

Hypercapnia potentiates HIF-1 α activation in the brain of rats exposed to intermittent hypoxia

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ABSTRACT

The mechanisms and signalling pathways of the neuroprotective effect of hypercapnia and its combination with hypoxia are poorly understood. The study aims to test the hypothesis about the potentiating effect of hypercapnia on hypoxia adaptation systems directly related to hypoxia-induced factor 1 α (HIF-1 α).

In this study we assessed HIF-1 α content in hippocampal extracts and astrocytes obtained from Wistar male rats exposed to different respiratory conditions (7- or 15-fold of hypoxia and/or hypercapnia). In addition, HIF-1 α content in astrocytes was assessed in *in vitro* model of chemical hypoxia as well as in the cerebral cortex after photothrombotic damage of this brain region.

This study indicates increased levels of HIF1 α in hippocampal extracts, astrocytes, and in cells of the near-stroke region of the cerebral cortex in rats exposed to hypoxia and hypercapnic hypoxia, but not hypercapnia alone. In *in vitro* study, hypercapnia facilitates the effects of acute chemical hypoxia observed in astrocytes.

Thus, hypercapnia does not increase the level of transcription factor HIF-1 α . However, the combined effects of hypercapnia and hypoxia in *in vitro* simulations of acute chemical hypoxia potentiate the accumulation of HIF-1 α .

1. Introduction

Mild or brief intermittent hypoxia has been proven to be effective in increasing the ischemic tolerance of individual organs/tissues (Chen et al., 2005; Yang et al., 2009). There are numerous data on the preventive and therapeutic efficacy of hypoxia against cardiovascular pathology (Shatilo et al., 2008; Neckár et al., 2002) and ischemic brain injury (Sharp et al., 2004). However, the practical application of hypoxic therapy is limited due to the necessity of long-term exposure (1-6 hours) and several sessions (at least 7) (Chen et al., 2005; Yang et al., 2009; Shatilo et al., 2008; Neckár et al., 2002).

However, it is the therapeutic use of carbon dioxide that is gaining increasing interest. The pronounced neuroprotective efficiency of mild permissive hypercapnia has previously been established (Zhou et al., 2010; Tao et al., 2013). In the scientific literature, there has even emerged a special term, "therapeutic hypercapnia" (Tao et al., 2013; Laffey et al., 2000). The main neuroprotective mechanisms of hypercapnia are probably the carbon dioxide-induced inhibition of the apoptosis of nerve cells (Zhou et al., 2010; Tao et al., 2013), its

antioxidant effect (Zakynthinos et al., 2007), and the stimulation of angiogenesis (Siafakas et al., 2001).

We have shown in our previous studies that the effects of mild hypercapnia (especially in combination with oxygen deficiency) are superior to the effect of hypoxia in respect of increasing the body's tolerance to hypoxia and the development of cerebral ischemic tolerance (Tregub et al., 2013; Tregub et al., 2015). However, to date the mechanisms and signaling pathways of the neuroprotective efficiency of hypercapnia and its combination with hypoxia remain poorly understood.

The mechanism of increased tolerance of organs and tissues to hypoxia is known to be largely based on the effects of the hypoxia-inducible factor 1 α (HIF-1 α) (Murphy, 2004). The concentration and stability of HIF-1 α , its localization in the cell, as well as the activity of transcription directly depend on the cellular level of oxygen (Semenza, 1999). With a lack of oxygen in the cells, this transcription factor accumulates, followed by an increase in the expression of genes that enhance the synthesis of erythropoietin (Marti et al., 2000; Sugawa et al., 2002), VEGF (vascular endothelial growth factor) (Siafakas et al., 2001)

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and neuroprotective proteins (Kleindienst et al., 2004; Lin et al., 2011). Therefore, an assessment of the role of HIF-1 α in the protective effect of a combination of hypoxia and hypercapnia is of particular interest.

We found that permissive hypercapnia and hypercapnic hypoxia stimulated angiogenesis in the brain and activated VEGF production (Bespalov et al., 2014), which was commonly associated with HIF-1 α activation (Siafakas et al., 2001).

In one of our trial experiments, we demonstrated an increase in HIF-1 α level in the brain homogenate not only under the conditions of oxygen deficiency, but also as a result of increased CO₂ concentration under the conditions of normal oxygen level (Kulikov et al., 2015). In another study, we showed that a course exposure to hypercapnic hypoxia causes HIF-1 α accumulation in the hippocampal neurons in CA1 region. However, this was not observed in the case of exposure to hypercapnia and hypoxia alone (Kulikov et al., 2018). Due to limited experimental data, these studies did not allow us to draw unambiguous conclusions regarding the role of hypercapnia in the mechanism of the development of cerebral ischemic tolerance.

We chose the hippocampus as the main object of the study (extracts, astrocytes for cultivation) due to the fact that the nerve cells of this brain structure are most sensitive to oxygen deficiency [Zhou et al., 2004; Zhao et al., 2012]. Therefore, an increase in the content of HIF-1 α under the influence of hypoxia in this region of the brain will occur earlier than in other areas and will more accurately state the development of ischemic resistance [Zhang et al., 2014].

It should be noted that Xu et al., 2009 and Benderro et al., 2013 also showed in their studies an increase in HIF-1 α concentration in the brain tissue under the conditions of hypercapnia, although these effects were not directly associated with carbon dioxide effects.

