Prenatal Hyperhomocysteinemia as a Model of Oxidative Stress of the Brain

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We found that methionine added to the ration of pregnant rats (1 g/kg body weight) induced sustained hyperhomocysteinemia and led to the formation of sustained oxidative stress in the brain of their progeny. Newborn animals were characterized by lower body weight, SOD deficiency in the brain, increased neuronal death, and desensitization of NMDA receptors. These factors are associated with impaired cognitive capacity in the Morris test.

Key Words: *prenatal hyperhomocysteinemia; N-methyl-D-aspartate (NMDA); homocystein; homocysteinic acid*

High level of homocystein (HC) in the peripheral blood is a known risk factor for neurodegenerative and cardiovascular diseases [4,5], because it induces oxidative stress in organs and tissues [2,6]. The neurotoxic effect of HC and homocysteinic acid (HCA) is determined by long-term activation of glutamate NMDA receptors in the brain [11] and in immune system cells [8]. All these facts necessitate the search for natural metabolites that can protect the organism under conditions of hyperhomocysteinenia.

Here we describe the model of prenatal hyperhomocysteinemia in rats created by addition of methionine to drinking water (1 g/kg body weight) during pregnancy and nursing. The progeny of animals maintained under these conditions had all symptoms of oxidative brain damage detected by physiological tests and biochemical parameters.

MATERIALS AND METHODS

Experiments were carried out on adult rats weighing 220-250 g and their progeny. During trimester II of pregnancy, elevated content of HC in the blood was created by adding methionine in a daily dose of 1.00±0.01 g/kg body weight to drinking water. The rats were maintained on this ration also after delivery; the progeny received methionine with breast milk and then with drinking water. The control and experimental groups (n=6 each) were maintained under standard vivarium conditions. The number and body weight of rat pups in both groups were determined; at the age of 10-12 days, properties of neurons were studied; physiological characteristics were determined on day 45.

The content of HC and its metabolites in the peripheral blood was measured by HPLC using Perkin Elmer HPLS system according to manufacturer's instructions.

Activity of Cu/Zn-SOD in brain tissue samples was determined as described previously [7].

Primary suspension of neuronal cells was obtained by the previously described method [3] in our modification. The cerebellums from 3-4 animals at the age of 10-12 days and the prepared sections were pooled, which excluded individual differences within the litters. For isolation of neuronal suspension, brain tissue samples were placed into Tyrode solution (148 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4)

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containing collagenase (2 mg/ml, Wako, 400 U). The samples were incubated at 34°C for 30 min without agitation, repeatedly washed with Tyrode solution, and suspended using a Pasteur pipette with smoothened edge. The cells separated from the tissue formed an opalescent suspension. The suspension was filtered through a Teflon filter (pore size 43 μ). Before the experiment, the cells were left at 34°C for 30 min and then prepared for cytological analysis. The samples for cytometry were prepared at scattered light.

For evaluation of the content of reactive oxygen forms (ROS), the neurons were loaded with $DCFHA_2$-DA fluorescent probe (2,7-dichlorodihydrofluorescein acetate, Sigma) in a final concentration of 100 μM (dark incubation) [1,3]. Measurements were performed at excitation and emission wavelengths of 530 and 485 nm, respectively. $DCFHA_2$-DA was dissolved in DMSO, the final concentration of the solvent did not exceed 0.1%.

The percent of dead cells in the studied population was determined in the presence of propidium iodide (PI, Sigma) at excitation and emission wavelengths of 485 and 610 nm, respectively; the dye was added to the suspension in a concentration of 10 μM 1 min before measurements.

The cells were incubated with the ligands for 30 min at 37°C (in dark). Agonists of glutamate receptors NMDA, HC, and HCA (Sigma) and antagonists of ionotropic (MK-801 and D-AP5) and metabotropic receptors MSOP (-(RS)-a-methylserine-D-phosphate) or AIDA (UPF 523/(RS)-1,5-dicarboxylate; "Tocris") were used.

