Role of HIF-1 in Neuronal Mechanisms of Adaptation to Psychoemotional and Hypoxic Stress

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ABSTRACT:Using quantitative immunohistochemistry, neuronal expression of α-subunit of the transcriptional factor HIF-1 was studied in the hippocampus and the neocortex of rats in response to pathogenic psychoemotional stress (model of posttraumatic stress disorder, PTSD) and hypoxic stress (severe hypobaric hypoxia, 180 mm Hg, 3 h), as well as following neuroprotective hypoxic pre- and postconditioning. Prolonged overexpression of HIF-1α in the hippocampus and the neocortex of rats in response to the psychoemotional stress in PTSD paradigm, but not hypoxic stress, has been observed. Hypoxic pre- and postconditioning with mild hypobaric hypoxia (360 Torr, 2 h, 3 trials spaced at 24 h), which promotes adaptation to the psychoemotional stress, was shown to abolish the prolonged HIF-1 α overexpression. In addition, hypoxic postconditioning up-regulated HIF-1α expression in the brain neurons of rats survived severe hypoxia by improving the structure and functional rehabilitation after severe hypoxic stress. The results obtained indicate that a transcription factor HIF-1 is particularly involved in the processes of adaptation/maladaptation to the action of injurious stresses, but its role depends upon the nature of a stressor.

KEY WORDS: hypoxia-inducible factor HIF-1α, brain, psychoemotional stress, hypoxic stress, adaptation, hypoxic preconditioning, hypoxic postconditioning

I. INTRODUCTION

The damaging effect of severe hypoxia on brain neurons is well known. Hypoxia-induced structural and functional abnormalities, and neuron death in vulnerable structures of the brain (hippocampus, cortex) are considered as one of the most common causes of neurological and neurodegenerative disorders.[19] However, based on our own studies and literature, information has been accumulated that mild hypoxia can have beneficial effects instead of deleterious ones on the brain, helping to mobilize endogenous neuroprotective processes and mechanisms.[7,8,17] In particular, mild hypobaric hypoxia (MHH, 380 Torr, for 2h, in three episodes) increased the resistance of brain neurons to damaging impacts, leading to the formation of hypoxic/ischemic brain tolerance. The process involved specific hypoxia-inducible transcription factor 1 (HIF-1).[7]

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However, mild hypoxia also exerts significant anxiolytic and antidepressant effects on behavior by preventing the development of anxiety and depression, induced by intense psychoemotional stress in rats.[3-5] A similar anxiolytic effect has been recently described for a mild normobaric hypoxia (8% O_2, 2 h, three episodes) in mice,[14] wherein in the brain, up-regulation of mRNA expression of the vascular endothelial growth factor (VEGF) and adrenomedullin, HIF-1 target genes, was observed. On this basis, we can assume that HIF-1 is likely to play an important role in the formation of the brain tolerance not only to hypoxia-ischemia, but also to the psychoemotional stress, but this question has been almost unexplored yet.

HIF-1 is a key component in the cellular and systemic homeostatic responses to hypoxia.[2,9,26] The α-subunit of the heterodimer (HIF-1α) is oxygen-sensitive, and it is stabilized under hypoxia, performing a specific function in the regulation of genome expression, while the β-subunit of the latter (HIF-1β) is constitutively expressed.[11] HIF-1α is activated at physiologically important sites that regulate oxygen metabolism pathways, providing quick and adequate response to hypoxic stress by having an effect on target genes that regulate the process of angiogenesis, vasomotor control, energy metabolism, erythropoiesis and apoptosis.[10,13,18,22,23,26,27,31] Along with that, HIF-1 is involved in the processes of death / cell survival, glucose metabolism, cell migration and invasion, cell remodeling of microenvironment, carcinogenesis, metastasis, and many others, thus presenting a promising therapeutic target in many diseases.

