Hypoxic Preconditioning Modifies Activity of Pro- and Antioxidant Systems in the Rat Hippocampus

M. S. Kislin, S. A. Stroev, T. S. Gluschenko, E. I. Tyulkova, M. Pelto-Huikko, and M. O. Samoilov

Pavlov Institute of Physiology, Russian Academy of Sciences, nab. Makarova 6, St.-Petersburg, 199034 Russia
tel.: (812)3280701; fax: (812)3280501; e-mail: anoxia@pavlov.infran.ru
Tampere University, Tampere, Finland

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Abstract — The effects of repetitive mild hypobaric hypoxic preconditioning on pro- and antioxidant systems in rat hippocampus have been studied. It was found that three-trial preconditioning by mild hypobaric hypoxia (360 mm Hg, 2 h) induced moderate oxidative stress immediately after the last preconditioning trial. In addition, it down regulated the levels of protein antioxidants (Trx-1, Trx-2, Cu,Zn-SOD) and also decreased several lipid peroxidation products 24 h after the preconditioning.

Keywords: preconditioning, hypobaric hypoxia, antioxidants, lipid peroxidation, hippocampus

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INTRODUCTION

Mild hypobaric (altitude) hypoxia is widely used in clinical practice for prophylaxis and therapy of various cardiovascular, endocrine, and allergic diseases, bronchial asthma, chronic fatigue syndrome, etc. [1–3]. Hypobaric hypoxia positively differs from other treatment by high efficiency of its adaptogenic action and lack of side effects. The method of hypobarotherapy is based on adaptation to the effect of mild hypobaric hypoxia, which is similar to hypoxia naturally occurring under mountain climate conditions at the altitude of 3.5 km and is known by its positive effect on the organism. At the same time, results of recent basic studies have stimulated the development of the other direction, which employs hypobaric hypoxia in the preconditioning mode. In this case the protective effect is achieved by rapid activation of brain protective mechanisms rather than long term adaptation to rarefied air [4]. Analysis of literature data suggests that hypoxic preconditioning triggers a cascade of signal transduction, which involved intracellular regulatory systems, mitochondria, genome, neuromodulator peptides, and stress proteins providing increased resistance of brain neurons to severe forms of hypoxia [5]. Adaptive activation of glutamategic, calcium, and phosphoinositide regulatory systems, transcription factors and activation of de novo synthesis of antiapoptotic proteins (Bcl, p53, etc.), protein antioxidants, heat shock proteins, glucose and glutamate transporters, aquaporins, connexins, various neurotrophins, growth factors and their receptors play an important role in this process [4–7].

Preconditioning is now used in various experimental systems as an effective non-medicated approach inducing the protective state and also as the method for prophylaxis of post-stress depressive episodes [8]. Hypoxic preconditioning may be also effective for general prophylaxis of post-stress depressive pathologies in humans (especially predisposed to depression) and also for extension of remission and prevention of repetitive episodes in patients with recurrent forms of diseases.

Taking into consideration results of basic studies demonstrating the cross-tolerance effect and clinical observations that those patients with brain infarction who had preliminary episodes of transitory ischemic attack (TIA) were characterized by better recovery of lost functions than patients without such episodes it is reasonable to use hypoxic preconditioning as a therapeutic tool in patients with high risk of ischemic stroke [9].

Results obtained in our laboratory using the model of hypobaric hypoxia have demonstrated that severe hypobaric caused significant disorders in behavior of exposed rats and caused long-term impairments in key molecular/cell processes and oxidative stress [4, 10–12]. We have also demonstrated that three-trial preconditioning by mild hypobaric hypoxia was the best way to compensate alterations induced by subsequent severe hypobaric hypoxia [4, 12].

Intensive aerobic oxidative processes are the characteristic feature of brain metabolism. The central nervous system is characterized by well equilibrated...
balance between pro- and antioxidant systems and this allows free radicals and various oxidation products to operate as signal and regulatory molecules involved in functioning of brain structures and in processes of learning and memory. In addition, oxidative modification of membrane molecules is one of the fastest ways for changes of their physicochemical parameters compensating homeostatic changes induced by dramatic environmental changes.

The aim of this study was to investigate effects induced by three-trial hypoxic preconditioning (mild hypobaric hypoxia) on pro- and antioxidant systems of the rat hippocampus. This included analysis of protein antioxidant expression and time course of lipid peroxidation (LPO) in cell membranes.