Cytometric studies were performed on a FACStar device (Becton Dickinson). In each sample, 10,000 events were analyzed. The gait limited the area corresponding to neuron localization. The data obtained by flow cytometry were analyzed using WinMDI software. The mean fluorescence (X_m) was calculated in percent of control.

Learning capacity was studied using Morris water maze [10] allowing quantitative evaluation of the cognitive capacities and spatial memory of experimental animals. To this end, a round basin (d=1.7 m) filled with water (depth 30 cm) was used. A platform with a diameter of 15 cm was positioned at a depth of 0.5-1.0 cm not close than 30 cm from the wall, so that the animals could not accidentally find it during their swimming along the basin walls. Water temperature was maintained at 21±1°C.

In the Morris test, 45-day-old progeny from rats of both groups was used. On day 2 after the rats were trained to find the platform (after 4-5 attempts), the time of finding the platform (sec), the path (m), and the mean rate (m/sec) were determined under conditions of free search.

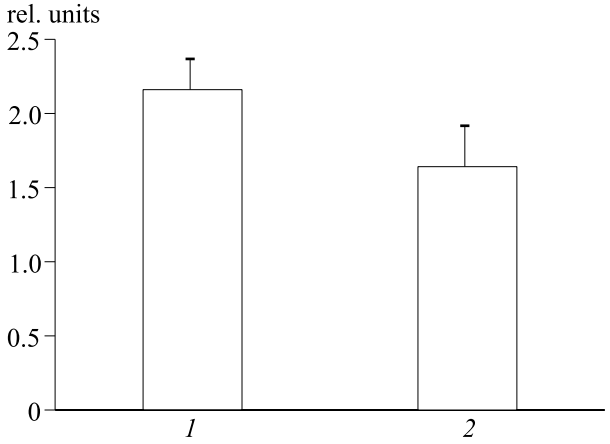

Fig. 1. SOD activity in brain tissue of control animals (*1*) and rats with homocysteinemia (*2*).

RESULTS

Gradual increase in methionine content in the ration led to sustained increase in serum HC level (from 5.9±1.8 to 33.0±3.9 μmol/liter; $p<0.01$), *i.e.* the fetus developed under conditions of hyperhomocysteinemia starting from the early terms of pregnancy. These conditions did not affect the number of pups in groups (14±2), but reduced their body weight: 23.3±0.4 and 18.9±0.5 g ($p<0.05$) in groups 1 and 2, respectively, on postnatal day 12.

SOD activity in the brain of group 2 rats on postnatal day 12 was significantly lower than in group 1 (Fig. 1). The percent of dead neurons in the suspension obtained from the cerebellum in animals with homocysteinemia was 2-fold lower than in the control group (30.3±2.5 and 15.5±1.5%, respectively). Increased neuronal death is directly related to impairment of the antioxidant enzyme system.

Cerebellar neurons from animals developed under conditions of hyperhomocysteinemia also considerably differ by their response to ligands activating

TABLE 1. Results of Morris test

Parameter	Group	
	1	2
Time of finding the platform, sec	20±7	140±18
Path, m	5±2	20±5
Mean velocity, m/sec	0.24±0.02	0.18±0.02

Note. Data of the 4th session are presented.

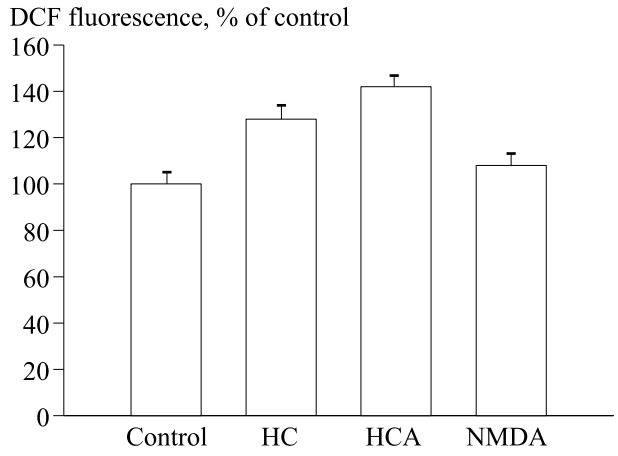

Fig. 2. Production of ROS by neurons isolated from animals of group 2 in response to activation with HC, HCA, and NMDA (concentration of the ligands 500 μmol/liter).

