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Localization and Quantitative Assessment of Oxygen-Sensitive Hypoxia-Inducible Factor 1α in the Brain of the Mitten Crab *Eriocheir Japonica* in Normal Conditions and Acute Anoxia (an immunohistochemical study)

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Western immunoblotting and immunohistochemistry methods were used to study the expression of hypoxia-inducible factor 1α (HIF-1α) in the brain of the mitten crab *Eriocheir japonica* in normal conditions and anoxia lasting 2, 4, 6, and 12 h using a water deprivation model. The number of immunopositive neurons was small in intact crabs, and increased with increases in the duration of anoxia. There was a particularly marked increase in the proportion of neurons expressing HIF-1α in cells of group 6. In group 9/11, the highest values were seen at 2–6 h of anoxia. In group 17, significant changes in the proportion of immunopositive cells were noted only at 2 h of anoxia. After anoxia lasting 6 h, the proportion of neurons expressing HIF-1α decreased in all cell groups, though the reaction appeared in hemocytes. It is suggested that the increase in the proportion of immunopositive neurons and the suppression of HIF-1α expression in brain hemocytes in anoxia play important roles in mediating compensatory and protective processes, increasing the adaptive potential of the mitten crab in conditions of hypoxic stress.

Keywords: central nervous system, mitten crab, hypoxia-inducible factor 1α, anoxia.

A family of highly conserved transcription factors – hypoxia-inducible factors (HIF) – plays an important role in the regulation of oxygen homeostasis. The key role in this process has now been established to belong to oxygen-sensitive transcription factor HIF-1α [11, 17, 19]. Evidence has been obtained showing that HIF-1α in mammals takes part in neuroprotective mechanisms in hypoxia/ischemia and oxidative stress [4, 11, 15]. Recent studies have demonstrated that hypoxia stimulates HIF-1α expression in a number of organs in invertebrates [7, 8, 12]. Studies in the CNS of invertebrates have used only biochemical methods, which do not provide any assessment of the distribution of such cells in individual brain structures [9]. Furthermore, reports on the topography and numbers of HIF-1α-positive cells in the brain are of fundamental importance for understanding the mechanisms of formation of tolerance to hypoxia in different classes and species of animals, including the Japanese mitten crab *Eriocheir japonica*, which is characterized by its exclusive ability to adapt to various extreme conditions, including hypoxia.

The aim of the present work was to study the locations and quantitative content of HIF-1α in brain cells in the crab *E. japonica* in normal conditions and in acute anoxia.

Materials and Methods

Experiments were performed on adult male mitten crabs *Eriocheir japonica*, with carapaces of width 61–62 mm, caught in Peter the Great Gulf (Sea of Japan) in April, 2013. After adaptation over a few days, the animals were divided into two groups. Animals of the control group (five crabs) were kept in an aquarium with aerated sea water at a temperature of 20°C; the experimental group was exposed to anoxia induced by water deprivation for 2, 4, 6, or 12 h (five animals per group)

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at 20°C as described previously [5]. Studies with hydrobionts were guided by Directive 86/609/EC of the Council of the European Union of November 24, 1986.

For western blotting, freshly prepared brain samples were homogenized at a ratio of 1:5 in buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonylfluoride (PMSF). After centrifugation (15000 *g*, 4°C, 20 min), supernatants were collected and total protein levels were assayed. Protein electrophoresis was run in 12% polyacrylamide gels. The quantity of total protein added to electrophoresis slots was 40 μg. After electrophoresis, proteins were transferred to nitrocellulose membranes (Santa Cruz Biotechnology, Inc.). Non-specific antibody binding to membranes was blocked by overnight incubation in tris-HCl buffer containing 4% bovine serum albumin. Immunodetection was performed using monoclonal mouse antibodies against HIF-1α (diluted 1:1000, Abcam, UK) for 3 h at 4°C, followed by secondary antibodies conjugated with horseradish peroxidase (Vector Labs, USA). Peroxidase reactions were visualized using a VIP Substrate Kit (Vector Labs, USA). Membranes were then washed and scanned.

