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The Role of ATP-Sensitive Potassium Channels and Nitric Oxide in the Protective Effect of Preconditioning of the Brain

O. G. Deryagin,[1] S. A. Gavrilova,[1] S. V. Buravkov,[1] V. V. Andrianov,[2,3] G. G. Yafarova,[3] Kh. L. Gainutdinov,[2,3] and V. B. Koshelev[1]

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Objective. The role of ATP-dependent potassium (K^+_{ATP}) channels in the neuroprotective effect of ischemic (IPre) and pharmacological (PPre) preconditioning and changes in blood levels of nitric oxide (NO) metabolites were studied in conditions of cerebral ischemia. **Materials and methods.** Ischemic stroke (IS) was modeled in male rats (n = 86) by electrocoagulation of a branch of the middle cerebral artery (MCA). The nonselective K^+_{ATP} channel blocker glibenclamide and the K^+_{ATP} channel activator diazoxide were used. IPre and PPre were performed one day before MCA occlusion. Blood concentrations of NO, nitrates (NO_3^-) and nitrites (NO_2^-) were determined in experimental animals at 5, 24, and 72 h after MCA occlusion. Results. IPre decreased the lesion zone by 37% ($p < 0.05$), while prior administration of glibenclamide countered the action of IPre. The protective effect of PPre was analogous to that of IPre. Decreases in blood levels of oxygenated R-conformers of hemoglobin-bound NO (Hb-NO) were seen 5 h after MCA occlusion, with an inversely proportional increase in the concentration of nonoxygenated T-conformers; there were also increases in NO_3^- and NO_2^- concentrations. NO_3^- and NO_2^- levels showed normalization by one day after MCA occlusion, along with changes in the concentrations of Hb-NO complexes – R-conformers dominated, while the blood level of T-conformers reached a minimum. Furthermore, by 24 h there was a correlation between blockade of K^+_{ATP} channels and decreases in serum NO_3^- and NO_2^- levels ($p < 0.03$). **Conclusions.** The neuroprotective effect of preconditioning was due to activation of K^+_{ATP} channels. Analysis of blood levels of NO metabolites in rats with IS showed that Hb-NO complexes in the R-conformation stored and carried NO to the tissues, releasing NO on occurrence of the R → T transition in ischemic conditions.

Keywords: cerebral ischemia, ischemic tolerance, neuroprotection, preconditioning phenomenon, ATP-dependent potassium channels, nitric oxide, hemoglobin, spectrophotometry, electron paramagnetic resonance.

The resistance of the brain to deficient blood supply can increase in response to brief episodes of ischemia-reperfusion or hypoxia [1], transient hypothermia [2], or other moderate stressors able to activate endogenous defense mechanisms and increase tissue resistance to subsequent severe ischemia [3–5]. This phenomenon is termed preconditioning. Activation of adenosine triphosphate-dependent potassium (K^+_{ATP}) channels is regarded as the main component of responses in models of preconditioning [3]. Decreases in ATP levels during ischemia lead to opening of K^+_{ATP} channels in the plasma membrane; the role of these channels is to restore low concentrations of Na^+ and Ca^{2+} ions in the cytosol and prevent depolarization. Activation of K^+_{ATP} channels in the inner mitochondrial membrane is associated with protecting mitochondria against Ca^{2+} ion overload [6]. The leading role in the development of preconditioning is played by the mitochondrial pool [7].

The role of nitric oxide (NO) in the development of ischemic cell damage is just as important. The nature of the action of NO depends on the intensity and location of its

[1] Lomonosov Moscow State University. Moscow, Russia; e-mail: olegderyagin@gmail.com.
[2] Kazan Physicotechnical Institute, Kazan Scientific Center, Russian Academy of Sciences, Kazan, Russia.
[3] Institute of Basic Medicine and Biology, Kazan (Volga) Federal University, Kazan, Russia.