glutamate receptors. Activation of NMDA glutamate receptors with N-methyl-D-aspartate leads to enhanced ROS generation and this response is attenuated by NMDA receptor antagonist MK-801. HC and HCA activate neurons via both NMDA receptors and metabotropic receptors, whose activity can be blocked with antagonists AIDA and MSOP [11]. In light of this, ROS generation in cerebellar neurons from control animals in case of NMDA was suppressed by MK-801, but not by AIDA or MSOP. Activation of neurons with HC or HCA was suppressed by both MK-801 and metabotropic receptor antagonists.

In neurons isolated from the cerebellum of group 2 animals, the initial level of ROS was higher. These neurons were practically not activated by NMDA, but responded to HC and HCA by enhanced ROS generation (Fig. 2). The effect of these ligands did not depend on MK-801, but was abolished by AIDA and MSOP. These findings suggest that long-term hyperhomocysteinemia induces sustained oxidative stress in brain neurons associated with desensitization of NMDA glutamate receptors.

Group 2 animals demonstrated less pronounced learning and memory capacities (Table 1).

The results suggest that prenatal development under conditions of sustained hyperhomocysteinemia is characterized by oxidative stress in the brain manifested in decreased SOD activity, increased vulnerability of neurons, desensitization of NMDA receptors, and resulting in deep disturbances of cognitive functions.

During preparation of the manuscript we found a report where similar processes were analyzed: it was found that long-term hyperhomocysteinemia impairs memory and simultaneously inhibits expression of specific brain proteins (S-100 protein and cell adhesion protein [9]), which confirms the development of deep disturbances in the formation of the brain and its functions observed in long-term hyperhomocysteinemia.

The described model can be considered as a tool for studying the mechanisms of oxidative stress and protection of the brain under conditions of hyperhomocysteinemia.

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REFERENCES

1. A. A. Boldyrev, *Neirokhimiya*, **23**, 165-172 (2006).
2. A. V. Makhro, E. R. Bulygina, and A. A. Boldyrev, *Ibid.,* pp. 179-184.
3. S. L. Stvolinskii, T. N. Fedorova, M. O. Yuneva, and A. A. Boldyrev, *Byull. Eksp. Biol. Med*., **135**, No. 2, 151-154 (2003).
4. O. P. Shevchenko and G. A. Olefrienko, Laboratoriya, No. 1, 3-7 (2002).
5. G. Baydas, S. Koz, M. Tuzcu, *et al., Int. J. Devl. Neurosci.,* **25**, No. 3, 133-139 (2007).
6. A. A. Boldyrev, D. O. Carpenter, and P. Johnson, *J. Neurochem*., **95**, No. 4, 913-918 (2005).
7. A. A. Boldyrev, R. Song, V. A. Dyatlov, and A. D. Lawrence, *Cell. Mol. Neurobiol*., **20**, 433-449 (1999).
8. J. Loscalo, *J. Clin. Invest*., **98**, 5-7 (1996).
9. K. McCully, *Atherogenesis. Anal. Clin. Lab. Sci.*, **23**, 477-493 (1993).
10. R. G. Morris, P. Garrud, J. N. Rawlins, and J. O'Keefe, *Nature*, **297**, 681-683 (1982).
11. J. Tuneva, S. Chittur, A. A. Boldyrev, *et al., Neurosci. Res*., **9**, No. 4, 297-304 (2006).