GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

Variability of Neuronal Damage and Proportion of Activities of NO Synthase Isoforms during Cerebral Ischemia/Reperfusion in Rats

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We studied expression of endothelial and neuronal NO synthase isoforms and severity of ischemia/reperfusion-induced damage to neurons in different brain compartments in albino rats. The peculiarities of distribution of NO synthase isoforms in the cerebral cortex and medulla oblongata were determined by different sensitivity of these compartments to ischemic and reperfusion damage to neurons.

Key Words: *brain; ischemia/reperfusion; endothelial nitroxide synthase; neuronal nitroxide synthase*

Acute cerebrovascular diseases (stroke, transitory ischemic attack) are responsible for 45% acute vascular diseases, being even more incident than acute coronary disease [1,7,8]. Numerous experimental and clinical data indicate that the duration of the "therapeutic window" in acute cerebral ischemia is determined by many factors, including the size and location of the focus, individual characteristics of metabolism and regional microcirculation [3,4,9].

NO and NO synthase (NOS), enzyme catalyzing its synthesis from arginine, participate in the mechanisms of acute cerebral ischemia [2,10]. Study of NOS activity in different brain compartments will help to evaluate structural changes in neurons in the course of the disease.

We studied variability of the expression of endothelial and neuronal NOS isoforms and the severity of neuronal damage in different brain compartments during ischemia/reperfusion of the brain.

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MATERIALS AND METHODS

The study was carried out on 28 adult outbred rats of both sexes (180-250 g). Common carotid arteries were mobilized on both sides and clamped for 1 (n=5), 3 (n=6), 8 (n=5), and 12-14 h (n=6) in rats intraperitoneally narcotized with 30 mg/kg sodium ethaminal. The bloodflow in the carotid arteries was resumed for 30 min in all experimental rats, after which the animals were sacrificed by sodium ethaminal overdose. Controls (sham-operated animals; n=6) were narcotized for 12 h with mobilized, but not clamped carotid arteries and sacrificed similarly as experimental animals. All manipulations on animals were carried out in accordance with Regulations on Handling Experimental Animals.

Immediately after sacrifice the brain was removed using a sparing method, placed into 10% buffered formalin (pH 7.4) for 30 min, and then divided into 3 blocks. A total of 50-60 serial sections (5 μ) were sliced from each block. The sections were stained with hematoxylin and eosin and

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with thionin after Nissl (every 10th section). The following structures were identified and evaluated on sections: posterior frontal, parietal and occipital hemispheric cortex, thalamus, hypothalamus, striopallidum, cerebellum, midbrain, and medulla oblongata. Microphotographs were made with Canon digital camera (5.0 megapixels) and Axiostar plus microscope (Carl Zeiss). The severity of damage was evaluated by a semiquantitative method: formation of ghost cells and neuronophagia were considered as complete destruction, other pathological changes in neurons were considered as half-destruction. The analysis of compartments most important from clinical viewpoint was supplemented by original method of radial morphometry developed at Volgograd Research Center, Russian Academy of Medical Sciences. Coefficients characterizing graphic thionin distribution function in neurons were used: K (curve slope to function extremum), I_m (staining intensity in extremum determined as the mean length of a segment in 3D RGB coordinate system so that the maximum length of the segment at the maximum brightness of 3 color spectrum constituents was taken for 10 arb. units), R (distance from the center of measurement to the zone with I_m), and V_m (maximum variability of intensity for sectors) [5].

Immunohistochemical reaction was carried out by indirect immunoperoxidase method with monoclonal antibodies to neuronal NOS-1 (NOS125 clone; DakoCytomation) and endothelial NOS-3 (RN5 clone, DakoCytomation). The neurons were classified with consideration for expression of immunopositive material: negative, slightly positive, positive, and hyperexpressive, the percentage of each class was calculated. The ratio of expressions was evaluated by specific brightness in comparable regions of the brain in serial sections on the same slide [6].

The significance of differences between the samples was evaluated using Student's *t* test.