TABLE 1. Experimental Groups

Experimental series	Group names	Manipulations
Series I	OMCA (n = 10)	Control: 400 μl/kg DMSO i.p. 30 min before OMCA
	Glib (n = 9)	20 mg/kg (5 mg/100 μl DMSO) glibenclamide (Sigma) i.p. 30 min before OMCA
Series II	OMCA (n = 10)	Control: 400 μl/kg DMSO i.p. 24 h 30 min before OMCA
	IPre (n = 10)	400 μl/kg DMSO i.p. 30 min before IPre, OMCA 24 h after IPre
	IPre + Glib (n = 10)	20 mg/kg (5 mg/100 μl DMSO) glibenclamide i.p. 30 min before IPre, OMCA 24 h after IPre
Series III	OMCA (n = 6)	Control: intraventricular administration of 5 μl of DMSO + 5 μl buffer solution 24 h before OMCA
	PPre (n = 7)	Intraventricular administration of 10 μl of 6 mM diazoxide solution (in 5 μl DMSO + 5 μl buffer solution) 24 h before OMCA
Series IV	Int (n = 6)	Intact animals
	OMCA (n = 18)	Electrocoagulation of the frontal branch of the MCA and adjacent veins with simultaneous ligation of the ipsilateral carotid artery, six rats at each time point

DMSO – dimethylsulfoxide.

production and the state of the surrounding tissue. Overproduction of NO in ischemic stroke (IS) induces damage to the structural and regulatory components of cells [8], while binding of NO to enzymes of the mitochondrial transport chain inhibits cellular respiration [9]. Moderate activation of the NO system during preconditioning can have neuroprotective effects, activating enzymes of the antioxidant system, triggering antiapoptotic mechanisms, and increasing cerebral blood flow [8]. The protective effect of moderate NO production may also be mediated by activation of K^+_{ATP} channels [10].

Increases in local cerebral blood flow after hypoxic/ischemic preconditioning (IPre) can probably occur only in the first early phase of preconditioning. Blood flow undergoes centralization during the first hours after hypoxic preconditioning. A significant role in the adaptive response of brain vessels induced by oxygen deficit is played by compensatory increases in oxygen extraction from venules [11]. Then, after several days, IPre has no effect on basal blood flow in the cerebral cortex [12–14]. At the same time, IPre affects the rate of restoration of blood flow in the penumbra zone after occlusion of the middle cerebral artery (OMCA), which makes a contribution to producing the protective effect of preconditioning [15].

The aims of the present work were to study the role of ATP-dependent potassium K^+_{ATP} channels in mediating the neuroprotective effects of IPre and pharmacological preconditioning (PPre) and to assess changes in blood levels of NO metabolites in conditions of cerebral ischemia.

Materials and Methods. Experiments were performed on white male mongrel rats (n = 86; weight 300–500 g). Studies were performed in compliance with the requirements of the Bioethics Commission of Lomonosov Moscow State University. The study involved two experimental stages (Table 1). The first stage addressed the effects of blockade of K^+_{ATP} channels (glibenclamide) on the size of ischemic lesions (series I), the protective effect of the delayed phase of IPre (series II), and the possibility of modeling the effects of preconditioning by pharmacological (diazoxide) activation of K^+_{ATP} channels (series III).

The second stage addressed the effects of cerebral ischemia on components of the NO system: levels of NO metabolites were measured in experimental animals of series I–III, as were the effects of OMCA on the content of hemoglobin-bound NO (Hb-NO) in venous blood in rats of experimental series IV.

Modeling of IS. **I**n experimental series I–IV, IS was modeled under general anesthesia with chloral hydrate (400 mg/kg, i.p.) using an advanced method with electrocoagulation of right frontal branch of the MCA and adjacent vein with simultaneous ligation of the ipsilateral carotid artery [16–18].

Modeling of the phenomenon of preconditioning of cerebral IPre in experimental series II was performed 24 h before OMCA by transient clamping of the right and left common carotid arteries for 5 min with 5-min reperfusion over 1 h. Cerebral PPre was induced in series III by intraventricular administration of 10 μl of a 6 mM solution of the nonselective K^+_{ATP} channel blocker diazoxide (Sigma) into the right lateral ventricle (AP –1.0 mm, L 2 mm, V –4.5 mm) 24 h before OMCA. Prior blockade of K^+_{ATP} channels in series I and II was with glibenclamide at a dose of 20 mg/kg.

Assessment of the severity of ischemic damage. The extent of necrosis in series I and II was determined as the proportion (%) of damaged tissue to the total area of the hemisphere cortex in brain sections 1–2 mm thick stained with 2,3,5-triphenyltetrazolium chloride.

