Perinatal Hypoxic-Ischemic Brain Injury Affects the Glutamatergic Signal Transduction Coupled with Neuronal ADP-Ribosyl Cyclase Activity

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Agonists of glutamate ionotropic (NMDA) and metabotropic receptors (mGluRI and mGluRIII) had the regulatory effect on ADP-ribosyl cyclase/CD38 activity in cerebellar granular cells of newborn rats. Perinatal hypoxic-ischemic brain injury was followed by dysregulation of this mechanism.

Key Words: *CD38; ADP-ribosyl cyclase; glutamatergic signaling; cerebellar granular cells; perinatal brain injury*

The excitotoxic cascade due to hyperactivation of glutamate receptors forms a mechanism of brain damage under conditions of hypoxia/ischemia. This cascade has specific features in the developing (immature) brain, which determines specific pathogenesis and therapeutic approaches to perinatal hypoxic-ischemic injury. Ionotropic and metabotropic glutamate receptors play an important role in the formation of synaptic contacts, regulate neuronal viability and cell response to neurotrophic factors, and modulate the process of neuritogenesis and myelinization [7]. Dysregulation of glutamatergic neurotransmission in developing brain has a strong effect not only under conditions of acute damage, but also in the formation of delayed consequences of chronic neurodegenerative processes due to impairment of tissue reparation.

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Some specific features of the glutamatergic system in the developing brain include changes in the ion flux through ionotropic receptor channels, ratio between the expressed subunits of receptors, and components of signal transduction systems in the neuron. These characteristics of glutamate receptors provide high neurotrophic potential in the developing brain and contribute to increased sensitivity of brain tissue in newborns to hypoxic-ischemic injury and induction of excitotoxic mechanisms [15].

Much attention was paid to the role of inotropic and metabotropic glutamate receptors in the pathogenesis of perinatal hypoxic-ischemic brain injury. These data allowed us to develop new approaches to pharmacological correction of associated diseases [7]. However, studying the mechanisms of intracellular signal transduction associated with activity of these receptors in brain cells is an urgent problem. It is important to evaluate the relationship and interaction between signal pathways of inotropic and metabotropic glutamate receptors under normal and pathological conditions.

ADP-ribosyl cyclase/NAD^+-glycohydrolase/CD38 is a transmembrane glycoprotein, a component of cell

signaling systems associated with the receptors for some neurotransmitters (bradykinin receptors, adrenergic receptors, purinergic receptors, histamine receptors, muscarinic receptors, acetylcholine receptors, *etc*.). It catalyzes the formation of cyclic ADP-ribose (cADPR) from NAD^+ and hydrolysis of cADPR [6].

cADPR is a main catalytic product of CD38 modulating activity of type II and III ryanodine receptors due to specific binding to FKBP12.6 protein. This process is followed by Ca^{2+} mobilization from the intracellular store and regulation of M-type ion channels (involved in regulation of cell proliferation and differentiation). Receptor-mediated regulation of ADP-ribosyl cyclase activity was shown to exist in CNS cells. Stimulation of subtype 1, 3, 5, and 6 metabotropic glutamate receptors in retinal cells and neuroblastoma-glioma hybrid cells (NG108-15) causes activation of ADP-ribosyl cyclase. Enzyme activity is inhibited upon activation of subtype 2 receptors. Subtypes 4 and 7 have no effect on activity of this enzyme [6]. Experiments on dopaminergic neurons showed that stimulation of metabotropic receptors causes Ca^{2+} mobilization from intracellular stores, which is mediated by the inositol 1,4,5-triphosphate-dependent and cADPR-dependent mechanisms [10].

Cerebellar granular cells express various classes of neurotransmitter receptors and serve as a convenient model to study the mechanisms of signal transduction in neuronal cells. The expression of ADP-ribosyl cyclase and cADPR hydrolase was verified in these cells. Cyclase activity was shown to be most pronounced in cerebellar granular cells. The membrane enzyme provides an extracellular synthesis of cADPR, which is hypothesized to undergo internalization in the cytosol [3]. Our previous studies showed that N-methyl-D-aspartate (NMDA) causes the increase in intracellular Ca^{2+} concentration in cerebellar granular cells, which is related to activation of ryanodine receptors [13]. However, the role of NMDA receptors in this process remains unknown.

