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EXPERIMENTAL ARTICLES

The PI3K/Akt System Is Involved in the Neuroprotective Preconditioning of Rats with Moderate Hypobaric Hypoxia

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Abstract—Protein kinase B (Akt) is a key enzyme in one of many neuroprotective signaling cascades in neurons that are activated during preconditioning by episodes of moderate hypoxia/ischemia. However, the data on the involvement of this anti-apoptotic pathway in mechanisms of the hypoxic tolerance induced by different experimental protocols are incomplete and contradictory. We exposed rats to moderate hypobaric hypoxia (3MHH) corresponding to an altitude of 5000 m above sea level three times (once a day for 2 hours). The 3MHH treatment is known as an effective stimulus for hypoxic brain tolerance. We used immunocytochemistry to study the dynamics of phosphorylation of Akt caused by 3MHH in various areas of the brain. It was found that each of the three 3MHH episodes quickly (within 3 hours) activated Akt phosphorylation in the neocortex, piriform cortex, and dentate gyrus of the hippocampus. Severe hypobaric hypoxia (SHH), the equivalent of a rise to 11000 m above sea level, did not produce this effect. Behavioral experiments on 3MHH-preconditioned rats subjected to subsequent SHH have shown that blocking the activity of PI3K/Akt with wortmannin during preconditioning had an anxiogenic effect typical of non-preconditioned animals that survived the SHH. Thus, we established the details of the involvement of the PI3K/Akt pathway in the neuroprotective mechanism of 3MHH-preconditioning for the first time.

Keywords: hypobaric hypoxia, preconditioning, PI3K/Akt, brain, cortex, hippocampus, immunocytochemistry, behavioral tests

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INTRODUCTION

During last 20 years, the phenomenon of the neuroprotective effects of hypoxic preconditioning on brain neurons has been repeatedly confirmed in experiments using different in vivo and in vitro models [1–3]. However, there is still no consensus on whether the mechanism of this phenomenon is non-specific and common to all types of moderate hypoxia/ischemia, or each model reveals different components of this apparently complex mechanism. Moderate hypobaric hypoxia (MHH) is a preconditioning factor that is well established in experiments both in vivo and in vitro [4]. The neuroprotective efficacy of MHH has been proven in a series of long-term studies where the effect of MHH was subsequently tested by severe hypobaric hypoxia (SHH). In particular, it was demonstrated that rats subjected to SHH had structural damage of neurons in the neocortex and hippocampus, impaired learning and memory, altered expression of early genes and transcription factors, and activation of pro-apoptotic factors. In animals preconditioned with series of 3 MHH sessions prior the SHH the subsequent negative effects were significantly reduced or prevented [5–8].

In studies on the possible mechanisms of hypoxic tolerance induced by 3MHH-preconditioning, the phosphoinositide system of intracellular regulation draws special attention [9]. We have previously studied the phosphoinositide-dependent signaling pathway mediated by stimulation of group I metabotropic glutamate receptor (mGluRs1,5) as one of the possible components of the induced neuronal tolerance mechanism. It was found that 3MHH activates this pathway and leads to a moderate adaptogenic increase in the intracellular Ca^{2+} [10, 11]. Another pathway, which does not involve hydrolysis but the additional phosphorylation of polyphosphoinositides with phosphoinositide 3-kinase (PI3K), triggers the activation of protein kinase B phosphorylation (Akt), and triggers a cascade of proadaptive and antiapoptotic signals [12, 13]. According to many authors the signaling precursors of PI3K activation include mGluRs1,5, whose

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Abbreviations: MHH, moderate hypobaric hypoxia; 3MHH, a procedure of three MHH episodes with 24 h intervals; SHH, severe hypobaric hypoxia; Akt, protein kinase B; pAkt, phosphorylated (active) Akt, PI3K, phosphoinositide 3-kinas; EPM, elevated plus maze; NC, the number of entries to closed arms; NCO, the number of entries to the central and open parts; TCO, time spent during performance of NCO; NR, the number of rearings; OF, open field; VMA, vertical motor activity; HMA, horizontal motor activity; FD, freezing duration.

activation leads to the organization of the protein complex mGluRs/PIKE-L/Hommer/IP3Rs and activation of PI3K/Akt-signalling in addition to the moderate release of Ca^{2+} [14, 15].

