Perinatal Hypoxic and Ischemic Damage to the Central Nervous System Causes Changes in the Expression of Connexin 43 and CD38 and ADP-Ribosyl Cyclase Activity in Brain Cells

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The expression of connexin 43 and CD38 and ADP ribosyl cyclase activity in brain cells were studied in rats with experimental hypoxic and ischemic damage to the CNS. Changes in the expression and activity of the enzyme were detected over the course of ischemic injury indicating a possible contribution of NAD^+-converting activity and NAD^+-transporting processes to the pathogenesis of acute cerebral ischemic injury.

Key Words: *brain; perinatal hypoxia/ischemia; CD38; connexin 43*

Mechanisms associated with disorders in electrical excitability of neurons, development of oxidative stress, microcirculatory disorders, cytokine hyperproduction, changes in neurotransmitter secretion and reception, development of apoptosis and necrosis play an important role in the pathogenesis of hypoxic/ischemic perinatal damage to the CNS [6]. In ischemia, the neurotoxic cascade is triggered by high concentration of free calcium in the cytoplasm, which is associated with reduced membrane potential, increased translocation of calcium into the cell, with opening of receptor-regulated Ca channels (for example, in stimulation of glutamate receptors), and with calcium release from cell depots [5]. An important component of the mechanism of excitotoxicity is the so-called glutamate stroke realized at the expense of interactions between neuronal and glial cells and leading to prolonged accumulation of calcium in the cytoplasm.

Astrocytes are usually detected in the immediate vicinity to synapses and perform some important functions. For example, they regulate neurotransmitter and ion transport and modify the neuronal mitochondrial function by regulating astrocyte-neuronal lactate shuttle mechanism mediated through astrocyte glycolytic activity; they release bioactive molecules modulating endotheliocytes, microgliocytes, and neurons [9]. Co-culturing of astrocytes and neurons leads to an appreciable increase in the expression of astrocytic CD38 on the plasma membrane and in cells, which is associated with the effects of glutamate released from activated neurons on astrocytes [3].

One of the main NAD^+-converting enzymes in different cells is CD38 combining ADP-ribosyl cyclase (ADPR) and cADPR hydrolase activities (NAD^+ converting glycohydrolase, EC 3.2.2.6). By the present time, our understanding of the identification of the key characteristics and main regulators of the

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expression of this bifunctional enzyme was appreciably improved [8]. Receptor-mediated regulation of ADPR activity in CNS cells is proven [7]. The product of CD38 enzyme activity, cADP ribose, modulates types 2 and 3 ryanodine receptors via specific binding to FKBP12.6 protein and provides mobilization of calcium from intracellular depots into the cytoplasm.

Neurons and astrocytes express CD38 [8], but the role of ADPR/cADPR hydrolase activity in the regulation of neuron-astrocyte interactions is virtually not studied. It is shown that functionally active type 3 ryanodine receptors are essential for astrocyte migration [10].

It was previously shown that connexins 43 mediating cell-cell communication and transport of some signal molecules (NAD^+, ATP, calcium) [3] functionally react with CD38 on membranes of some cells, providing NAD^+ availability as the substrate for catalytic reaction [2].

In CNS, connexins are expressed only by astrocytes, but not neurons [4,11] and participate in the regulation of cell-cell interactions and astrocyte survival, including that in oxidative stress and hypoxia. However, no data about the expression of connexins 43 and their possible functional interactions with CD38 in CNS cells in hypoxic/ischemic injury were reported.

We studied the expression of connexin 43 (Cx43), CD38, and ADPR activity of this enzyme in brain cells in experimental perinatal hypoxic ischemic injury.

MATERIALS AND METHODS

Experiments were carried out on outbred newborn albino rats (n=67).

Perinatal hypoxic/ischemic injury to the brain was induced as described previously [12] on day 7 of postnatal development: extravasal occlusion of the right common carotid artery with subsequent exposure of rat pups under total ketamine anesthesia (25 mg/kg) in an atmosphere with low (8%) oxygen content. Material for the study (brain) was collected 8 and 72 h and 10 days after surgery (days 7, 10, and 17 of postnatal development) and during delayed periods (weeks 3, 4, 5, and 6 after brain injury). Control group consisted of sham-operated rats.