Recently, we have demonstrated HIF-1α induction in response to severe psychoemotional stress, and the profile of HIF-1α activation turned out to be quite complex.[1] In an experimental model on rats, severe psychoemotional stress, which resulted in the development of post-stress depression, induced delayed expression of HIF-1α in the hippocampus and the hypothalamus, which is probably involved in the mechanisms of the pathogenesis of depression. That fact was also confirmed in experiments using preconditioning mode of MHH, when the preliminary MHH action in three episodes prevented the development of post-stress depression and significantly modified the expression profile of HIF-1α in the brain structures in response to stress. The latter enhanced the activation of this factor expression in the early period (24 h after stress) and neutralized its delayed overexpression (5 – 10 days after stress). Thus, it appears that HIF-1 is involved in improving the sustainability of the brain (and the body as a whole) to psychoemotional stress and in the formation of post-stress disadaptive states, but its role in the mechanisms of adaptation / pathology is quite complex and requires more detailed study. The present study, devoted to further analysis of just this question, was conducted in an experimental model of post-stress anxiety disorder (post-traumatic stress disorder, PTSD), emerging as a distant response to stress, and being characterized by a specific delayed dynamics.[16] Previously, we have found that an exposure to MHH in the modes of pre- and postconditioning (i.e. before or after the pathogen stress) exerts a significant protective effect, completely correcting the development of PTSD in this experimental model.[4,5]

The work was aimed at studying the possible role of HIF-1 in the development of PTSD-induced anxiety pathology and in stress-protective and anxiolytic effects of hy-

poxic pre- and postconditioning, as well as a comparative analysis of the features of HIF-1α expression in the rat neocortex and the hippocampus in the remote period after psychoemotional and severe hypoxic stress and in neuroprotective effects of MHH.

II. METHODS

The experiments were carried out on male Wistar rats weighing 200 – 250 g with the requirements, set out in the Directives of the Council of the European Communities (86/609 / EEC) on the use of animals for experimental research. Protocols of experiments were approved by the Commission on the Humane Treatment of Animals, I.P. Pavlov Institute of Physiology, Russian Academy of Sciences, Russia. An experimental PTSD was made using "stress – restress" model in rats,[16] which is currently considered the most adequate model of PTSD. The rats were subjected to severe combined stress, consisting of two-hour long immobilization, the forced swimming (20-min) and, after a 15-min long break, the ether-induced stress up to total immobility. This superintense psychoemotional (traumatic) stress evokes anxiety psychopathology in rats, which is based on the impaired stress reactivity in conditions that are associated with the reminiscence of traumatic stress. In the paradigm of "stress – restress", restress performs the "reminiscent" role, or the role of the trigger stimulus to which individuals with developed PTSD-specific pathology, based on post-traumatic stress, can not adapt, therefore falling into anxiety and depression. Restress lies in the 30-minute long immobilization, to which rats were exposed 7 days after traumatic stress. During an exposure of experimental groups to psychoemotional stress and restress, the control animals were also taken from the home cage and transferred for 5 min in a new situation, to eliminate possible impacts of non-specific influences, in particular, handling and novelty stress.

Protective effect of moderate hypoxia with an exposure of animals to hypobaric hypoxia in a flow barochamber (360 Torr, 2 hours, 3 episodes, equivalent to lifting to an altitude of 5 km) was studied using pre- and postconditioning. An exposure of rats to three sessions of MHH with intervals of 24 h was performed the day before the psychoemotional (pathogen) stress using the PTSD model (hypoxic preconditioning, HP) or on the 4^{th}, 5^{th}, and the 6^{th} days after psychoemotional stress (hypoxic postconditioning, HPost). The brain tissue for immunohistochemical analysis was taken in 1, 5 and 10 days after restress.