It appears that the activation of Akt reaches the maximum when the phosphorylation of two of its sites, S473 and T308, occurs [16]. However, detection of Akt phosphorylation only at S473 is apparently sufficient to confirm its activation as a key enzyme that protects cells from ischemic damage and apoptosis [13, 17]. An analysis of the literature showed that there is no established opinion on the extent and kinetics of Akt system involvement in the mechanisms of hypoxic tolerance of neurons and the specificity of the mechanism for different parts of the brain. Using different in vivo and in vitro models of hypoxic and ischemic impacts and preconditioning types, different authors have shown the involvement of the Akt system in the mechanisms of induced tolerance of neurons to ischemia and described the latency of its activation from hours to several days depending on the studied model [18–20]. Other authors have questioned the participation of the Akt system in the mechanisms of ischemic preconditioning of the brain [1]. In the 3MHH-preconditioning in vivo model, the dynamics and intracerebral localization of Akt phosphorylation have not been fully studied.

The purpose of our study was to establish the fact and dynamics of the PI3K/Akt system involvement in the formation of hypoxic tolerance of neurons in the brain induced by preconditioning in vivo using the 3MHH procedure. Therefore, here we (1) determined changes in the levels of immunoreactivity of Akt phosphorylated at S473 (pAkt) in different brain regions of rats after 3MHH, SHH, and 3MHH + SHH and (2), in a separate series of experiments, blocked the phosphorylation of PI3K during the 3MHH-preconditioning procedure, and then applied SHH to assess the impact of the blockade on the effectiveness of preconditioning in behavioral tests.

MATERIALS AND METHODS

Animals. The study was performed with adult male Wistar rats weighing 180–200 g, grown under standard vivarium conditions of the Pavlov Institute of Physiology of the Russian Academy of Sciences. Under laboratory conditions, they were kept in standard cages of six animals with free access to food and water and a 12/12 hours lighting mode. All procedures related to the animals keeping, surgical interventions, and material sampling were performed in compliance with the directives of the Council of the European Community (86/609/EEC) regulating the animal use for experimental research. Experimental protocols were approved by the Commission of the Pavlov Institute of Physiology of the Russian Academy of Sciences on humane treatment of animals.

Hypobaric procedures. To accomplish the first task, the hypobaric exposure was performed as a daily MHH procedure repeated three times with 24 h intervals. Each MHH session was a 2-hour exposure of animals in a flow type vacuum chamber (KG 2-760/450 Taganai, Barogipoksiya, Russia) at the atmospheric pressure of 360 mmHg, which corresponds to an altitude of 5000 m above the sea level. A day after 3MHH, some groups of animals were exposed to SHH (3-hour exposure at a pressure of 180 mmHg equivalent to an altitude of 11000 m) or to SHH without 3MHH-preconditioning. To achieve hypobaric conditions, a slow stepwise decrease in pressure was used, when each 1000 m of altitude was accompanied by an increasing pause (from 1 minute at 1000 m to 20 min at 10000 m). The speed of "ascent" and "descent" did not exceed 4 m/s [21]. Decapitation and gathering brain samples for immunohistochemical studies were carried out at different time points after the completion of hypobaric procedures.

To accomplish the second task, animals were injected with an antagonist of PI3K, wortmannin, during 3MHH preconditioning to block the Akt activation system; 1 day later they were placed in SHH conditions; behavioral tests were then conducted.

Immunocytochemical study. To identify the dynamics of Akt phosphorylation under various combinations of hypobaric effects, brain samples of the control group and 11 experimental rats were analyzed by immunocytochemistry. The control group was not exposed to hypobaric effects. Experimental groups 1–7 included rats that underwent one, two, or three MHH sessions with extracting brain samples 0.5 and 24 hours after MHH (and at additional 3 hours after 3MHH); groups 8 and 9 included rats exposed to both 3MHH and SHH at 1 day intervals (samples gathered 3 and 24 h after SHH), and groups 10 and 11 included rats exposed to SHH only (samples gathered 3 and 24 h after SHH) (Table 1).