Detection of CD38 in brain cells was carried out using frozen fixed sections of the brain by the standard immunhistochemical protocol using antibodies to CD38 (Sorbent). Due to high homology of human, rat, and mouse CD38, we used antibodies to human antigen. After incubation of the sections with primary antibodies (1:50, 2 h at 37°C) and secondary FITC-labeled antimurine antibodies (1:200, 1 h at 4°C), the antigen-antibody complex was visualized under a fluorescent microscope (×900). The percentage of cells diffusely expressing the antigen in the membrane or perimembrane area or cytoplasm was evaluated.

The expression of Cx43 on glial cells was evaluated by double indirect immunofluorescent analysis using monoclonal antibodies to Cx43 (Chemicon International). The percent of cells carrying the fluorescent label (per 200 cells) was evaluated under immersion (×900).

Co-expression of CD38 and Cx43 was evaluated by double immunofluorescent staining using monoclonal antibodies to Cx43 (Chemicon International) visualized by FITC label and antibodies to CD38 (Sorbent) visualized by PE label. The numbers of cells expressing each antigen and the $CD38^+Cx43^+$ cells were counted.

Enzyme activity of ADPR/CD38 was evaluated by fluorometry with nicotinamide guanidine dinucleotide (NGD) as the fluorogenic substrate according to the standard protocol. Tissue specimens were homogenized at 4°C. Protein concentration in the resultant homogenate was measured by the method of Lowry. Enzyme activity was measured by incubating 100 μl tissue homogenate with the reaction mixture containing 100 μM NGD in 20 mM Tris-HCl (pH 7.4) for 20 min at 37°C. The supernatant fluorescence was recorded during minutes 0 and 20 of incubation on an SM2203 spectrofluorometer (Solar) at λ_{ex}=300 nm and λ_{em}=410 nm. Enzyme activity was estimated from the difference in fluorescence amplitudes during minute 20 and minute 0 of incubation per 1 mg protein per minute.

The severity of neurological symptoms in experimental animals was evaluated by the International NSS score for laboratory animals.

Statistical analysis included methods for statistical description and verification of statistical hypotheses. The arithmetic mean, mean square deviation, and error of the mean were evaluated for each sampling. For data corresponding to normal distribution, the significance of differences between the means was evaluated using Student's t test and T test. The results were statistically processed using Statistica 6.0 and Biostatistica software.

RESULTS

Development of neurological deficiency after hypoxia/ischemia of the brain on day 7 of life evaluated in experimental animals by the progress of neurological symptoms (NSS score) indicated that the

peak of dysfunction was attained within the first 8 h after surgery (9.700±0.716 compared to the control, $p<0.001$). Manifest clinical symptoms persisted until day 10 of postnatal development (7.900±0.611; $p<0.005$) and gradually leveled by week 6 of observation (0.900±0.314).

Dynamic changes in ADPR/CD38 activity in the cerebral cortex were paralleled by unidirectional changes in enzyme activity in the frontal and occipital areas of the involved hemisphere. Enzyme activity of ADPR increased significantly from the 8th to 72nd hours of acute ischemia in the occipital cortex and decreased significantly in the frontal cortex. Importantly catalytic activity of ADPR in controls decreased significantly by day 17 of postnatal development, this fact reflects characteristic dynamics of enzyme activity during the early postnatal period. During this period, activity of ADPR in animals subjected to perinatal injury to the CNS virtually did not differ from the control (Table 1).

Presumably, higher availability of NAD^+ as the substrate for catalytic conversion is one of the most probable mechanisms of high ADPR activity during the early ischemic period. Therefore, in order to evaluate the contribution of Cx43 expressed on astrocytes into this mechanism, we evaluated the expression of this molecule during the early period of ischemia/hypoxia.

TABLE 1. Activity of ADPR in the Cerebrocortical Cells after Perinatal Hypoxia/Ischemia (*M*±*m*)

Day of postnatal development		Enzyme activity, U/mg protein/min	
		frontal area	occipital area
Control	7	0.12±0.05	0.16±0.07
	10	0.45±0.31	0.05±0.02
	17	0.020±0.006	0.010±0.003
Perinatal hypoxia/ischemia	7	0.058±0.040*	0.244±0.150*
	10	0.005±0.003*	0.15±0.14*
	17	0.013±0.009	0.05±0.04$^+$

Note. $p<0.05$ compared to: *control, $^+$previous term.

