies [28, 65]. On the other hand, high levels of blood HC can be not the cause but the consequence of these pathologies. In such a case, toxic effect of HC will enhance the pathological state resulting in appearance of more severe symptoms. In any case, consequences of hyperhomocysteinemia are very substantial, and there is a need to develop protocol(s) for protection of the body from toxic effects of HC.

An important aspect of hyperhomocysteinemia is its aggravated effect in pregnancy. It was found that HC levels in mother's blood are in inverse correlation with mass of the neonate [66]. HC easily penetrates the blood–brain barrier and may render teratogenic and fetotoxic effects.

The mechanisms described above may participate in complications of pregnancy. Formation of microthrombi and disordering of microcirculation will induce defects of fetoplacental blood circulation and raise reproductive deficiency [67].

The teratogenic effect of hyperhomocysteinemia might be the result of disordering of DNA and RNA methylation and polyamine synthesis. Hyperhomocysteinemia at early steps of pregnancy has been demonstrated to be one of the causes of anencephalia and a nonclosure of *spina bifida* [68]. The former results in 100% lethality, and the latter to serious neurological problems, like motor paralysis of the newborn, permanent invalidization, and early death. At the later steps of pregnancy, hyperhomocysteinemia causes chronic fetoplacental deficiency and chronic fetus hypoxia. All these reasons result in birth of neonates with low body mass and decreased functional activity of all life-supporting systems, and appearance of a number of complications [66]. Thus, increased HC levels in mother's blood at early steps of pregnancy induces disordering of the neural system in the fetus and at the later steps of pregnancy may result in severe intoxication of both mother and fetus and finally in abortion. All these factors make it important to develop protection from toxic effects of hyperhomocysteinemia.

A widely accepted approach to prevent hyperhomocysteinemia is systemic administration of vitamins of group B and folic acid. In many cases, however, especially when hyperhomocysteinemia is induced by genetic defects in expression of enzymes of HC metabolism, this is not effective enough. In such cases, it is useful to analyze the possibility of decreasing the toxicity of the HC molecule.

Because the effects of HC and HCA on cells expressing NMDA receptors result in accumulation of ROS and disordering of cell signaling mechanisms, clinical use of ligands modulating these receptors can be accompanied with undesirable consequences. At the same time, use of natural metabolites able to regulate intracellular ROS levels and thus keep the cells viable could be potentially effective. With this in mind, we have turned attention to carnosine, a natural dipeptide being a specific component of excitable tissues and supporting function of brain neurons under conditions of oxidative stress [69, 70]. Carnosine demonstrates apparent ability to protect neurons from damage induced by the excitotoxic effect of NMDA [71, 72].

Carnosine is easily accumulated in brain after administration into blood of animals and is characterized by low toxicity [73-75]; its excess is easily hydrolyzed by serum and kidney carnosinases, which guaranty the absence of side effects of carnosine with possible over dosage [76, 77]. Moreover, carnosine has been found to protect the brain when administered into blood under hypoxic conditions [78, 79]. Recently, it was shown that carnosine protects red blood cells from acidic hemolysis (not accompanied by ROS accumulation) in the presence of HCA [62], which possibly indicates its action other than direct antioxidant effect.

To estimate possible protection of animals from toxic action of HC, an experimental model of prenatal

**Table 2.** Characterization of experimental animals (from [81] with permission)

| Group of animals | Number of rat families under study | Average number of pups in litter | p1 | Weight, g (for 10-day-old pups) | p2 |
|---|---|---|---|---|---|
| Group 1 (intact animals) | 6 | 12 ± 2 | | 23.3 ± 0.4 | < 0.05 |
| Group 2 (methionine treated) | 4 | 7 ± 1 | < 0.05 | 18.9 ± 0.5 | |
| Group 3 (methionine + carnosine treated) | 6 | 13 ± 2 | > 0.05 | 24.1 ± 0.6 | < 0.05 |

Note: p1 corresponds to statistically significant difference from group 1; p2, the same from group 2.

**Table 3.** Results of Morris water test for experimental animals (from [81] with permission)

| Parameter | Group 1 ($n$ = 18) | Group 2 ($n$ = 18) | Group 3 ($n$ = 18) |
|---|---|---|---|
| Time of reaching platform, sec | 20 ± 7 | 140 ± 18<br>p1 < 0.01 | 45 ± 6<br>p2 < 0.01 |
| Average rate of swimming, m/sec | 0.24 ± 0.02 | 0.18 ± 0.02 | 0.25 ± 0.04<br>p2 > 0.05 |
| Time of swimming in the central region of the pool (% of total time of search) | 20 ± 7 | 7 ± 5<br>p1 < 0.01 | 35 ± 5<br>p2 < 0.01 |

Note: Designations as in Table 2.

hyperhomocysteinemia was used [80, 81]. Concerning this model, increased HC levels in blood of pregnant rats was created by alimentary loading with methionine (1 g per kg body mass daily). The rats started treatment with methionine (given with drinking water) from the end of the first trimester of pregnancy. Thus, the prenatal development of the fetus occurred under constant hyperhomocysteinemia; in this case, the number of progeny and the weight of the pups were decreased notably (Table 2).