Here we studied the effects of ligands for ionotropic and metabotropic glutamate receptors on ADP-ribosyl cyclase/CD38 activity in cerebellar granular cells under normal conditions and after hypoxic-ischemic brain injury.

MATERIALS AND METHODS

Experiments were performed on male and female outbred albino rats (n=48) aged 10-12 days and weighing 10-18 g. The study was conducted in accordance to the animal welfare guidelines.

Hypoxic ischemic brain injury in animals was induced by extravasal occlusion of the right common carotid artery (under general anesthesia) on the 10th day of postnatal development. In the follow-up period, the animals were exposed to low-oxygen atmosphere (8% O_2) at 28-30°C for 1 h [12]. Brain tissue (cerebellum) was sampled from animals of the treatment group and sham-operated controls 4 h after injury.

Cerebellar granular cells were isolated by the standard method [2]. The cells were then maintained in Tyrode medium containing 10 mM HEPES, 148 mM NaCl, 2 mM $CaCl_2 \times 2H_2O$, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM D-glucose.

Activity of glutamate receptors was *in vitro* modulated by NMDA receptor agonist, NMDA (concentration range 100-1000 μM, Sigma); selective agonist of class I metabotropic receptors ($mGluR_1$ and $mGluR_5$), 3,5-dihydroxyphenylglycine (DHPG, final concentration 100 μM, Sigma); agonist of class III metabotropic receptors ($mGluR_4$, $mGluR_6$, $mGluR_7$, and $mGluR_8$) phospho-L-serine (L-SOP, final concentration 100 μM, Sigma); and competitive antagonist of NMDA receptors, D-2-amino-5-phosphopentanoate (DAP-5, final concentration 100 μM, Sigma). The cells were incubated with these agents for 30 min [1].

Activity of ADP-ribosyl cyclase/CD38 in cerebellar granular cells was estimated by the standard fluorometric method with a fluorogenic substrate nicotinamide guanine dinucleotide [5].

The results were analyzed by means of variation statistics (Statistica 6.0 and Biostatistica softwares). Each parameter was tested for normal distribution. Quantitative data were described by the method of descriptive statistics (arithmetic mean and standard deviation at $p<0.05$). The results were analyzed by Student's t test and T-test (when consistent with normal distribution). Nonparametric statistical tests were used to analyze the data not described by normal distribution.

RESULTS

We evaluated the possible involvement of ADP-ribosyl cyclase/CD38 in signal transduction from activated glutamate receptors. The effect of various agonists and antagonists on enzyme activity was studied in cerebellar granular cells of rats. Basal enzyme activity of ADP-ribosyl cyclase in control and ischemic cells was 0.32±0.14 and 0.64±0.20 U, respectively (p=0.66).

ADP-ribosyl cyclase activity in cerebellar cells from control animals was suppressed after treatment with NMDA in concentrations of 100-500 μM (Fig. 1). It should be emphasized that the inhibitory concentration of NMDA is similar to that inducing the excitotoxic effect in cultured neuronal cells [14]. The inhibitory effect of NMDA on ADP-ribosyl cyclase was also observed with an increase in the concentration of this agonist to 750-1000 μM.

NMDA in concentrations of 100-500 μM did not produce the inhibitory effect on ADP-ribosyl cyclase in cerebellar granular cells from animals of the treatment group (Fig. 1). As differentiated from control animals, NMDA in a concentration of 750 μM had a stimulatory effect on enzyme activity. ADP-ribosyl cyclase activity decreased significantly under the influence of this agonist in a concentration of 1000 μM (Fig. 2). Therefore, NMDA should be used in a higher concentration to suppress ADP-ribosyl cyclase in cells after hypoxia-ischemia. These data reflect the existence of functional coupling between ionotropic glutamate receptors and ADP-ribosyl cyclase.

We studied whether NMDA has a receptor-mediated specific effect on enzyme activity. The influence of NMDA was evaluated in the presence of DAP-5 (100 μM). DAP-5 partially abolished the inhibitory effect of NMDA in cerebellar cells from control animals. However, this effect was not observed in animals of the treatment group (Fig. 3).