RESULTS

Screening evaluation of the severity of ischemic and reperfusion damage to the brain showed augmenting severity and gradual cessation of the increment in the percentage of damaged neurons and extension of the volume of injury with prolongation of ischemia (Table 1).

The most pronounced neuronal damage was observed in reperfusion after short-term ischemia in the frontal cerebral cortex and hypothalamic nuclei, the maximum increment of lesions between the 3rd and 8th hours of ischemia being characteristic of the limbic and occipital cortex. Reperfusion after 12-h ischemia maximally damaged frontal cortical neurons and less so (but more than by 50%) neurons in virtually all other cortical regions and subcortical structures. The cerebellum, midbrain, and medulla oblongata were less sensitive to this exposure.

Differences in the radial profiles of tinctorial distribution of thionin in the neuronal perikaryon were detected in the most sensitive (frontal cortex) and least sensitive structures (reticular nuclei of the medulla oblongata) (Table 2).

Reduction of total chromatophilia and staining homogeneity, shift of the staining density peak from the neuron center to the periphery developed in cortical neurons during ischemia/reperfusion. The sum of these changes characterizing disorganization of neurons reflects irreversible ischemic injuries. The only sign of injury in neurons of the

TABLE 1. Severity of Neuronal Damage in Total Ischemia of Different Duration and 30-min Reperfusion of Rat Brain (%; *M*±*m*)

Structure		Duration of ischemia, h			
		1 (*n*=5)	3 (*n*=6)	8 (*n*=5)	12-14 (*n*=6)
Cerebral cortex	frontal	15.8±1.0	29.4±1.8*	64.9±3.7*+	72.3±4.1*
	parietal	11.3±0.5	20.0±1.7*	48.3±2.5*+	61.0±3.7*+
	occipital	8.8±0.6	22.6±1.9*	38.4±2.4*+	58.5±3.4*+
		10.0±0.8	28.5±2.2*	45.5±3.3*+	60.1±3.5*+
Striopallidum		7.1±0.8	17.6±1.3*	6.6±1.8*+	55.1±3.0
Thalamus		12.5±0.9	18.6±1.4*	30.5±2.0*+	65.1±5.8
Hypothalamus		15.0±0.8	29.6±2.4*+	37.5±3.1*+	67.1±5.2*+
Cerebellum		6.6±0.5	8.6±0.7*	16.9±1.5*+	39.4±2.7*+
Midbrain		4.9±0.4	18.0±1.1*	26.5±1.5*+	45.7±2.6*+
Medulla oblongata		4.5±0.4	9.5±0.8*	22.1±1.7*+	39.2±4.0*+

Note. Here and in Tables 2, 3 $p<0.05$ compared to: *control (0%), +previous term of the experiment.

TABLE 2. Parameters of Radial Morphometry of Neurons in Total Ischemia of Different Duration and 30-min Reperfusion of Rat Brain (*M*±*m*)

Parameter	Control (*n*=6)	Duration of ischemia, h		
		3 (*n*=6)	8 (*n*=5)	12-14 (*n*=6)
Posterior frontal cortical neurons				
K tangent	0.72±0.05	0.66±0.05	0.47±0.03*+	0.24±0.03*
I_m, arb. units	8.55±0.62	8.12±0.54	7.23±0.49*	6.32±0.46
R	0.44±0.03	0.30±0.03*	0.27±0.03*	0.22±0.02*
V_m	3.24±0.27	2.17±0.20*	1.55±0.14*+	1.37±0.07*
Neurons of lateral reticular nucleus of medulla oblongata				
K tangent	0.60±0.04	0.54±0.05	0.48±0.04*	0.37±0.03*
I_m, arb. units	11.32±0.75	12.18±0.88	11.03±0.70	11.27±0.65
R	0.38±0.08	0.35±0.07	0.30±0.02	0.31±0.07
V_m	4.29±0.36	5.52±0.40*	3.16±0.22*+	2.65±0.19*

reticular formation in the medulla oblongata was increased peripheral tinctorial density of perikaryons (abscissa coordinates 9-10 in radial morphometry). These changes were characterized as reversible.