For immunohistochemical detection of HIF-1α, crab brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4°C. Cryostat sections of thickness 30 μm were cut. HIF-1α was detected on serial sections of the cerebral ganglion by indirect avidin-biotin-peroxidase labeling. After inhibition of endogenous peroxidase with 1% H_2O_2 solution and suppression of nonspecific antibody binding with 1% normal horse serum, sections were incubated for 18 h at 4°C with mouse monoclonal antibodies to HIF-1α (diluted 1:500, Abcam, UK). Sections were then washed in several changes of 0.1 M phosphate-buffered saline (PBS) pH 7.2 and incubated for 2 h in secondary biotinylated antibody solution (Vector Labs, USA) diluted 1:200. After washing, sections were incubated for 1 h with avidin-biotin-peroxidase complex (Vectastain ABC Kit, vector Labs, USA) at a temperature of 22–24°C in the dark, followed by three washes with PBS. Reaction product was detected using substrate (VIP Substrate Kit, Vector Labs, USA), the staining process being monitored under the microscope. Sections were then washed in three changes of PBS, dehydrated using standard methods, and embedded in balsam. The suitability of mouse monoclonal antibodies to HIF-1α to identify this molecule in the various organs and tissues of invertebrates, including the brain in crabs, was confirmed by previous molecular-genetic and biochemical investigations [7, 9, 12, 17].

The brains of all animals were studied in relation to cell groups 6, 9/11, and 17, whose topography was defined by Sandeman et al. [13]. Experiments were performed using serial sections, one of which was treated with 0.5% methylene blue to determine the total number of neurons, every other section being used for immunohistochemical studies.

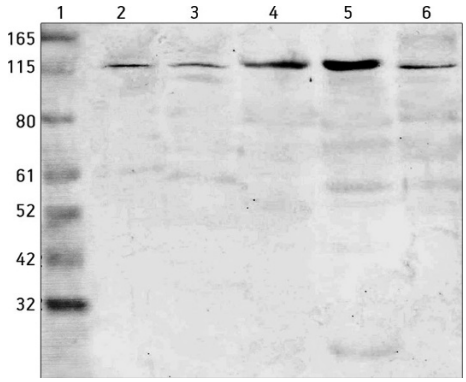

Fig. 1. Western immunoblotting of HIF-1α in *E. japonica* brain homogenates in controls and anoxia. *1*) Molecular weight markers; *2*) control group; anoxia for *3*) 2 h; *4*) 4 h; *5*) 6 h; *6*) 12 h.

Specimens were examined under an Axiovert 200M microscope (Zeiss, Germany). The number and sizes of neurons were determined in montages of sections formed in AxioVision 4.8, only in those cell groups of the ganglion in which immunopositive neurons were detected consistently and in sufficient numbers for statistical analysis. Only those neurons with clearly visible nuclei were included. Reaction specificity was verified by processing sections without primary or secondary antibodies. Control sections produced no immunopositive reactions.

Neurons were quantitated using programs from the automatic image analysis system Allegro-MC. Proportions of labeled cells among all neurons in the cell group of interest were determined, including only those neurons whose staining density was at least 1.5 times background. Quantitative results obtained from processing at least 10 serial sections of ganglia from each study animal are presented as mean and standard error. Differences were assessed using Student's *t* test and were regarded as significant at $p < 0.05$.

Results

Western blotting of total brain protein extracts from both intact *Eriocheir japonica* crabs and animals after anoxia induced by water deprivation demonstrated a polypeptide antigen with a molecular weight of about 116 kDa which cross-reacted with antibodies to mouse HIF-1α (Fig. 1).

Immunocytochemical studies of the brains of intact crabs showed the presence of HIF-1α in cell groups 6 and 9/11. HIF-1α-expressing neurons were consistently not detected in group 17. Most immunopositive cells were oval, pear-shaped, or polygonal in shape and were relatively large in size (about 800 μm^2) and had low reaction intensity (Fig. 2, *a*). However, precipitate density increased significantly in small neurons with body areas of 70–250 μm^2. These were generally round and pear-shaped cells, with large nuclei and a narrow rim of cytoplasm. The analyses showed that the proportion of HIF-1α-positive neurons was highest in cell group 6, though the content here was no more than 4% of