Cerebellar granular cells from animals of the control and treatment groups were incubated with NMDA in a concentration, which decreased significantly activity of ADP-ribosyl cyclase (500 and 1000 μM, respectively). These cells were also incubated in the presence of 100 μM DHPG. We found that functional modulation of metabotropic receptors has various effects on the NMDA-induced cell response. The inhibitory effect of NMDA was abolished in control animals, but increased in treated specimens (Fig. 3).

Similar results were obtained after coincubation of cells with NMDA and L-SOP (100 μM). The inhibitory effect of NMDA on ADP-ribosyl cyclase was completely abolished in control animals, but increased in treated specimens (Fig. 3).

The presence of agonists of class I and III metabotropic glutamate receptors in the incubation medium had a strong effect on the cell response to NMDA. Activity of ADP-ribosyl cyclase was shown to increase under control conditions and, particularly, after hypoxia/ischemia (Fig. 3). Addition of DAP-5 to the incubation medium after pretreatment with NMDA and mGluR agonists was followed by similar changes. ADP-ribosyl cyclase activity under these conditions was higher compared to that in NMDA-treated and intact cells (not exposed to the effect of glutamate receptor ligands). Therefore, prevention of NMDA-induced changes facilitates the effect of metabotropic receptor agonists on ADP-ribosyl cyclase activity.

These data indicate that ADP-ribosyl cyclase in cerebellar granular cells is inhibited by NMDA receptors, but stimulated by class I and III metabotropic receptors. The sensitivity of ADP-ribosyl cyclase in cerebellar granular cells to an inhibitory action of NMDA decreases 4 h after hypoxic-ischemic brain

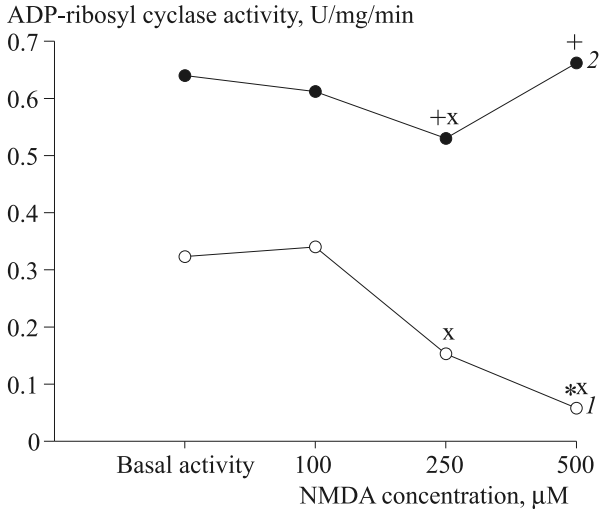

Fig. 1. ADP-ribosyl cyclase activity in cerebellar granular cells during the early postnatal period: control animals (*1*) and specimens with hypoxic-ischemic brain injury after NMDA treatment (*2*). Here and in Fig. 2 and 3: $p<0.05$: *compared to the basal activity (Mann–Whitney test); +compared to the control; xcompared to the parameter observed at 100 μM (control and treatment).

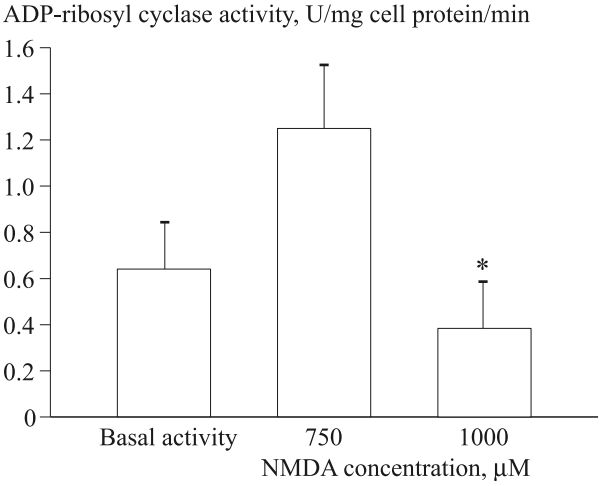

Fig. 2. ADP-ribosyl cyclase activity in cerebellar granular cells from early postnatal animals with hypoxic-ischemic brain injury after NMDA treatment.