Another group of animals was subjected to stress using a pattern of severe hypoxia in a barochamber (SH, 180 Torr, 3 hours). The rats, SH survivors, were exposed to MHH (360 Torr, 2 hours, with an interval of 24 h) on the 1^{st} – 3^{rd} days after hypoxic stress. Brain tissues in that experimental series were removed 24 hours after the last MHH treatment (96 h after SH,, respectively). Previously, it was shown that the SH induced structural and functional damage to the sensitive brain structures,[6] while hypoxic post-conditioning effectively corrected the damaging SH effect on the neurons of the brain and exerted anxiolytic effects on the behavior of animals.[24]

After decapitation of the rat, brain was rapidly removed, and regions of the hip-

pocampus with the adjacent frontoparietal neocortex were isolated. The samples were fixed with molecular fixative FineFix ("Milestone", Italy) for 24 hours at + 4^0 C. Next, preparations were washed in running water for 2 hours, and they were dehydrated by dipping through ethanol solutions of increasing concentrations (50, 70, 80, 96, 96%, 1 h each) and butanol (1 h and a night). The material was then dipped through four xylene portions (15 min), 3 portions of paraffin (45 min), and embedded in paraffin blocks. Next, series of the alternating brain sections in the frontal plane (with thickness of 7 μm) were made at the level of-2.80 mm from the bregma using a microtome. They were used for quantitative immunocytochemical evaluation of protein content, as early gene HIF-1α product, in the neocortex and the hippocampus. Following standard procedures of deparaffination, rehydration, and antigen unmasking, slices were incubated with primary rabbit polyclonal antibodies against HIF-1α ("Santa Cruz Biotechnology, Inc", USA, dilution. 1: 100) overnight at +4°C, and further avidin-biotin detection system ("Vector Laboratories, Inc", UK) was applied. To visualize the reaction, diaminobenzidine was used. After dehydration and placing sections in gelatin, quantitative analysis of immunoreactivity of neurons was conducted using a system consisting of a light microscope Jenaval ("Carl Zeiss", Germany), a digital camera Baumer CX05c ("Baumer Optronic", Germany) and the IBM PC computer with software Videotest Master Morphology. Based on the evaluation of the optical density, immunopositive cells were divided into 2 classes: the weak and intense immunopositive cells. The total number of immunoreactive cells (N) and the number of intensely immunopositive cells (Ni), reflecting the changes in the intensity of expression of the protein, were analyzed. The results were statistically processed using the data analysis package Statistica 7.0 Stat Soft, Inc and Microsoft Excel'2002, and ANOVA ($P < 0.05$). All results are presented as arithmetic mean ± SEM. The results and SEM are expressed as a percentage of control, and the control value is taken as 100%.

III. RESULTS AND DISCUSSION

With the development of the PTSD-induced pathological state, considerable and sustained induction of HIF-1α in the rat hippocampus and the neocortex was observed, despite the fact that the total number of immunopositive neurons changed slightly. A sustainable (1 – 10 days) increase in the total number of HIF-1α expressing neurons was found in the hippocampus and the layer V of the neocortex (Fig. 1, 2). The maximum amplitude of changes in this parameter (up to 270%) was found in layer V of the neocortex (Fig. 2). In contrast to the total number of immunopositive cells, the intensity of HIF-1 expression increased significantly. The maximum increase in the latter was detected in layer II of the cortex (over 6.000%, Fig. 1, 2). These results confirm previously obtained ones, indicating that maladaptive state in response to the psychoemotional stress is accompanied by overexpression of HIF-1α in the rat brain structures in remote post-stressor period.[1] In previous studies, we used the model of "learned helplessness" in rats, where the depressive-like pathology was formed immediately after an exposure to stress, and it persisted for at least 10 days. Persistent stimu-

lation of HIF-1α in the hippocampus and the hypothalamus was detected by the 5th – 10th days after stress, whereas HIF-1α induction in the early post-stressor period was expressed weakly. At that, HP, preventing disorders in adaptation to "learned helplessness" stress, intensified HIF-1α induction in the early period (the 1st day), thus neutralizing the delayed overexpression. For the interpretation of the results obtained, you should take into account the specificity of PTSD-induced pathology, especially the fact that the latter develops hidden during an extended period of time (up to restress), therefore all the studied periods (1 – 10 days after re-stress) characterize the remote period after the pathogenic effect (Traumatic Stress). In connection with this, persistent stimulation of the HIF-1α expression, detectable in the brain of experimental animals at all stages, also suggests that sustained activation of the transcription factor in the remote period after psychoemotional stress is obviously important in the pathogenesis of stress-induced maladaptive states. It is also believed that an excessive and prolonged activation of the HIF-1 forms the pathogenic basis of Alzheimer's disease.[28,33]