**MATERIALS AND METHODS**

Male Wistar rats (200–220 g) were used in experiments. Animals were subjected to hypobaric hypoxia by hypoxic preconditioning at 20–22°C using a flow type barochamber. Animals of the experimental group were exposed to three-trial preconditioning by mild hypobaric hypoxia (360 mm Hg, 2 h/day). The mode of simulated altitude has been described in details by S.A. Stroev et al. [13]. Control animals were exposed to three sessions in the barochamber (2 h/day) at normal atmospheric pressure. Animals were decapitated immediately after the last session of preconditioning, 3 h, and one day after the trial.

**Analysis of Dynamics of Lipid Peroxidation (LPO)**

Changes in various LPO components were evaluated after hippocampus isolation from the brain followed by homogenization and use of various methods [12, 14] applicable for evaluation of activities of prooxidant proteins and key steps of free radical lipid peroxidation. All procedures were performed in the cold and included: spectrophotometric determination of conjugated dienes and trienes and calculation of the Klein oxidation index \( I = \frac{E_{233}}{E_{215}} \); colorimetric determination of lipoperoxides; spectrophotometric determination of thiobarbituric acid reactive substances (TBARS); fluorimetric determination of Schiff bases.

The lipid fraction was extracted by the Folch method. The extract was washed once with cold 0.09% NaCl and twice with the mixture methanol : H₂O : chloroform (47 : 48 : 3) to remove gangliosides and non-lipid contaminations. Results were expressed as units of optical density/fluorescence per 1 mg of phospholipids determined by the Bartlett method or per 1 mg of protein determined by the Lowry method.

**Analysis of Protein Antioxidant Expression**

The number of neurons expressing Trx-1, Trx-2, and Cu,Zn-SOD was determined by the immunocytochemistry method 24 h after the final preconditioning session. Primary antibodies used in these experiments were generous gifts from Dr. Yumiko Nishinaka and Professor Junji Yodo (Trx-1), Professor Giannis Spyrou (Trx-2), Dr. Ling Yi and L. Chang (Cu,Zn-SOD).

For immunocytochemistry analysis anesthetized animals were perfused transcardially with 100 mL of saline, then with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3 (for 4–5 min). After the perfusion animals were decapitated and removed brain was fixed during 60 min in the same fixation solution. Before analysis brain samples were kept at +4°C in 0.1 M phosphate buffer containing 15% sucrose, pH 7.3.

Brain tissue was frozen in a Tissue-Tek® O.C.T™ (Sakura Finetek, USA) and immediately sectioned at −20°C using a microtome cryostat; brain frontal sections (11 µm thick) were made at the level of hippocampus and basolateral amygdala (bregma −2.80 mm) [15]. Sections mounted on poly-L-lysine (Sigma, USA) coated slides were initially preincubated with 1% bovine serum albumin (BSA, Boehringer Mannheim GmbH, Germany) for 15 min and then overnight (at +4°C) with primary polyclonal affinity purified rabbit antibodies to the investigated antioxidants: mouse Trx-1, rat Trx-2 or bovine Cu,Zn-SOD [16]. After a triple wash with 0.1 M phosphate buffer, pH 7.3 (15 min each), sections were incubated with biotinylated goat secondary antibodies (Vector Labs, USA) for 30 min at room temperature. After the repeated triple wash in the phosphate buffer sections were incubated with the avidin–biotin complex (Vector Labs) for 30 min at room temperature. Immune reaction and localization of the investigated antioxidants were visualized using diaminobenzidine.

Quantitative analysis of neuron immunoreactivity was performed using the system consisted of a Nikon Microphot–FXA microscope (Japan), a PCO Computer Optics GmbH camera (Germany) and an IBM PC computer with downloaded programs Image-Pro Plus (Media Cybernetics, USA) and Morphix [17]. The levels of antioxidant expression were determined in neurons of the hippocampus regions CA1, CA2 and CA3 and dentate gyrus (DG) by the number of neurons expressing each antioxidant protein. Neurons were considered as expressing ones when their optical density on digitalized microimages exceeded optical background. The image analysis was performed at the distance of 500 µm in six sections of each brain.

All experimental results were treated statistically using the Origin 6 software package (OriginLab Corporation, USA). Neurochemical data obtained for rats from different groups were compared using the Student’s t test. Differences were considered as statistically significant at \( p = 0.05 \). The statistical treatment of immunohistochemistry data was performed by one-way ANOVA.