Samples of the brain were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (V/V) for 24 hours. Then, after routine histological processing, samples were embedded in paraffin and cut into slices of 7 μm on a microtome. After deparaffinization, sections were incubated at 4°C with polyclonal rabbit antibodies for pAkt (S473). This site is specific for cytoplasmic protein kinase Akt1 isoforms [22]. The sections were then treated with secondary antibodies using a Rabbit ABC Staining System kit. To visualize immunopositive areas or individual cells, diaminobenzidine staining was performed. Sections were examined under a light microscope (Carl Zeiss, Germany) using a video camera (Baumer Optronic, Germany) and ImageJ MacBiophotonic program (NIH, USA). The main objects of interest were various areas of the hippocampus and cortex.

In the hippocampus, the pAkt immunoreactivity level was quantified by optical density at the selected areas in the DG and CA (1–4) that contain principal neurons in comparison with immunonegative regions of neuropil. In the neocortex (layer V) and the piriform cortex (layer II), intensely stained cells were counted, whose immunoreactivity exceeded a predetermined threshold, at an area of 1200 × 400 μm and 600 × 160 μm, respectively. Immunoreactivity values obtained in the symmetric parts of the two hemispheres for each slice were averaged.

Behavioral studies. At the beginning of the experiments, 28 rats were placed in cages where they originally stayed during all phases of the experiment. After implantation of microcannulas (see below) and a 6-day recovery period, 26 animals that successfully underwent surgery were subjected to the 3MHH series, during which 14 rats received microinjection of wortmannin (WORT group) and 12 were treated with a placebo (SHAM group). At 1 day after 3MHH the animals were subjected to SHH; six animals of the WORT group and three animals of the SHAM group died during SHH. Starting from the following day, the behavior of the surviving animals (eight from the WORT and nine from the SHAM group) was evaluated in the "elevated plus maze" and "open field" tests using the appropriate standard tools (Open Science, Russia).

Implantation of microcannulas and microinjection protocol. Microcannulas were made from an injection needle with an external diameter of 0.7 and 10 mm in length. For implanting and fixing microcannulas, animals were anesthetized with Zoletil (3 mg/kg) and Rometar (0.05 mg/kg). After exposure of the skull, a hole 1 mm in diameter was drilled using the stereotactic atlas coordinates for rats (S0.8, L1.8 from bregma) for insertion of the cannula. In addition, a 1.2 mm diameter hole was drilled in 1.5 mm from the cannula to insert a stainless steel screw (1.6 mm in diameter and 3 mm in length). Microcannulas fixed in the vertical arm of a manipulator were then implanted to a depth of 2.5 mm from the dura mater. Its outer parts were connected with the head of the screw using a light-curing plastic material (filling material Valux Plus, 3M ESPE, USA); after solidification, microcannulas were freed from the fix, and the musculocutaneous cut was sutured. Wortmannin microinjection into the lateral ventricles of the brain was carried out according to the standard protocol [23, 24]. Either a 3 μL wortmannin 2 mM solution in 2% DMSO, or the same volume of 2% DMSO were injected. The injections were carried out for 20–30 min before each of the 3 MHH sessions without immobilization of the animal. After the end of the behavioral experiments the animals were decapitated; the brains were extracted to confirm the position of the tip of microcannulas in the lateral cerebral ventricle.