TABLE 2. Changed Expression of Cx43 in Brain Cells after Perinatal Hypoxia/Ischemia of the CNS (*M*±*m*)

Day of postnatal development		Cx43-immunopositive cells, %	
		frontal area	occipital area
Control			
	7	11.8±1.7	11.9±0.7
	10	5.9±1.5	11.5±1.4
Perinatal hypoxia/ischemia	7	11.3±0.5	9.2±0.6
	10	13.9±1.4*	12.9±1.4

Note. $p<0.05$ compared to the control.

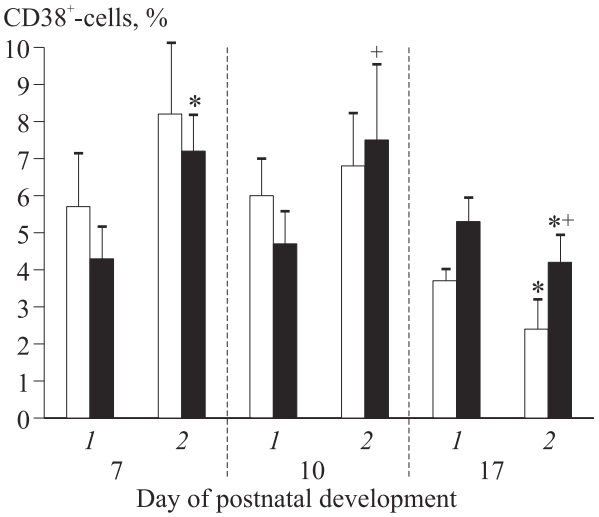

Fig. 1. Expression of CD38 on cells of brain cortex during the early postnatal period in sham-operated animals and after hypoxia/ischemia of the CNS. *1*) control; *2*) ischemia. Light bars: frontal cortex; dark bars: occipital cortex. $^+p<0.05$ compared to previous term of observation; $*p<0.05$ compared to the control.

Evaluation of Cx43 expression as astrocyte marker showed that the count of $Cx43^+$ cells increased significantly by the 72nd hour after hypoxia/ischemia of the brain, this indicating an increase in the count of $Cx43^+$ astrocytes in the focus of brain injury (Table 2). Double immunofluorescent assay showed that the majority (up to 80%) of cells expressing Cx43 were also CD38-positive (11.7±1.8 and 10.0±2.2 in the frontal and occipital cortex, respectively). No appreciable differences in the distribution of Cx43-immunopositive material in the frontal and occipital cortex were detected in the control group and after CNS injury.

Analysis of $CD38^+$ cell counts in brain preparations from control newborn rats showed that the level of CD38 expression in the newborns was much lower than in the brain cells of adult animals [1] and the counts of CD38-expressing neuronal and glial cells virtually did not change from day 7 to day 10 of postnatal development. Perinatal brain injury led to a significant increase in enzyme expression during the first 3 days after hypoxia/ischemia. This parameter decreased in the frontal and occipital cortex by day 10 after prenatal exposure (day 17 of postnatal development) (Fig. 1). No appreciable differences in the distribution of $CD38^+$ cells in the frontal and occipital cortex were detected. The distribution pattern of immunopositive material in

cells did not change much with the progress of ischemia: the cells expressed CD38 diffusely in the cytoplasm, perinuclearly, and towards the axons.

These data suggest that astrocytes are the main cells expressing CD38 during the early period after perinatal CNS injury. High expression of Cx43 in these cells can be indicative of a functional relationship between activity of ADPR catalyzing NAD^+ conversion into cyclic ADP ribose and connexins regulating NAD^+ availability as the reaction substrate, similarly as in cells of different origin [2].

Since high expression of CD38 on astrocytes is characteristic of the development of the so-called glutamate stroke (including that of ischemic origin), when glutamate released from the neurons stimulates the enzyme expression on glial cells, we think that changed expression of CD38 and Cx43 on brain cells is a marker of disorders in the neuroglial interactions in perinatal injury to the CNS.

Detection of the molecular mechanisms of cell death after hypoxia/ischemia in immature brain can serve as a prerequisite for the creation of new effective neuroprotective drugs. The regularities of changes in the expression and activities of two molecules regulating the transport and utilization of NAD^+ in brain cells and NMDA-mediated regulation of ADPR activity in the cerebellar granular cells of newborn animals revealed in our study suggest that the mechanisms of neuroglial interactions mediated by activities of NAD^+ converting enzymes and Cx4 can be regarded as pathogenetically justified targets for drug correction.

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