Comparison of the expression of NOS-1 and NOS-3 showed differences between brain areas with different sensitivity to ischemic and reperfusion injuries (Table 3).

The expression of NOS-1 and NOS-3 increased in the cerebral cortex starting from 3 h of ischemia, the increment in the neuronal isoform expression being many times more rapid in comparison with the expression of endothelial NOS. After 12-h ischemia, the majority of layers 5-6 neurons were classified as hyperexpressing NOS (Fig. 1, *a*). The increase in the percentage of sharply immunopositive endotheliocytes in dilated vessels feeding the cerebral cortex (Fig. 2, *a*) was similar or somewhat less pronounced.

NOS expression in the medulla oblongata increased after 8-h ischemia, the expression of both isoforms increased similarly. Even after 12-h ischemia and 30-min reperfusion, more than one-quarter of neurons were NOS-1 negative (Fig. 1, *b*), endotheliocytes of up to 50% open functioning capillaries were NOS-3-negative or slightly positive (Fig. 2, *b*).

TABLE 3. Distribution of Neurons by Expression of NOS and Proportion of NOS-1/NOS-3 Expression after Total Ischemia of Different Duration and 30-min Reperfusion of Rat Brain (*M*±*m*)

Neurons, characteristics of NOS expression	Control (*n*=6)	Duration of ischemia, h		
		3 (*n*=6)	8 (*n*=5)	12-14 (*n*=6)
Posterior frontal cortical neurons				
negative, %	78.4±5.5	22.7±1.5*	7.3±0.5*	6.9±0.5
slightly positive, %	17.5±0.8	45.8±3.1*	42.0±3.3	32.0±2.8*+
positive, %	4.1±0.3	28.7±1.8*	33.5±2.2*+	36.1±2.7*
hyperexpression of NOS, %	0	3.0±0.2*	17.2±1.1*+	25.0±1.3*+
NOS-1/NOS-3	1.8±0.2	5.1±0.4*	6.6±0.5*	7.7±0.5*
Medulla oblongata lateral reticular nucleus neurons				
negative, %	86.0±7.3	65.5±4.3*	48.6±3.0*+	27.0±1.9*+
slightly positive, %	11.4±0.6	25.1±1.9*	32.7±2.1*+	39.2±2.8*
positive, %	2.6±0.2	9.4±0.7*	12.5±0.9*	26.2±1.2*+
hyperexpression of NOS, %	0	0	5.6±0.3*+	12.6±0.7*+
NOS-1/NOS-3	2.9±0.2	2.7±0.4	3.1±0.5	3.3±0.4