Table 1. Treatments and corresponding groups of histologic samples for the immunocytochemical analysis

Group	Number of rats	Hypobaric treatment	Time of sample collection, hours after treatment
Control	12	–	–
1	4	1MHH	0.5
2	4	1MHH	24
3	4	2MHH	0.5
4	4	2MHH	24
5	4	3MHH	0.5
6	6	3MHH	3
7	10	3MHH	24
8	6	3MHH + SHH	3
9	6	3MHH + SHH	24
10	6	SHH	3
11	6	SHH	0.5

Behavioral testing in the elevated plus maze. The elevated plus maze behavioral test (EPM) was performed using a standard EPM apparatus for rats (arm length 50 cm, width of arms 14 cm, height of the walls of the closed arms 30 cm, height above the floor 90 cm). After placing a rat into the central part of the EPM the following parameters were recorded for 5 min: the number and duration of the time spent in the closed, open, and central regions; the number of defecations; the number of rearings and head dips, and the duration of grooming. Four valid indicators were chosen during the preliminary assessment of the results of the experiment: (1) the number of visits to closed arms (NC); (2) the number of visits to the central part and the open arms (NCO); (3) the relative time spent in the central and open parts (TCO); and (4) the number of head dips (NH). To represent the parameters on the same graph, TCO values were multiplied by 10. The EPM test was carried out once, 1 day after the completion of the 3MHH + SHH procedure. The averaged parameters of the SHAM and WORT groups were compared.

Behavior in the open field test. The behavioral open field test (OF) was performed using a standard circular arena made of white plastic 97 cm in diameter, with walls 42 cm in height, and 100 lx lighting. The OF arena was divided into sectors, so that it consisted of a central circular region, with 6 sectors in the middle and 12 sectors near the walls. The area of the parts was approximately 130 cm^2. At the beginning of the test, an animal was placed in the center circle and the following parameters were recorded for 5 min: the number of

crosses of the central field, middle, and peripheral sectors; the latency of an exit from the center of the field; the number of rearings with and without climbing; the number and duration of acts of grooming and freezing; the number of defecations; the preferred area (one-third of the field) and the proportion of the total activity that occurred there. Out of these parameters, we selected only parameters that were changed after the 3MHH + SHH procedure for the analysis: (1) the total number of rearings as a parameter of the vertical motor activity (VMA); (2) the total number of crossings of the sectors as a measure of horizontal motor activity (HMA); and (3) the total freezing duration (FD) as a parameter of the anxiety level. The OF test was performed four times in both groups: before implantation of microcannulas (the initial testing) and 2, 3, and 4 days after the 3MHH + SHH procedure. For each of the surviving rats, values from the initial testing were subtracted from the post-hypoxic test performance values. These differences were averaged for each of the two experimental groups and compared with each other.

Mathematical analysis of the results. The statistical analysis of immunocytochemical data was performed using one-way ANOVA (Statistica 7.0, Statsoft Inc., United States) with the significance level $p < 0.05$. All of the results and the standard error of the mean from the experimental groups with the analysis of either the optical density in a selected area (for the hippocampus) or the number of immunopositive neurons (for the cortex) are presented on the figures as a percentage of the mean in the respective control group, which was taken as 100%. In behavioral experiments in the EPM, mean normalized parameters of the experimental group were compared with the parameters of the control group. The results are presented as the mean ± SEM. The significance of the differences was established with a probability of $p < 0.05$. In OF experiments, the individual differences between the values from the repeated tests and values measured in the initial test were calculated for each animal. In two groups of animals (WORT and SHAM), we compared the mean values of the differences of repeated tests with the value of the initial test, which was taken as 0. The analysis was performed the same way as in the EPM test.

RESULTS AND DISCUSSION

The effect of 3MHH-preconditioning on Akt phosphorylation was evaluated by analyzing pAkt immunoreactivity in brain samples taken at different time intervals after the 3MHH procedure. We found significantly increased pAkt levels in the neocortex and the dentate gyrus of the hippocampus within 3 hours after 3MHH, and 1 day after pAkt level decreased to the baseline level (Fig. 1a). The same tendency was found in the hippocampus but the changes were not significant (data not shown). This result showed that Akt is activated in certain areas of the brain for a short period after a series of MHH procedures (Fig. 1b).