The results of experiments using neuroprotective modes of SHH (HP and Hpost) serve an indirect confirmation of the pathogenetic role of HIF-1 excessive induction. It turned out that the HP, increasing resistance to psychoemotional stress in the model of PTSD, largely prevented stress-induced overexpression of HIF-1 in the neocortex, and it normalized the levels of HIF-1α in the hippocampus. The total number of neurons in the dorsal hippocampus (CA1) and the ventral one (dentate gyrus), which contain HIF-1α, remained within the range of control values for the whole registration period (up to 10 days; Fig. 1). A similar pattern was detected for the index of the expression intensity-the number of intensive HIF-1-immunopositive cells in the hippocampus did not differ from the control values at all stages, i.e. HP completely prevented persistent overexpression of HIF-1α in that brain structure, which was consistent with the results obtained previously using a model of psychoemotional stress "learned helplessness".[1] In the neocortex, the HP in the PTSD model resulted in a significant reduction of the number of immunoreactive neurons with intensely expressed HIF-1α relative to non-preconditioned group of animals, but nevertheless moderate induction of that factor remained, and it was several times higher than the basal level (Fig. 2).

HPost, completely neutralizing pathological consequences of psychoemotional stress in the PTSD model, also suppressed the persistent overexpression of HIF-1α in the hippocampus and the neocortex (Fig. 1, 2). The effect was observed with respect to the total number of immunoreactive cells, and particularly in relation to the intensity of HIF-1α expression. In the hippocampus and the upper layers (layer II) of the cortex, immunoreactivity in post-conditioned animals did not differ much from the basal one, whereas in the area of the projection neurons in the neocortex (layer V), moderate HIF-1α expression for the entire registration period (the 1st – 10th days after re-stress) was preserved, which corresponded to the 8th – 18th days after the pathogenic psychoemotional stress; Fig. 2). Thus, the development of anxiety pathology in PTSD model in rats was accompanied by a significant and prolonged up-regulation of HIF-1α in the hippocampus and the neocortex. Stress-protective and anxiolytic effects of HPand HPost were accompanied by a full or partial suppression of the HIF-1α overex-

pression in the hippocampus and the neocortex. It is known that there is close functional interaction of HIF-1 with glucocorticoid receptors, playing a key role in the stress responses and adaptation to damaging impacts both at the level of protein-protein interactions, and at the level of regulation of the genome activity.[12,15,29] PTSD pathology, resulting from the action of superintensive psychoemotional stress, is associated with impaired activity of the hypothalamic-pituitary-adrenocortical system and the reduction of the content of glucocorticoid hormones in the blood plasma.[32] The latter, in turn, have a suppressive effect on the transcriptional activity of HIF-1, reducing the levels of α-subunit.[29] Thus, persistent excessive induction of HIF-1α, observed in the hippocampus and the neocortex (brain structures that have high levels of corticosteroid receptors), may probably be the result of decreased glucocorticoid tone, characteristic of this pathology.