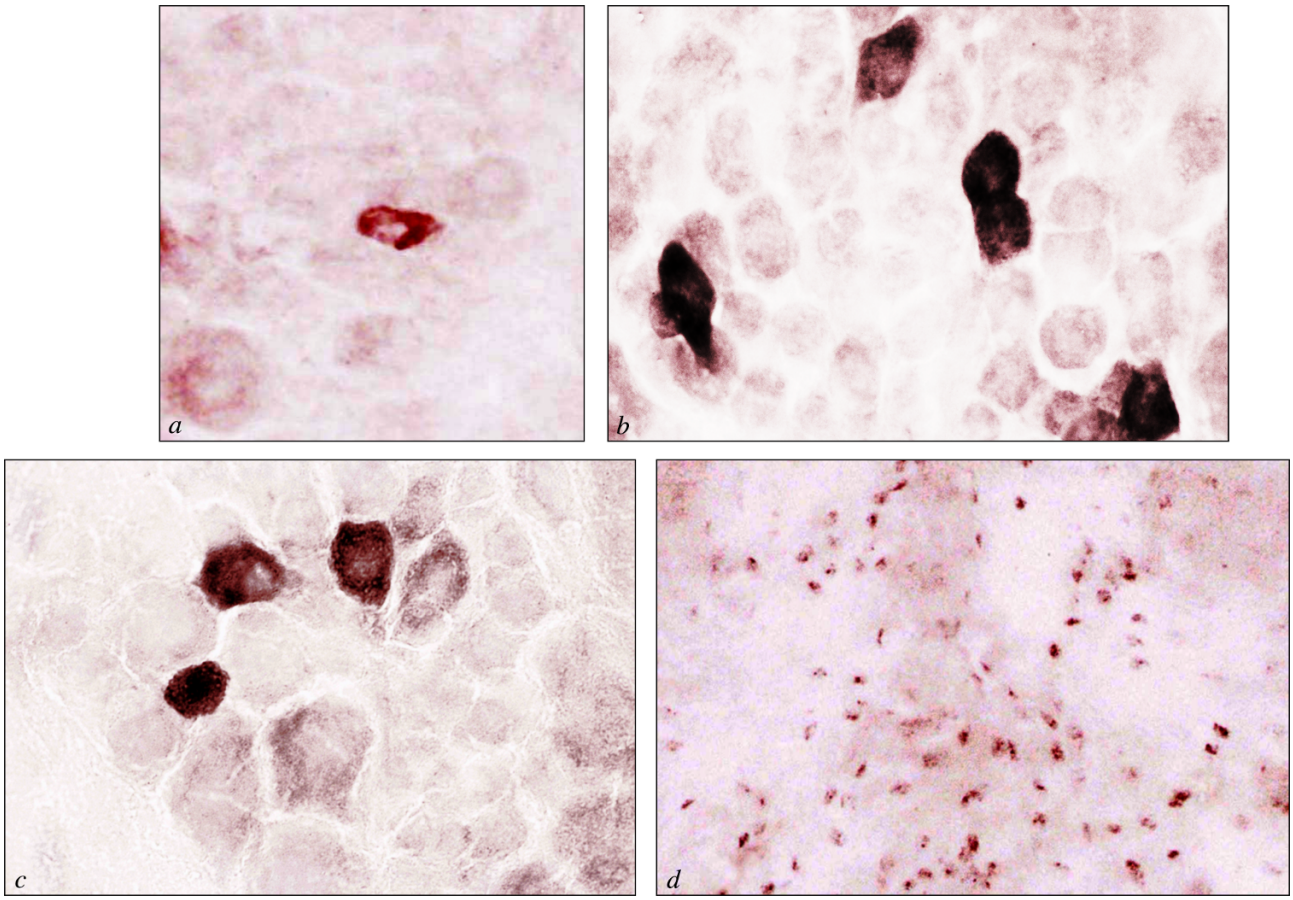

Fig. 2. HIF-1α-positive neurons (*a–c*) and hemocytes (*d*) in the brain of *E. japonica*. HIF-1α-positive neurons in cell group 6 in intact crabs (*a*) and after anoxia for 2 h (*b*) or 6 h (*c*) induced by water deprivation; *d*) hemocytes in cell group 17. Immunohistochemical reaction. *a*, *d*) Objective ×20, ocular ×15; *b*, *c*) objective ×40, ocular ×15.

the total number of cells detected in this group by methylene blue staining (Fig. 3, *a*). An even smaller proportion of immunopositive neurons was seen in cell group 9/11 ($p \leq 0.05$).