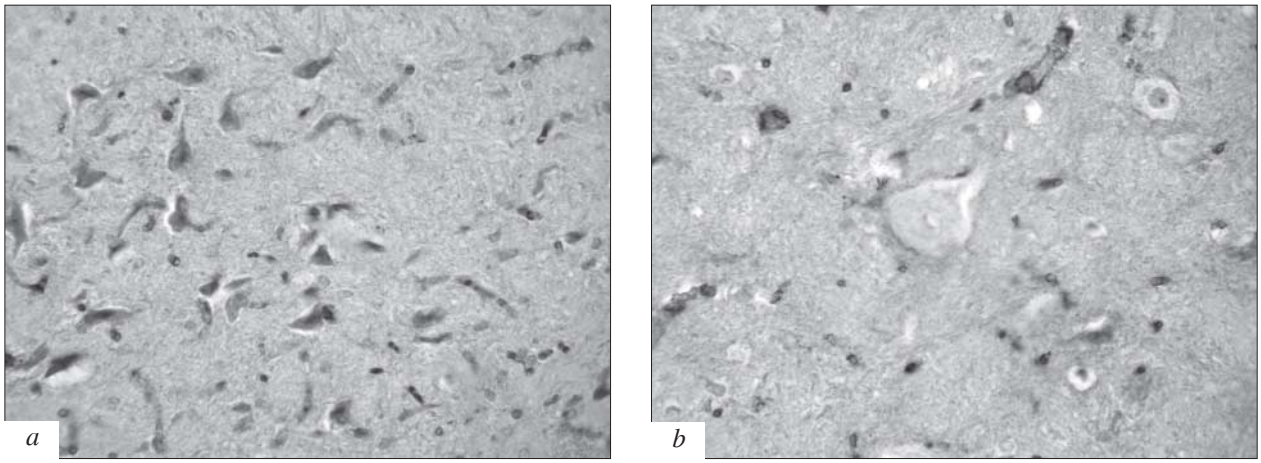

Fig. 1. Differences in NOS expression in the frontal cortical and medulla oblongata neurons after 12-h ischemia and 30-min reperfusion of rat brain. *a*) hyperexpression in the majority of frontal cortical neurons of layers 4-5; *b*) moderate expression in solitary small neurons of the medulla oblongata reticular nuclei. Staining with monoclonal antibodies to NOS-1, ×400.

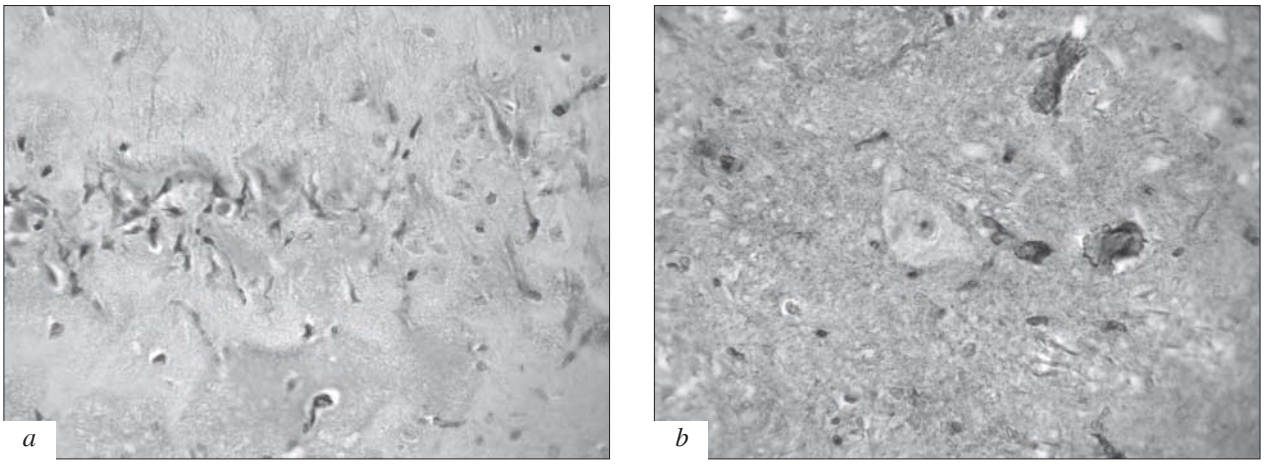

Fig. 2. Differences in NOS expression in frontal cortical and medulla oblongata vascular endothelium after 12-h ischemia and 30-min reperfusion of rat brain. *a*) hyperexpression in the majority of endotheliocytes in frontal cortical vessels dilated after reperfusion; *b*) moderate and slight expression in medulla oblongata vessels. Staining with monoclonal antibodies to NOS-3, ×400.

Published data on the role of NO in the development and mediation of excitotoxicity, free-radical and cytokine damage of neurons in cerebral ischemia/reperfusion [2,4,10] suggest that this distribution of NOS in neurons and endothelium of the studied brain compartments is caused by their principally different sensitivity to this injury. It seems that initially low expression of endothelial NOS isoform in the medulla oblongata underlies the subsequent dynamics of ischemic and reperfusion neuronal lesions in this brain compartment and should be taken into consideration when developing new approaches to neuroprotection.

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