Determination of venous serum levels of NO and its metabolites. Levels of nitrates (NO_3^-) and nitrites (NO_2^-) were

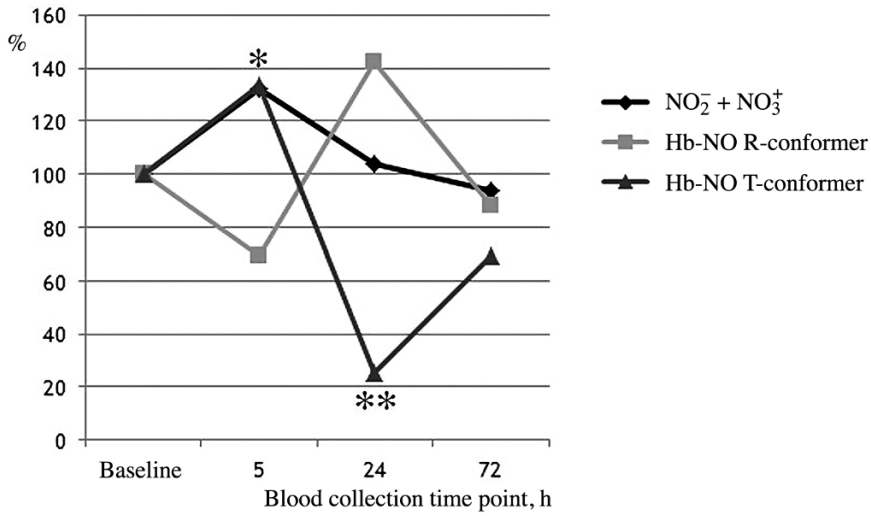

Fig. 1. Dynamics of changes in total NO_3^- and NO_2^- contents and R and T conformers of Hb-NO complexes in the blood of rats with ischemic stroke. Data are shown as % of NO metabolite levels in intact animals (baseline). $p < 0.05$: *Wilcoxon's test, comparison of NO_3^- and NO_2^- levels (OMCA vs. baseline); **Wilcoxon's test, comparison of the level of Hb-No T conformers (OMCA vs. Int).

TABLE 2. Dynamics of Changes in Serum NO_3^- and NO_2^- Concentrations and Lesion Sizes in the Cerebral Cortex in Rats with IS

Time of blood collection		% of baseline NO_3^- and NO_2^- concentrations			Lesion area, %
		5 h	24 h	72 h	
Series I	OMCA	146 ± 62¤	124 ± 56	101 ± 45	12.7 ± 5.9
	Glib	107 ± 64	69 ± 38*	62 ± 54	15.1 ± 8.2
Series II	OMCA	134 ± 58¤	109 ± 60	103 ± 42	14.9 ± 7.2
	IPre	110 ± 45	81 ± 24	71 ± 40	9.4 ± 3.7#
	IPre + Glib	136 ± 39	73 ± 49*	81 ± 30	16.8 ± 9.2
Series III	OMCA	107 ± 26	61 ± 27¤	64 ± 13¤	13.4 ± 3.4
	PPre	110 ± 28	63 ± 22	75 ± 22	9.1 ± 4.0

$p < 0.05$: *Mann–Whitney *U* test for experimental groups compared with controls; #Fisher's test, IPre vs. IPre + Glib; ¤Wilcoxon's test for control groups compared with baseline.

determined in venous serum collected from animals of series I–III one week before modeling focal ischemia and then 5, 24, and 72 h after OMCA. NO_3^- was reduced to NO_2^- with vanadium (III) chloride; total concentrations were determined using the Griess color reaction. Optical densities of solutions were measured using a Multiscan EX Primary EIA V 2.1-0 spectrophotometer at a wavelength of 492 nm.

Measurements of hemoglobin-bound NO (Hb-NO) levels in animals of series IV were made by electron paramagnetic resonance (EPR) using a Bruker ER 200E SRC spectrometer in range X (9.50 GHz) at a temperature of 77 K [19]. Two types of paramagnetic complexes were detected in venous blood: complexes of NO with hemoglobin heme in different planes, i.e., the R and T conformers.

Results were analyzed statistically in Excel 2010, SPSS 17.0, and Statistica 8.0. Data are presented as means (*M*) ± standard deviations (σ). Studies of the relationship between dependent sets were performed using the Spearman rank correlation coefficient (*r*). Dependent sets were compared using the nonparametric Wilcoxon test. Pairs of independent sets were compared using the nonparametric Mann–Whitney test (*U* test) and Fisher's test. Differences were regarded as significant with error probabilities of less than 0.05.