injury (*i.e.*, under conditions of "glutamate-induced stress" in these cells). Agonists of class I and III metabotropic glutamate receptors produce the stimulatory effect under conditions of combined treatment and, particularly, after blockade of NMDA action. It should be emphasized that stimulation of mGluRI and mGluRIII in hypoxic-ischemic cells after pretreatment with NMDA receptor antagonist was accompanied by a 10-fold increase in enzyme activity. These changes were not observed in the control group under various experimental conditions.

Hypoxic-ischemic brain injury can also trigger another mechanism for the regulation of ADP-ribosyl cyclase activity. The availability of NAD^+ (substrate)

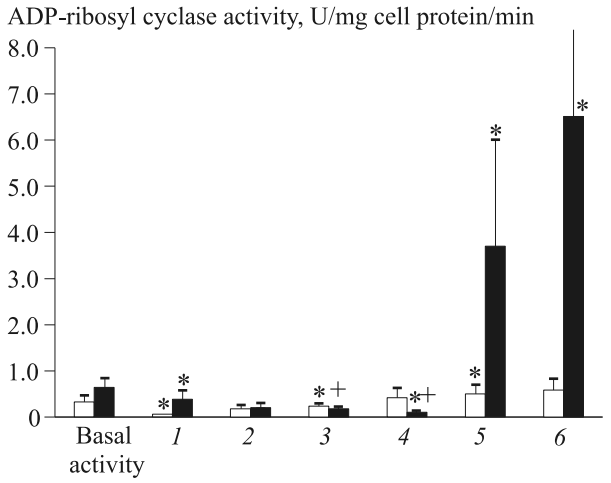

Fig. 3. ADP-ribosyl cyclase activity in cerebellar granular cells during the early postnatal period: control animals (light bars) and specimens with hypoxic-ischemic brain injury (dark bars) under the influence of glutamate receptor agonists and antagonists. NMDA (*1*); NMDA+DAP-5 (*2*); NMDA+DHPG (*3*); NMDA+L-SOP (*4*); NMDA+DHPG+L-SOP (*5*); NMDA+DHPG+L-SOP+DAP-5 (*6*).

decreases due to increased catabolism of this compound by poly(ADP-ribosyl)polymerase. This enzyme is activated due to increased mitochondrial production of free radicals upon the stimulation NMDA receptors [4].

The involvement of ADP-ribosyl cyclase in the phenomenon of excitotoxicity should be taken into account in evaluating the calcium-mobilizing effect of cADPR (catalytic product of this enzyme). Our results suggest that ADP-ribosyl cyclase activity in cerebellar granular cells decreases during signal transduction induced by excitotoxic concentrations of glutamate and mediated by NMDA receptors. Metabotropic mGluR agonists produce a stimulatory effect on enzyme activity, which reflects coupling of these agents with the ryanodine-dependent mechanisms of Ca^{2+} release from the intracellular store. We revealed a phenomenon of ADP-ribosyl cyclase desensitization to inhibitory influence of NMDA. Moreover, metabotropic receptor ligands possess stimulatory activity in hypoxic-ischemic cells only under conditions of NMDA receptor blockade. These data suggest the existence of the receptor-mediated or post-receptor mechanism, which provides the opposite effects of mGluRI/mGluRIII and NMDA receptors on the cADPR-dependent release of Ca^{2+} from the intracellular store. It is similar to the direct relationship between NMDA receptors and $mGluR_5$ in hippocampal neurons [11].

Evaluation of molecular mechanisms for cell death after hypoxia/ischemia in the immature brain hold much promise for the development of new efficient neuroprotectors. Taking into account the results of our study, it is necessary to revise some data on the prevention of glutamate toxicity by $mGluR_5$ activation in cerebellar neurons [9] and neuroprotective action of antagonists of class I metabotropic glutamate receptors under conditions of stroke or perinatal brain injury [8].

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