Effect of *In Vivo* Hypoxic Preconditioning on Changes in Intracellular Calcium Content Induced by Long-Term Anoxia in Rat Brain Slices

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We studied changes in intracellular calcium content induced by 10-min anoxia in olfactory cortex slices from rats exposed to single or 3-fold moderate hypobaric hypoxia ("ascend" at 5000 m). Repeated preconditioning with moderate hypobaric hypoxia produced a neuroprotective effect. This treatment abolished pathological calcium overload in brain slices induced *in vitro* by 10-min test anoxia.

Key Words: *hypobaric hypoxia; preconditioning; calcium; brain slices*

Much attention is paid to the phenomenon of brain resistance to ischemia/hypoxia [2,3,9,15]. Moderate hypoxia increases the resistance of brain neurons to further exposure to severe hypoxia or ischemia. The methodical procedure initiating tolerance of the brain to hypoxia received the name "hypoxic preconditioning". Our previous studies showed that long-term (10 min) anoxia of slices of the olfactory cortex from rats induces sustained hyperactivation of NMDA receptors and pathological calcium overload of neurons [13]. Preconditioning of slices with short-term anoxia performed 90 min before long-term anoxia mobilizes the immediate mechanisms of tolerance (including NMDA receptor-mediated mechanisms) and prevents pathological changes induced by long-term anoxia [3, 14]. *In vivo* moderate hypoxia is an adequate model of preconditioning. Successful preventive strategy requires estimating the "dose" of *in vivo* hypoxic preconditioning, *i.e.*, depth and duration of hypoxia and period of the development and persistence of a neuroprotective effect. These studies were previously performed *in vitro* [2-4,15].

Two-step experiment is suitable for the search of optimal regimen of hypoxic preconditioning and study the induced intracellular neuroprotective mechanisms. The animal is subjected to moderate hypobaric hypoxia, and then cultured brain slices are *in vitro* exposed to long-term anoxia [1].

Here we studied whether preconditioning with moderate hypobaric hypoxia can increase the resistance of brain neurons to calcium overload induced by test anoxia in brain slices from experimental animals. We also compared the neuroprotective effects of repeated and single hypobaric preconditioning. We recorded changes in intracellular calcium content induced by test anoxia in brain slices from rats exposed to different regimens of preconditioning with moderate hypobaric hypoxia.

MATERIALS AND METHODS

Experiments were performed on male Wistar-Kyoto rats weighing 190-210 g. The animals were divided into 2 groups. Group 1 rats were exposed to single preconditioning with hypobaric hypoxia. Pressure in a flow altitude chamber was maintained at a level of 380 mm Hg for 2 h (corresponds to an altitude of 5000 m above see level). In group 2 rats this procedure was repeated 3 times with 24-h intervals. Non-preconditioned animals served as the control.

The 2nd stage of the study was performed *in vitro* on the next day after preconditioning. The rats were decapitated and tangential slices of the olfactory cor-

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tex (400 μ) were prepared on an EMS-4000 vibrotome (Electron Microscopy Sciences). The slices were maintained in a flow incubation medium at 37.5°C. Intracellular calcium content was measured spectrofluorometrically in the incubation systems allowing estimation of the concentration of bound (Ca-b) and free intracellular calcium (Ca^{2+}_{cyt}). Changes in the content of Ca-b and Ca^{2+}_{cyt} were studied using a LYuMAM KF contact fluorescent microscope (LOMO) and Hitachi F-2000 spectrofluorometer, respectively. Chlortetracycline fluorescence probe (Sigma) was used to induce the fluorescence signal characterizing Ca-b content. The fluorescence quantum yield was measured in a spectral region with a maximum of 520 nm (excitation wavelength 410 nm). Relative changes in Ca^{2+}_{cyt} content were recorded using fura-2AM fluorochrome (Molecular Probes). These changes were characterized as the ratio between fluorescence signals recorded at 510 nm during excitation at 340 and 380 nm. Both techniques were described previously [13]. *In vitro* anoxia was modeled by replacement of the oxygen-containing medium for the nitrogen-containing medium. O_2 flux above the surface of incubation solution was substituted for N_2 flux. Normoxic conditions were achieved by the reverse manipulation after 10-min anoxic superfusion. The measurements were performed during anoxia and reoxygenation at intervals of 5 and 70 min, respectively. The data are presented as $M \pm m$. The results were analyzed by the Dunnett test (ANOVA).