Results

The role of K^+ channels in mediating the neuroprotective effect of preconditioning. The results of stage I of the study are presented in Table 2. In conditions of acute cere-

bral ischemia, blockade of K^+_{ATP} channels with glibenclamide (series I, Glib) had no effect on the size of the damage zone. IPre performed 24 h before OMCA (series II, IPre) significantly decreased infarct size, by 37%. In the group of rats receiving glibenclamide 30 min before IPre (series II, IPre + Glib), the size of the necrosis zone was comparable with that in control animals and was statistically significantly larger than that in rats with IPre. Activation of K^+_{ATP} channels with diazoxide 24 h before OMCA (series III, PPre) led to the development of a protective effect analogous to that of cerebral IPre.

Effects of activation and inhibition of K^+_{ATP} channels on the total content of blood NO_3^- and NO_2^- in rats with IS. All groups showed similar dynamics of changes in total venous serum NO_3^- and NO_2^- concentrations (see Table 2): at 5 h after OMCA, the control groups (series I and II) showed statistically significant increases in NO_3^- and NO_2^- concentrations (see Fig. 1); by 24 h and 3 days after modeling of IS, concentrations approached baseline levels or were below baseline (series III). Analysis of between-group differences revealed a link between inhibition of K^+_{ATP} channels with glibenclamide 30 min or 24 h before OMCA and decreases in NO_3^- and NO_2^- concentrations, regardless of preconditioning (series I, Glib; series II, IPre + Glib). Activation of K^+_{ATP} channels with diazoxide had no effect on serum NO_3^- and NO_2^- concentrations (series III, PPre).

Among the control groups, analysis of the relationship between the total concentration of NO metabolites and the size of the ischemic lesion zone demonstrated an inverse correlation. The animals with the smallest necrosis zones had high NO_3^- and NO_2^- levels 72 h after OMCA ($r = –0.556$, $p = 0.003$). However, rats given glibenclamide (the Glib and IPre + Glib groups) showed a direct relationship – the largest necrosis zones were accompanied by high NO_3^- and NO_2^- concentrations 24 h after IS ($r = 0.529$; $p = 0.024$). There were no significant correlations in the groups of animals with IPre or PPre.

Dynamics of changes in blood Hb-NO contents. The blood of rats with IS 24 h after OMCA showed at 42% increase in the level of the R-conformer of Hb-NO complexes (see Fig. 1), with no statistically significant difference from the group of intact animals ($p = 0.078$). For the T conformer, blood Hb-NO complexes in control animals showed the opposite situation (see Fig. 1) – levels were 75% lower than in intact animals at 24 h after OMCA ($p = 0.043$).

Discussion. Chloral hydrate was used as anesthetic – this is a long-acting aliphatic agent. This anesthesia has no neuroprotective action and allows for adequate assessment of the effects of test compounds on lesion size. The model used here stably reproduced IS; the level of MCA occlusion – at the frontal branch – was selected such that necrosis developed in the frontoparietal part of the cortex without affecting subcortical structures, avoiding complications related to visceral functions and death of the animals. Simultaneous coagulation of the veins adjacent to the MCA and ligation of the ipsilateral carotid artery stabilized necrosis zone size and decreased the number of animals used in the experiment.

These studies used the nonselective blocker of plasma membrane and mitochondrial K^+_{ATP} channels glibenclamide, at a dose of 20 mg/kg, and the activator of the same channels diazoxide, both dissolved in DMSO; doses and injection times were determined on the basis of published data [20, 21]. Selection of nonselective agents was based on the fact that in conditions of ischemia, both types of K^+_{ATP} channel are activated in cells. Our studies addressed the suggestion that the activity of different types of NO synthase is primarily affected by the state of plasma membrane K^+_{ATP} channels, while the state of the mitochondrial nitrite reductase systems and NO synthase is affected by the state of mitochondrial K^+_{ATP} channels. The task was to evaluate the complex contribution of K^+_{ATP} channels to regulating the contents of NO and its metabolites in the bodies of the experimental animals.

All experiments were performed 24 h after IPre, the effect being maximal at this time point [3]. Administration of glibenclamide 30 min before OMCA had no effect on necrosis zone size, so blockade of both cellular and mitochondrial K^+_{ATP} channels in conditions of IS is not important for generating damage and the drug itself eliminated the effects of IPre in our experiments. The model of PPre with intraventricular administration of diazoxide was selected as one of the most effective and reproducible, as only a fifth of the diazoxide circulating in the blood can cross the blood-brain barrier (the brain/plasma ratio is 0.20) and have direct actions on K^+_{ATP} channels in brain cells [22, 23].