The animals whose prenatal and early postnatal development was subjected to hyperhomocysteinemic conditions were tested in a Morris water pool to characterize their cognitive ability [82]. Efficiency of the search for a platform in the water pool was estimated the next day after preliminary learning of animals. Such test showed that learning capacity of methionine-treated animals is decreased (Table 3). Moreover, analysis of the neurons isolated from cerebellum of experimental animals showed desensitization of NMDA receptors expressed in suppression of response to high concentrations of NMDA, HC, or HCA [81].

Progeny of the animals receiving carnosine (100 mg/kg body mass daily) simultaneously with methionine sufficiently differed from the methionine-treated animals. First, pregnancy in the rats of this group was more successfully it terms of the number of pups and their weight (Table 2). Moreover, they were characterized by higher learning ability, thus demonstrating cognitive parameters similar to those in intact animals (Table 3). Finally, comparison of neurons isolated from cerebellum of 10-day-old animals of all three groups showed increase in the portion of necrotic neurons and stationary ROS levels, whereas in neurons isolated from (carnosine + methionine)-treated group these parameters were close to those in intact neurons (Fig. 5).

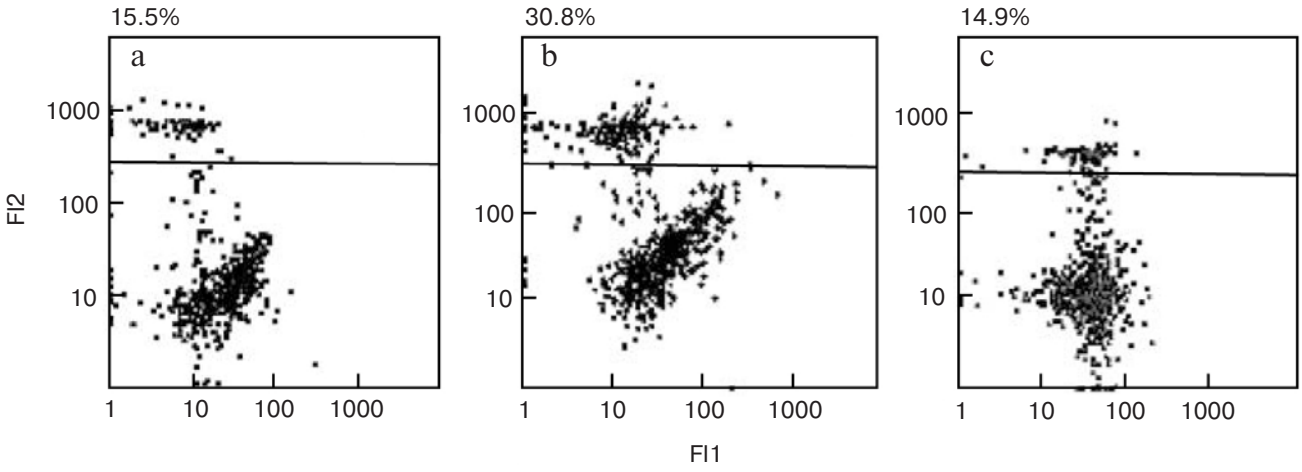


**Fig. 5.** Effect of carnosine on the cerebellum neurons of 10-day-old rats after prenatal hyperhomocysteinemia (from [81] with modification). Hyperhomocysteinemia was induced by dietary methionine (1 g/kg body mass daily) added to drinking water of the pregnant rats and their progeny; cerebellum of 10-day-old pups was used for preparation of neurons, which were characterized using flow cytometry. Double staining of the cells with propidium iodide, PI (marker of dead cells, ordinate, Fl2) and DCF (marker of viable cells, abscissa, Fl1) allows discrimination of dead from viable cells (dead cells are noted as % at the upper part of each panel) and mean DCF fluorescence corresponding to ROS levels in viable cells (comprises 27.1, 54.5, and 22.3 arbitrary units for panels a–c, respectively) for the three groups of animals—intact (a), treated with methionine (b), and treated with methionine and carnosine (100 mg/kg body mass, daily) (c). A typical experiment is presented.

All these facts suggest that regular administration of carnosine ameliorated conditions of pregnancy and protected the brain from toxic effects of HC. It is interesting to note that total HC in blood of animals treated with (carnosine + methionine) was as high (four-to-five times higher than in control) as in blood of animals treated with methionine alone. This means that the protective effect of carnosine is related not to improvements of HC metabolism and normalization of its levels, but to real protection of cells and tissues from toxic action of HC. It is still not clear what molecular mechanism(s) is(are) used by carnosine – whether it modulates affinity of glutamate receptors to HC or suppresses intracellular ROS levels increased after HC action or demonstrates other (still unknown) protective mechanisms. This should be the aim of the special research, while the protective action itself reflects the presence of natural mechanisms preventing HC toxicity and may be used in clinical practice.

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