Additionally, a series of comparative studies of the dynamics of Akt phosphorylation in animals subjected to SHH after 3MHH-preconditioning or without preconditioning was performed. This approach allowed us to characterize the state of the Akt system at the beginning of the behavioral experiments described below. The analysis of the samples revealed that in the non-preconditioned animals after SHH no significant differences in the levels of Akt phosphorylation were detected in the neocortex and the dentate gyrus in comparison with the baseline levels. In 3MHH-preconditioned animals, an increase in the pAkt level was found after SHH, although it was less pronounced and slower than after 3MHH. These data are also shown in the Fig. 1.

Figure 1 demonstrates a significantly increased level of pAkt in the first post-hypoxic sample (3 h). It was of interest to illuminate two issues: whether this is the earliest period for the peak of post-hypoxic phosphorylation and whether it was induced by the entire 3MHH procedure or the process had been developing during this process. To answer these questions, an additional series of experiments with exposure to 3MHH and taking samples 0.5 and 24 hours after each of the three sessions was performed. This study was also necessary for the development of the protocol for a further series of experiments with pharmacological inhibition of Akt phosphorylation. The analysis of the results led to the conclusion that in the dentate gyrus and piriform cortex the pAkt level increased in 30 min after each hypobaric session; in the neocortex this was observed only after the first session (Fig. 2). Moreover, this does not exclude the possibility that the most intense phosphorylation can occur not as a result but during the 2-hour MHH session. To confirm this possibility, a special investigation is required.

The results show that the first presentation of MHH caused a rapid and significant increase in the phosphorylation of Akt, which declined in 24 hours of reoxygenation. The next MHH session resulted in the same increase in immunoreactivity of pAkt in the piriform cortex, a decrease in the dentate gyrus, and no changes in the neocortex. After the third MHH session, no peak of phosphorylation was found at the 30 min point in all three regions of the brain; at 24 h, the pAkt levels did not exceed the initial values. However, previously, it was found that triple but not single MHH had a neuroprotective effect [25, 4]. We explain this apparent contradiction by the fact that the phosphorylation of Akt is a short-term trigger followed by the expression of effector "downstream" elements of