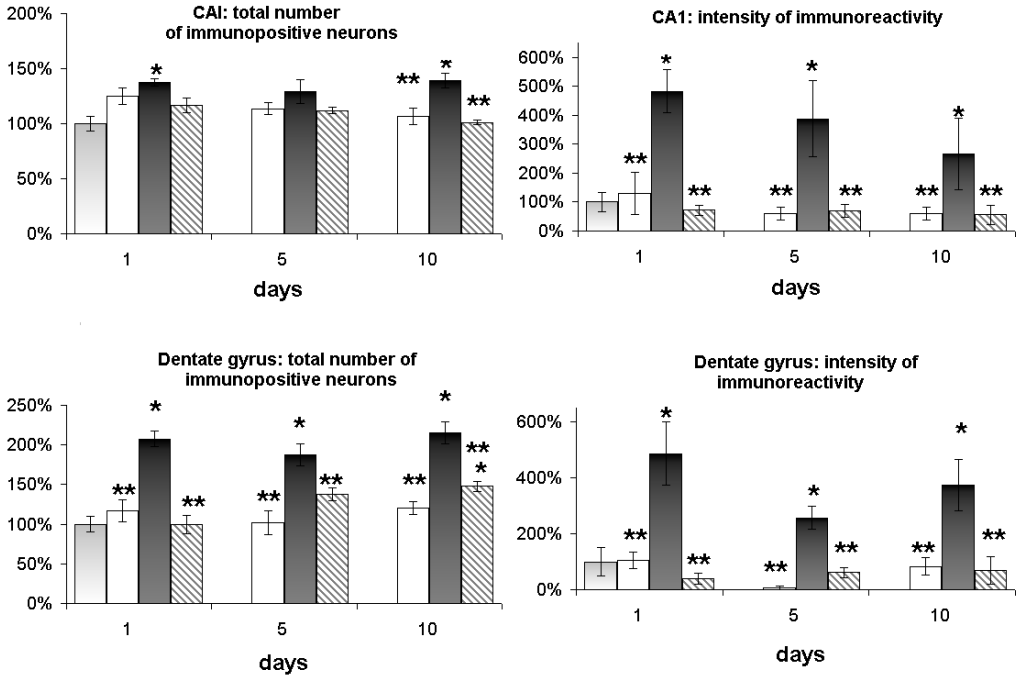

FIG. 1: Expression of specific hypoxia-inducible transcription factor (HIF-1α) in the dorsal (CA1) and ventral (dentate gyrus) areas of the rat hippocampus after an exposure to psychoemotional stress / restress in a model of post-traumatic stress disorder, as well as hypoxic pre- and postconditioning. Results are presented as a percentage of the control (gray bar, 100%). Black bar - animals forming post-stress pathology; white bar - preconditioned rats that do not develop pathology in response to stress; hatched bars - the rats exposed to postconditioning after psychoemotional stress-restress and also not forming pathology. Abscissa – days after restress. * – $P \leq 0.05$ compared with the control, ** – $P \leq 0.5$ compared with the group of rats with stress-induced disorders