Levels of NO_3^-, NO_2^-, and Hb-NO were measured in venous serum 5, 24, and 72 h after OMCA. These time points were selected on the basis of the activities of the constitutive and inducible NO synthases on development of IS: 5 h covers the period at which only constitutive NO synthases function, while measurements at 24 and 72 h allow assessment of inducible NO synthase [24].

Assessment of necrosis zone size after preconditioning 24 h before modeling of IS showed that IPre and PPre had identical protective effects. Prior administration of glibenclamide eliminated the protective effect of IPre, which is evidence of the key role of on K^+_{ATP} channels in mediating the protective effects of the delayed phase of preconditioning on the brain in rats.

Analysis of results from measurements of NO_3^-, NO_2^-, and Hb-NO complexes in the blood of rats with IS showed complex changes. Decreases in the levels of Hb-NO in the R conformation were seen during the first 5 h after OMCA; in normal conditions, this conformer accounts for about 90% of the total quantity of Hb-NO complexes. In conditions of decreased tissue oxygen levels and on exposure to various other regulatory factors (2,3-diphosphoglycerate, acidosis, hypercapnia), some of the R-conformers of Hb-NO complexes can release oxygen from the Hb-bound state and

convert to T conformers, which significantly weakens retention of the NO ligand [25–27]. Released NO can be oxidized to NO_3^- and NO_2^-, and consistent increases in these ions were seen in the blood of experimental animals in parallel with decreases in the concentrations of hemoglobin R conformers. Thus, Hb-NO complexes in the R conformation can simultaneously function as a depot and as a carrier and, with the R → T transformation in conditions of ischemia, as an NO donor; NO release can have regulatory actions. We cannot exclude the possibility that the increases in NO_3^- and NO_2^- levels were due to endothelial NO synthase activated by ischemia. NO_3^- and NO_2^- levels normalized by one day after experimental IS. The situation with Hb-NO complexes changed fundamentally: R conformers dominated and blood levels of T conformers became minimal. This T → R transition can be explained as reduction of accumulated NO_3^- and NO_2^- to NO with subsequent formation of Hb-NO complexes, while activation of inducible NO synthase occurs predominantly in damage zones.

At 72 h after OMCA, blood levels showed normalization of levels of Hb-NO complexes, with the R → T transition and release of NO molecules. NO_3^- and NO_2^- levels were not different from baseline. Correlation analysis revealed an inverse relationship between increases in serum NO_3^- and NO_2^- levels in control animals on post-IS day 3 and lesion size. This result indirectly points to the neuroprotective effect of moderate NO production in the vascular bed during formation of the ischemic necrosis zone, which may be linked with NO-mediated vasodilation and improvements in collateral circulation [8].

In animals given glibenclamide, the correlation between necrosis zone size and serum NO_3^- and NO_2^- concentrations one day after OMCA was direct. Published data indicate that microglial cells show increases in the expression of K^+_{ATP} channels in microglial cells [28], while glibenclamide significantly increases the extent of activation of microglial cells, leading to increases in the production of cytokines, NO, and reactive oxygen species (ROS) [29]. Excess production of ROS on the background of NO generation by inducible NO synthase and subsequent involvement of NO in free-radical reactions can explain the decreases in NO_3^- and NO_2^- concentrations one day after IS in rats which had received glibenclamide before OMCA. The NO molecule is a weak nitrosylating agent, though NO activity increases significantly on formation of more aggressive compounds, including superoxide [30], and in reactions with sulfhydryl radicals generated on oxidation of thiols [31]. This may lead to the fact that a proportion of NO molecules are not oxidized to low-toxic NO_3^- and NO_2^- but form complexes with cell components [32]. This results in cell damage and death in the inflammation zone. Further understanding of the mechanisms of these changes requires additional studies.

Thus, K^+_{ATP} channels occupy a key position in the development of the protective effects of preconditioning on the brain in rats with ischemic lesions. Apart from a role in the processes of preconditioning, K^+_{ATP} channels evidently have a special position in regulating the intensity of microglial inflammatory processes in ischemic tissues. Studies of the dynamics of changes in blood NO metabolite levels provide only an indirect view of the actual processes regulating the production of this short-lived molecule. A fuller concept is obtained by complex analysis of components of the NO system, which involves the concept of storage of NO by blood proteins. The link between the K^+_{ATP} channels system and the NO system at the cellular and subcellular levels, as well as the mechanisms controlling the free NO level in the circulation, requires further study.

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