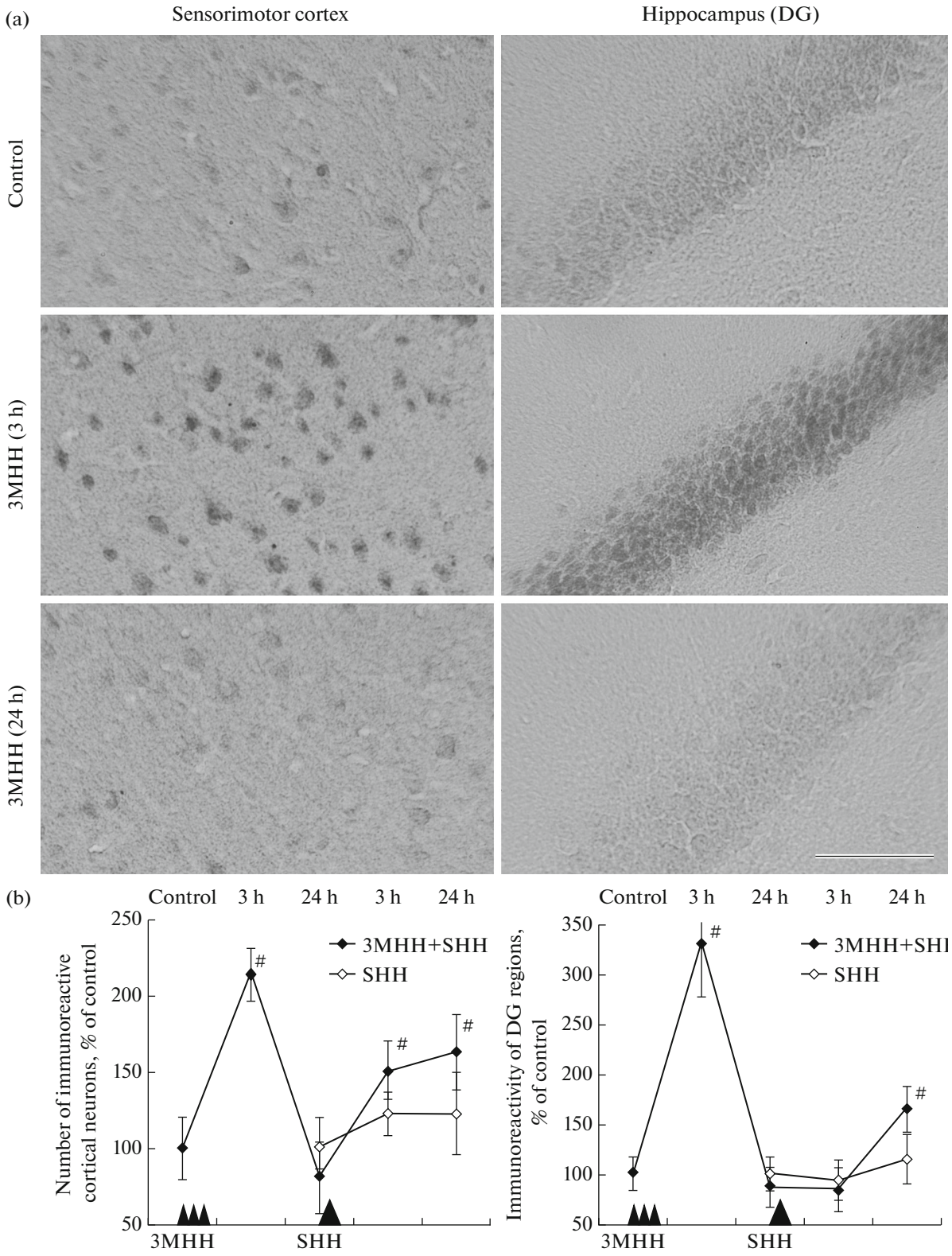

Fig. 1. (a) Representative microphotographs of pAkt immunoreactivity in neocortical areas (left) and the hippocampal dentate gyrus (right) in the controls and 3 and 24 hours after 3MHH. The scale is 100 μm. (b) The dynamics of pAkt immunoreactivity during preconditioning and subsequent exposure to SHH (3MHH + SHH group) in comparison with the dynamics after SHH without preconditioning (SHH group). Left panel, the neocortex (the number of immunoreactive neurons); right panel, the dentate gyrus of the hippocampus (immunoreactivity in the selected regions). The data are presented as the mean values ± standard error of the mean. # Marks the significant differences from the control at $p < 0.05$.

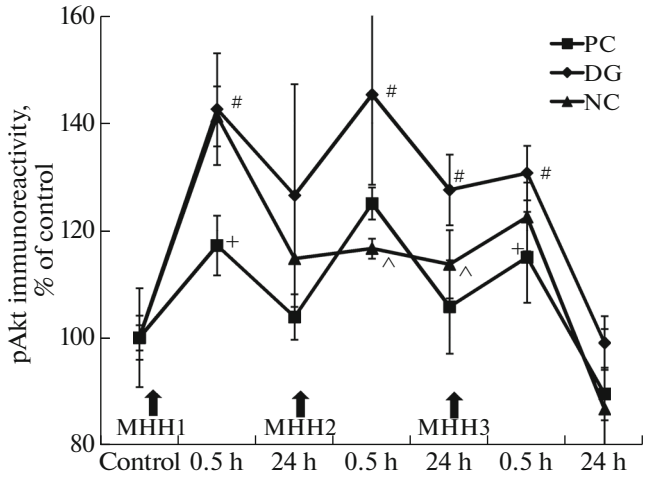

Fig. 2. The dynamics of pAkt immunoreactivity in separate 3MHH sessions in the hippocampal dentate gyrus (DG), piriform cortex (PC), and neocortex (NC). #, ^, + mark significant differences in each group compared to the respective control at $p < 0.05$.