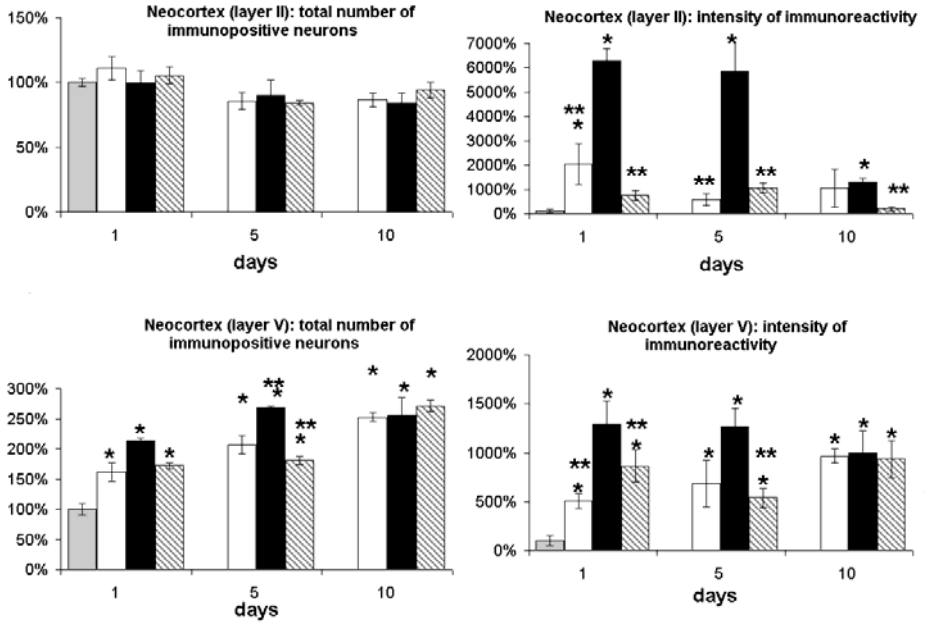

FIG. 2: Expression of specific hypoxia-inducible transcription factor (HIF-1α) in the rat neocortex after an exposure to psycho-emotional stress / restress in a model of post-traumatic stress disorder, as well as hypoxic pre- and postconditioning. Abscissa – days after re-stress. * – $P \leq 0.05$ compared with the control, ** – $P \leq 0.5$ compared with the group of rats with stress-induced disorders

In the model of hypoxic stress, changes in HIF-1α expression in the hippocampus and the neocortex of rats in the pathology and its correction by 3 sessions of MHH differed significantly from that in the model of psychoemotional stress. In the remote period, after SHH (96 h), significant changes in HIF-1α content in the rat hippocampus and the neocortex were not found (Fig. 3). HPost by three sessions of MHH had a powerful stimulating effect on the HIF-1α expression. Moreover, the number of neurons expressing the latter, and, particularly, the expression intensity increased (above 500% within CA1, and above 1.500% in the neocortex). Thus, hypoxic stress, unlike the psychoemotional, caused no remote overexpression of this factor in the hippocampus and the neocortex, and MHH-evoked neuroprotective effect in animals undergoing SH was accompanied by its activation in the rat brain sensitive structures. The results support the notion that HIF-1 is necessary for protection of the brain neurons against harmful action of the hypoxic factor, and it is obviously involved in neuronal protective processes through urgent mobilization of basic adaptation mechanisms to hypoxia and compensation of the neurotoxic effects of the latter. Thus, hypoxic stress, unlike the psychoemotional one, caused no remote overexpression of the factor in the hippocampus and the neocortex, and MHH-induced neuroprotective effect in animals undergoing SH was accompanied by an activation of the latter in sensitive structures of the rat brain. The results obtained support the notion that HIF-1 is necessary for protection

of the brain neurons against the harmful action of hypoxic factor. The latter is involved in neuroprotective processes, obviously, by urgent mobilization of the basic adaptation mechanisms to hypoxia and compensation of its neurotoxic effects.