In anoxia, all brain cell groups showed significant increases in the proportions of immunopositive neurons. In addition, there were differences in the dynamics of quantitative changes to HIF-1α-expressing neurons in the study groups. In cell group 6, anoxia for 2 h produced a sharp increase in the proportion of these neurons, the relative content reaching a peak at 6 h and then decreasing to 12 h (see Fig. 3, *b*). The increases in the numbers of HIF-1α-containing cells in this group were particularly significant in the central part of the dorsal surface and the anteromedial part of the ventral surface of the brain. These increases occurred predominantly because of small neurons, many of which were characterized by high levels of HIF-1α expression (see Fig. 2, *b*). However, starting at 4 h, the increase in the proportion of neurons also involved larger cells, some of which showed higher reaction product density (see Fig. 2, *c*). In cell group 9/11, changes in the proportion of immunopositive neurons occurred with a different dynamic (see Fig. 3, *b*). By 4 h of anoxia, there was a significant increase in the proportion of immunopositive cells, which was followed by a gradual decrease. In this group, the increase in the number of HIF-1α-positive neurons was not as significant as that in the previous group, and occurred as a result of similar increases in small and large neurons.

In cell group 17, immunopositive neurons were consistently detected at 2 h of anoxia. As a rule, these cells were round or polygonal in shape and had body areas of 600–900 μm^2 and were mainly located in the caudal part of the ventral surface of the brain. Although the number of these cells at this time point was small, there was a significant increase by 4 h, with a decrease at 6 h; by 12 h of anoxia, no HIF-1α-positive cells were seen in this group. However, by 6 h of anoxia, HIF-1α expression appeared in numerous small (area 50–70 μm^2) hemocyte-like cells located mainly along vessels in the medial antennular neuropil (Fig. 2, *d*).

Discussion

The material presented here provides evidence that hypoxia-inducible factor HIF-1α is detected in the brains of the crab *Eriocheir japonica* both in the animal's normal liv-

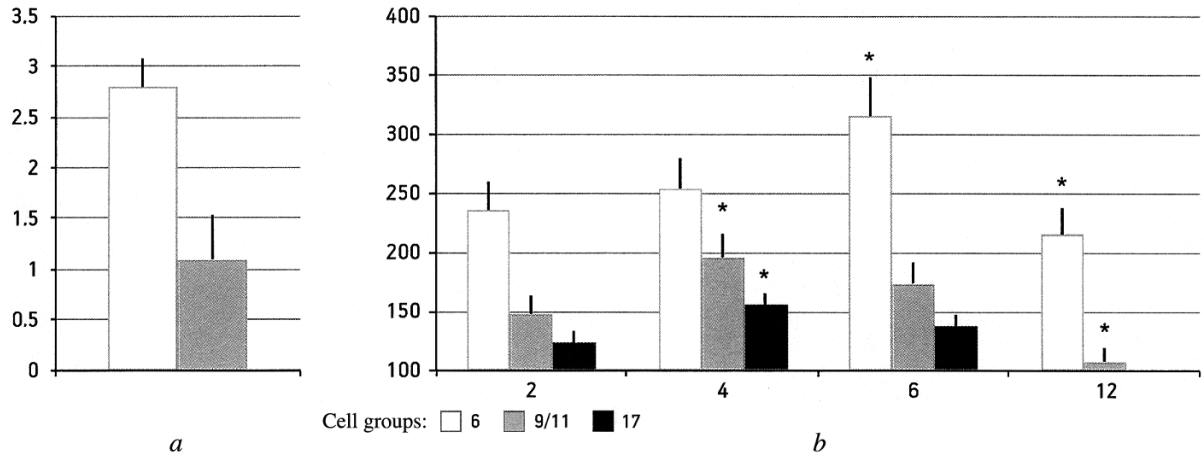

Fig. 3. Relative contents of HIF-1α-positive neurons in different cells groups in the brains of intact *E. japonica* (*a*) and after hypoxia (*b*). *b*) The horizontal axis shows the duration of hypoxia (h). Ordinates: *a*) proportion of HIF-1α-positive neurons amongst all neurons detected by methylene blue staining (%); *b*) changes in the proportion of HIF-1α-positive neurons (%) compared with values in the same cell group in the control group of animals, which was taken as 100%. *Significant differences, $p \leq 0.05$ compared with values in the preceding group; vertical bars – compared with values in the control group.