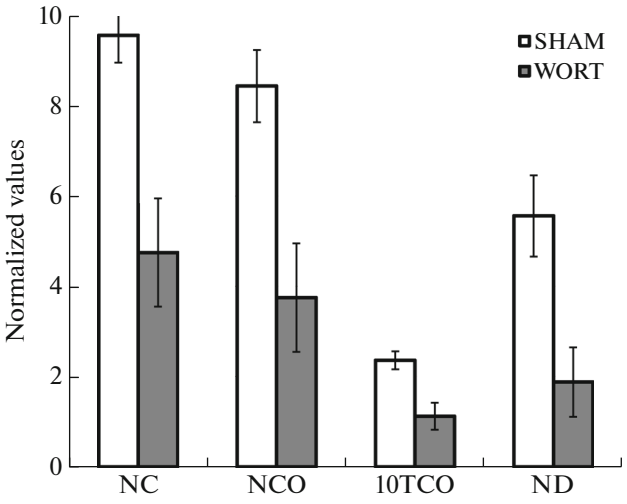

Fig. 3. A comparison of the four parameters of exploratory behavior in the EPM in two groups of animals subjected to 3MHH + SHH. Here and in Fig. 4: SHAM ($n = 9$) and WORT ($n = 8$) are groups of animals that received placebo or wortmannin microinjections during 3MHH, respectively. NC is the number of visits to closed arms; NCO is the number of visits to the central part and open arms; 10TCO is the total proportion of time spent in the central area and in the open arms multiplied by 10; ND is the total number of head dips from different parts of the EPM. The data is presented as the mean ± standard error of the mean. All values of both groups differ significantly from each other at $p < 0.05$.

the neuroprotective cascades [26], first of all, CREB/Bcl-2 [27]. With the development of their "full" expression, the role of Akt phosphorylation as a trigger mechanism is reduced.

Since the cause of the enhanced Akt phosphorylation during the 3MHH procedures may be the activation of its "upstream" PI3K kinase, we had to investigate the impact of the inhibition of this kinase with wortmannin on the effectiveness of 3MHH-preconditioning. The Akt phosphorylation dynamics both after and during the 3MHH procedure (Figures 1 and 2) suggests that wortmannin should be injected before each of the three MHH sessions.

In this series of experiments, some animals did not survive after SHH; the mortality in the WORT group was 43% and that in the SHAM group was 25%. These percentages indicate that the suppression of Akt phosphorylation during preconditioning (WORT group) apparently caused the attenuation, if not the elimination, of the neuroprotective effect of the 3MHH procedure (SHAM group). It should be noted that in previous studies with the same model of hypobaric exposure conducted on a significantly greater number of rats, the following average levels of mortality were found: after SHH it was 50%, while after 3MHH + SHH it was 15% [7].

A comparison of the behavior of SHAM and WORT animals that survived after the 3MHH + SHH procedure showed that the WORT group had significantly lower values of the parameters of exploratory behavior in the EPM (Fig. 3), which probably indicated a high level of anxiety of these animals compared with the SHAM group that received full preconditioning.

The testing of animals in the OF showed that the parameters of the exploratory behavior in the WORT group were lower than in the SHAM group. This difference in the HMA became significant by the third day after the SHH, and in the VMA, by the second day. However, freezing, as a parameter of the anxiety level, was significantly higher in the WORT group than in the SHAM group, starting from the third day after 3MHH + SHH (Fig. 4).

In a similar model of hypobaric preconditioning, its protective effect against the depressive behavior has recently been found in rats exposed to chronic unexpected mild stress. The effect occurred as prevention of depressive or anxious behavior in the EPM and OF, death of neurons in the hippocampus, reduced neurogenesis, and suppression of neurotrophin BDNF secretion [28], which is one of the "upstream" activators of Akt-signaling. In the described model, a decrease in the exploratory activity and an increase in anxiety in rats after SHH were found. These effects were inhibited by 3MHH-postconditioning [29]. In the model of post-traumatic stress disorder (PTSD) in rats, a decrease in BDNF levels in different parts of the brain was found and 3MHH-preconditioning prevented this effect and eliminated the symptoms of PTSD [30].