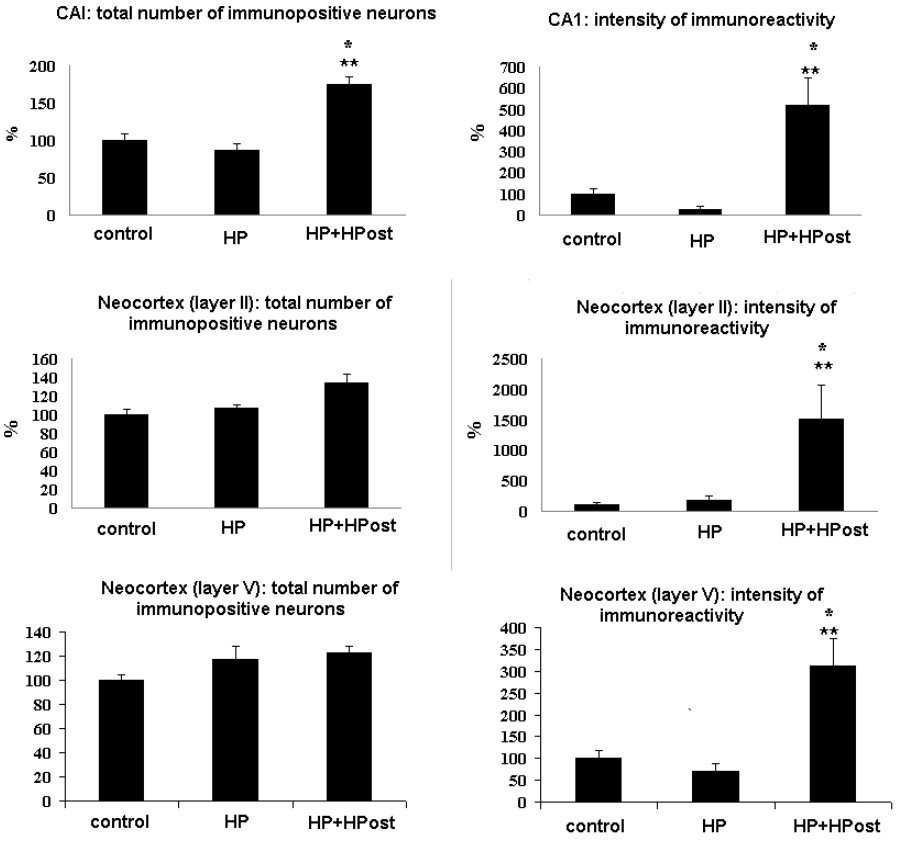

FIG. 3: The effect of severe hypobaric hypoxia (SH) and post-conditioned severe hypoxia (SH + HPost) on the expression of HIF-1α in the hippocampus and the neocortex of rats (4 days after SH). Results are presented as percentage of control. * – $P \leq 0.05$ compared with the control, ** – $P \leq 0.5$-compared to SH

Based on these studies and literature data, it is evident that HIF-1 in the brain may play a role as a pathogenic factor, steady excessive induction of which is associated with disorders in mechanisms of adaptation to stress, as well as a role of a neuroprotector, causing mobilization of pro-adaptive basic processes. At that, the role of HIF-1 is stress-specific, and it depends on the nature of a stressor. Adaptation to hypoxic stress was important for the life of unicellular and multicellular organisms already in the early stages of evolution, so the mechanisms of adaptation to hypoxia are ancient and rather conservative in the phylogeny. HIF-1 takes the central place in the intracellular hypoxic signalling, providing maintenance of oxygen homeostasis and

adaptation / cell survival under hypoxic conditions.[22,30] In recent years, an increasing number of studies on the neuroprotective role of HIF-1 in the brain has appeared. It has been reported that neuronal and glial cells produce not only the protein itself, but also cytokine erythropoietin, its transcriptional target, having a wide range of protective effects that are not associated with hematopoiesis.[20]

In contrast to hypoxic stress, psychoemotional stress is a disturbing factor that emerged in the late stages of evolution, and, consequently, adaptation mechanisms have been formed in the evolutionary aspect recently. Among them, a key role is played by the hypothalamic-pituitary-adrenocortical system,[25] whose function to a large extent is also regulated by HIF-1. Recently, in a model of regenerating skeletal muscle *in vitro*, it has been found that HIF-1-mediated response to hypoxia is independent on glucocorticoid-induced stress response,[21] which serves the basis to suggest that the pathway of adaptation to hypoxic stress, in which HIF-1 is a key element of the induction, and the system of non-specific response to stress are two different proadaptive mechanisms without functional interaction. However, our results on the participation of HIF-1 in the process of adaptation to psychoemotional stress, and available in literature data on the interaction of HIF-1 with glucocorticoid receptors demonstrate inconsistency of the assumption and the important stress-specific role of this factor in neuronal and systemic mechanisms of adaptation not only to hypoxia, but also to psychoemotional stress.

Thus, the data obtained indicate that HIF-1, localized in the neurons of the brain structures examined, is widely involved in the process of adaptation of the brain and the body as a whole to stress of different nature and compensation of post-stress disorders, but the role of the latter is specific as to the nature of a stressor. For the first time, our studies in rats have demonstrated the participation of HIF-1 in the pathogenesis of anxiety and depression, arising from the action of severe psychoemotional, but not hypoxic, stress. Stress-protective and proadaptive effects of MHH in the modes of pre- and post-conditioning are accompanied by enhancing of immediate activation of HIF-1α and netralizing prolonged excessive induction of the latter in the neurons of the brain.

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