RESULTS

In slices from control animals Ca-b concentration decreased to 89% of the baseline level during anoxia, increased in the postanoxic period, and attained 117.9±1.1% by the 70th minute of reoxygenation. These changes in Ca-b concentration reflect the initial stage of intracellular Ca^{2+} release in the anoxic phase, which is accompanied by Ca^{2+} entry from the extracellular space and its partial binding during reoxygenation [3,13]. Similar changes were observed in brain slices from group 1 rats. However, in group 2 animals the increase in Ca-b concentration typical of the postanoxic period was abolished after 20-min reoxygenation. Ca-b concentration in these rats increased to 110±0.8% by the 70th minute (Fig. 1, *a*). The anoxic and postanoxic phases of the increase in Ca^{2+}_{cyt} concentration in control slices reflected calcium overload during reoxygenation. Changes in Ca^{2+}_{cyt} concentration in group 2 rats were similar to those in control animals. Single hypoxic preconditioning was insufficient to initiate the neuroprotective mechanisms. However, the postanoxic increase in Ca^{2+}_{cyt} concentration in group 2 animals was abolished after 20-min reoxygenation. By the 70th minute this parameter corresponded to 9.2±0.6% (*vs*. 16.8±1.0% in the control, Fig. 1, *b*).

Our results show that 3-fold hypoxic preconditioning is more effective than single treatment. Probably, the neuroprotective effect became more pronounced with increasing the number of preconditioning procedures (to a certain limit).

In vivo preconditioning model used in our experiments (3-day interval between the first hypobaric session and the test anoxia) probably initiates the long-term mechanisms of neuronal resistance. According to modern notions, these mechanisms are associated with expression of the genetic apparatus in cells and in-

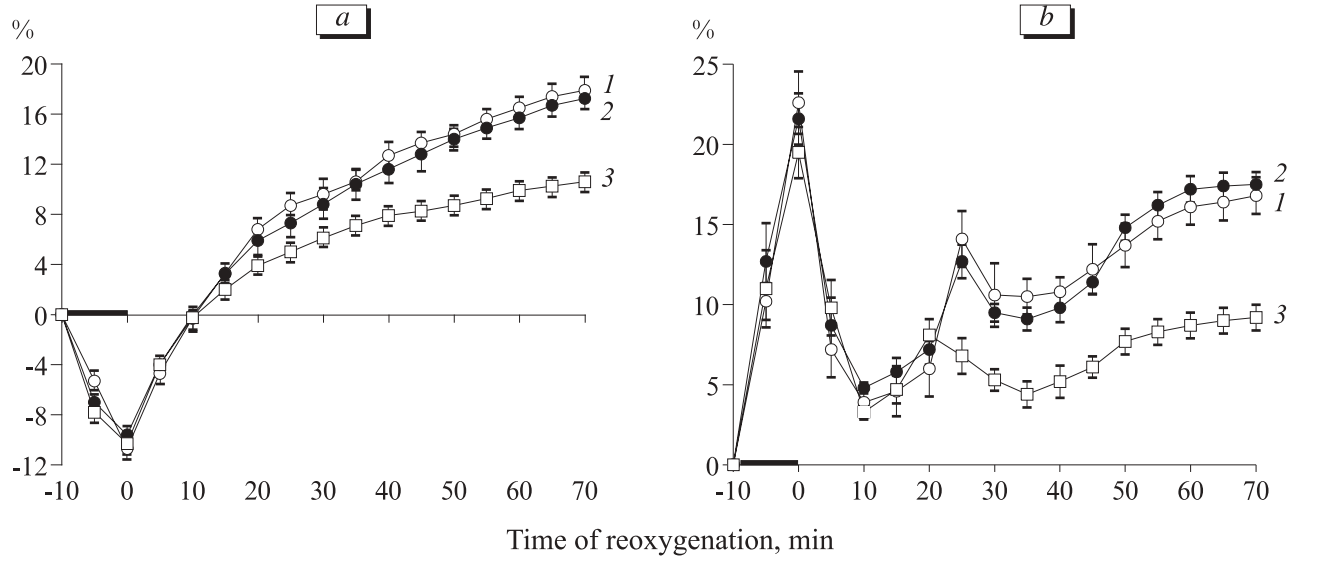

Fig. 1. Anoxia-induced (10 min) changes in the concentration of bound (*a*) and free calcium (*b*) in slices from control rats (*1*) and animals exposed to single (*2*) or 3-fold hypoxic preconditioning (*3*). Black rectangle: period of anoxia. Each curve: *n*=7-9.

creased synthesis of regulatory proteins and peptides [2,3,7]. The mechanism of long-term resistance of neurons to hypoxia involves early genes [5,12], heat shock proteins [6], neurotrophin peptides [11], corticotropin-releasing hormone [8], and opiate peptides [10]. Our results suggest that sufficient preconditioning has a neuroprotective effect and initiates long-term neuronal resistance to hypoxia. It decreases glutamate neurotoxicity mediated by NMDA receptors, typical of severe hypoxia and manifesting in calcium overload and death of neurons.

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