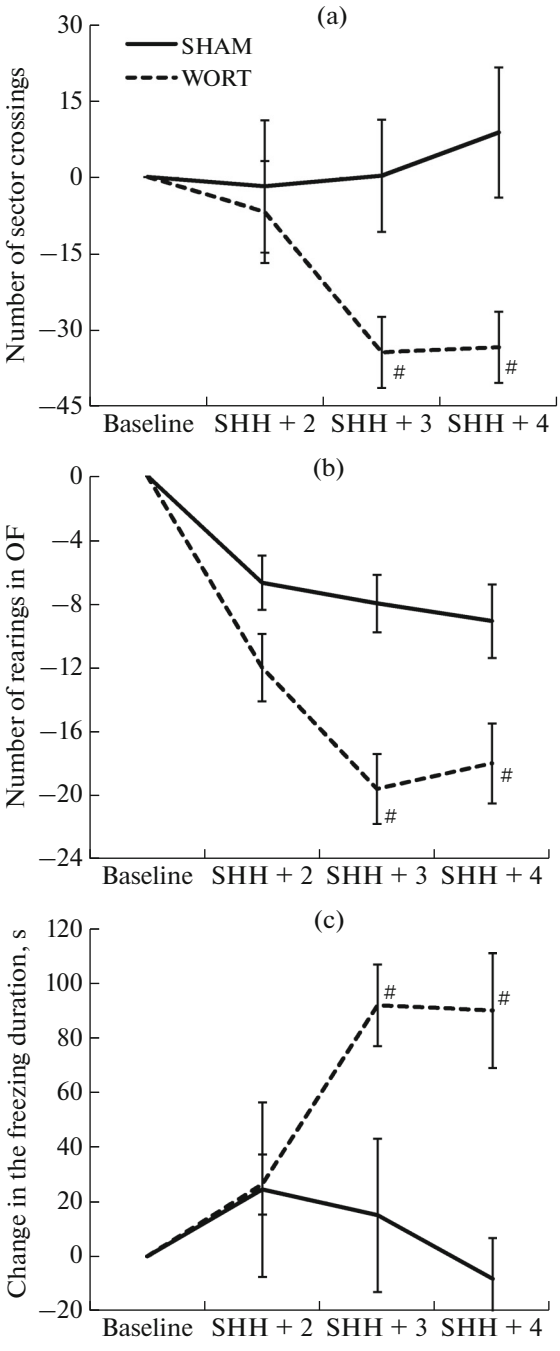

Fig. 4. The dynamics of horizontal locomotor activity (a), the vertical motor activity (b), and the freezing time (c) in the two groups of animals in the OF during 4 days after 3MHH + SHH. The mean values of the individual differences between the values of HMA, VMA, and FD determined after 3MHH + SHH and the values measured during the initial testing are shown. The data are presented as mean ± standard error of the mean. # Marks significant differences between the two groups at $p < 0.05$.

CONCLUSIONS

Our results showed for the first time that preconditioning of rats by three MHH sessions with 24-hour intervals caused early short-term activation of Akt phosphorylation. This phosphorylation was detectable by immunocytochemistry in the cortex and dentate gyrus of the hippocampus during the period from 0.5 to 3 hours after each session. This pattern of activation of the PI3K/Akt system may trigger known neuroprotective signaling pathways that form the hypoxic (ischemic) tolerance of the brain in preconditioned animals. Here, the hypothesis on the involvement of PI3K/Akt system in the mechanisms of hypobaric preconditioning is supported by the reduced survival of rats after SHH if 3MHH-preconditioning was performed in the presence of wortmannin, a blocker of the PI3K/Akt system. This hypothesis is also supported by the results of behavioral tests that showed the suppression of the exploratory activity and anxiety increase in WORT group compared to the SHAM group. Further studies of Akt-dependent mechanisms of 3MHH-preconditioning should be aimed at elucidation of signaling pathways that initiate the phosphorylation of PI3K in this model and identification of activated transcription factors, genes, and their encoded adaptive proteins.

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