ing conditions and in experimental conditions. Western immunoblotting demonstrated the presence in brain of a 116-kDa polypeptide antigen which cross-reacts with antibodies to mouse HIF-1α. Expression of this protein has been demonstrated in many organs and tissues in vertebrate and invertebrate animals in recent years [7, 10, 17], though the presence of HIF-1α in the CNS of crustaceans has been demonstrated only using biochemical methods [9]. Studies of the distribution of HIF-1α-containing neurons in various cell groups in the brain of the crab *E. japonica* showed that expression was seen in a small number of neurons in the cerebral ganglion of intact animals, with a significant increase in some brain areas in anoxia induced by water deprivation. It is entirely likely that the increase in the HIF-1α concentration in neurons in the experimental group of animals was linked with its neuroprotective action. A recent study showed that an increase in the level of HIF-1α expression in brain cells in rats protects them from death induced by oxygen starvation [11, 18]. A relationship was found between the HIF-1α concentration and the severity and duration of hypoxia: short-lived and moderate hypoxia produced a rapid and significant increase in the HIF-1α content in the brain, with a resultant triggering of a cascade of neuroprotective mechanisms protecting neurons, macro- and microglial cells, and the vascular endothelium in the brain from damage due to oxygen starvation [11, 15, 18]. In chronic and severe hypoxia, HIF-1α expression in the brain in rats decreases due to increases in the rate of its degradation and neuron apoptosis [4, 18].

In the CNS of marine mollusks, the mechanisms of activation of HIF-1α and its role in the formation of tolerance to hypoxia have received little study. We are aware of only one such study, which presented material showing increased HIF-1α expression in the brain of the mantis crab (*Oratosquilla oratoria*) in chronic hypoxia induced by anthropogenic environmental pollution [9]. In addition, there are reports showing changes in the content of this signal molecule in hypoxia in other organs and tissues in marine invertebrates [7, 8, 12]. Activation of HIF-1α in hypoxia is usually accompanied by the expression of genes regulating energy metabolism [10, 12, 17].

In contrast to vertebrates, an obligate condition for survival of invertebrates in hypoxia is a decrease in the total adenosine triphosphate consumption level due to metabolic depression linked with changes to protein metabolism [6, 19]. However, not all neurons decreases their metabolic needs in hypoxia to the same extent. In some hypoxia-resistant vertebrate species, such as the painted tortoise, most brain neurons show sharp reductions in their metabolic needs in conditions of restricted oxygen supply, while other neurons, controlling the critical body functions needed for survival not only do not decrease, but, conversely, increase their metabolic potential, acting in a way analogous to central oxygen chemosensors in mammals [3]. In these cells, termed "vigilance" neurons by the author, which are particularly important for both short-term and long-term adaptation to hypoxia, the level of HIF-1α expression was especially high.

Our data indicate that in *E. japonica*, these neurons are found mainly in group 6, whose cells play an important role in integrative CNS processes, particularly the formation of motor behavioral programs in extreme situations [16]. At 2 h of anoxia, this cell group showed a sharp increase in the number of immunopositive neurons, reaching a peak at 6 h of the experimental treatment. Some of these neurons were characterized by very high levels of HIF-1α expres-

sion. Between 2 and 4 h of anoxia, the number of HIF-1α-positive neurons in group 9/11 also increased, though not so significantly; this group contains cells involved in processing multimodal information arriving in the brain [14]. HIF-1α expression in mammal neurons is known to provide a significant level of support for rapid and appropriate responses to hypoxic stress due to stimulation of the respiratory and vasomotor centers and by activation of the genes responsible for energy metabolism, angiogenesis, and apoptosis, increasing resistance to hypoxia [18]. In cell group 17, involved in the innervation of the internal organs in crabs, the number of HIF-1α-positive neurons increased by 4 h of hypoxia, but then decreased rapidly. After 6 h of anoxia, the proportions of HIF-1α-positive neurons decreased in all cell groups (not detected in group 17 cells at this time point), though HIF-1α appeared in hemocytes. Evidence was recently presented showing that hemocytes in crustaceans have the properties of stem cells [1, 2]. Various experimental treatments, including hypoxia, prompt them to migrate from the organs of the immune system – where they are formed – to the brain, where they respond to decreases in the number of neurons by forming a population of precursor cells able to compensate for the deficit of neurons.

Thus, the brain of the crab *E. japonica* contains at least two cell populations expressing HIF-1α: neurons and hemocytes. These allow factor HIF-1α to take part in mediating the central mechanisms regulating oxygen hemostasis on adaptation to transient or long-term hypoxia by altering metabolism and locomotor behavioral reactions. Hemocytes not only support high HIF-1α contents in the brain, enhancing neuroprotection, but may also compensate for the proportion of neurons lost during development of chronic hypoxia.

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