Given the fact that that hypercapnia affects the systems of adaptation to hypoxia that are directly related to HIF-1 α , we hypothesized that hypercapnia alone and in combination with hypoxia could have a significant effect on this transcription factor thus increasing cerebral ischemic tolerance. The study was conducted to test this hypothesis.

2. Experimental Procedure

2.1. Animals

The studies were carried out using *in vivo* and *in vitro* models on 100 male Wistar rats (Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia) aged 5-6 months and the average weight of 250-300 g. The animals were randomized using SPSS version 11.5 (SPSS Inc, Chicago, IL). They were housed in cages at controlled room temperature ($\sim 23^\circ\text{C}$) in the room with a normal light cycle and had free access to food and water. Before and after the experiments, the animals were weighed.

2.2. Groups and experimental design

The study consisted of four series of tests with four groups of conditions in each one. The groups differed in the partial pressure of oxygen (PCO₂) and the partial pressure of carbon dioxide (PCO₂):

- NbH (normobaric hypoxia): PO₂ $\approx$ 90 or 35 mm Hg; PCO₂ $\approx$ 1 or 35 mmHg; the rest was N₂.
- PermH (permissive hypercapnia): PO₂ $\approx$ 150 mmHg; PCO₂ $\approx$ 50 mmHg; the rest was N₂.
- HyperH (hypercapnic hypoxia): PO₂ $\approx$ 90 or 35 mmHg; PCO₂ $\approx$ 50 mm Hg; the rest was N₂.
- Control (PO₂ $\approx$ 150 mmHg; PCO₂ $\approx$ 1 or 35 mmHg; the rest was N₂). The control group used the same experimental procedures with the exception of exposure to hypoxia and /or hypercapnia.

In the first series of tests, the rats (n = 40) were exposed to different respiratory conditions in a special chamber for 30 minutes daily over a

period of 7 days. Animals were randomized into four equivalent groups: exposure to NbH, exposure to PermH, exposure to HyperH, and the control group. 24 hours after the completion of the 'training' courses, the animals were euthanized by decapitation. The hippocampus was removed to prepare cytoplasmic and nuclear protein extracts followed by Western Blot analysis with an assessment of the HIF-1 α level.

In the second series of tests, the animals (n = 20) were exposed to different respiratory conditions in a special chamber for 30 minutes daily over a period of 15 days. 24 hours after the completion of the 'training' courses, 5 rats from each group were euthanized by decapitation and the hippocampus was removed to obtain the primary tissue culture of central nervous system progenitor cells and the initial culture of rat neurospheres for subsequent differentiation into astrocytes and neurons. Following neurosphere differentiation, an immunocytochemical technique was used to determine the HIF-1 α content in a culture of astrocytes under normoxic conditions and under the conditions of chemical hypoxia.

In the third series of tests, astrocytes obtained from the control group primary tissue culture of progenitor cells were placed in a special hypoxic chamber under NbH conditions (PO₂ $\approx$ 35 mmHg; PCO₂ $\approx$ 1 mmHg; the rest was N₂), or HyperH conditions (PO₂ $\approx$ 35 mmHg; PCO₂ $\approx$ 50 mmHg; the rest is N₂) for a period of 24 hours. Six hours later the cells were exposed to chemical hypoxia followed by immunocytochemistry staining to determine HIF-1 α content. Cells that were placed under standard conditions of a CO₂ incubator were used as control.

In the fourth series of tests, the rats (n = 40) were exposed to different respiratory conditions in a special chamber for 30 minutes daily over a period of 15 days. On the next day after completion of the exposure period, focal ischemia-induced brain damage was modeled in all animals. 72 hours after the surgery, a procedure of perfusion-fixation was carried out followed by decapitation and brain extraction.

2.3. Respiratory exposure

For respiratory exposure, we used a flow chamber described earlier (Tregub et al., 2015). The experimental groups of rats breathed different gas mixtures depending on the group purpose. The control group was placed in a chamber under similar conditions, but breathed atmospheric air pumped by a compressor instead of a gas mixture. The composition of the gas mixture in the chamber was controlled by a Microlux O₂ + CO₂ gas analyzer (Microlux Ltd., Russia).

2.4. Surgical procedures and photochemical thrombosis

All animals did not have access to food before the surgery used to simulate focal ischemic damage, but had unrestricted access to water. Before surgery, the animals were weighed. Each rat was anesthetized by intraperitoneal administration of chloral hydrate (400 mg/kg). A sterile incision was made in the left inguinal region. A 4% solution of pink Bengal rose (Sigma Aldrich, Germany) diluted in 0.9% NaCl (in a dose of 40 mg/kg) was injected into the left femoral vein.

Ischemic damage to the sensorimotor region of the cortex of the right cerebral hemisphere was induced using the method of transcranial photochemical thrombosis (Pevsner et al., 2001; Barth and Mody, 2011). An incision was made in the scalp, the periosteum was cleaned, and a 10-minute laser illumination of the bones of the skull was carried out using a green 532 nm laser with a power of 20 mW. An area of the parietal bone (2 mm in diameter) located in the middle between the bregma and the lambdoid suture and 2 mm lateral to the sagittal suture was illuminated.

Before decapitation, the animals underwent transcardial perfusion fixation (administration of 500 ml of PBS followed by 250 ml of 4% PBS-buffered paraformaldehyde). After the perfusion fixation, the brain was removed and stored for 2 days in 4% PBS-buffered paraformaldehyde and for 2 days in 30% sucrose. Later, the brain was partitioned on

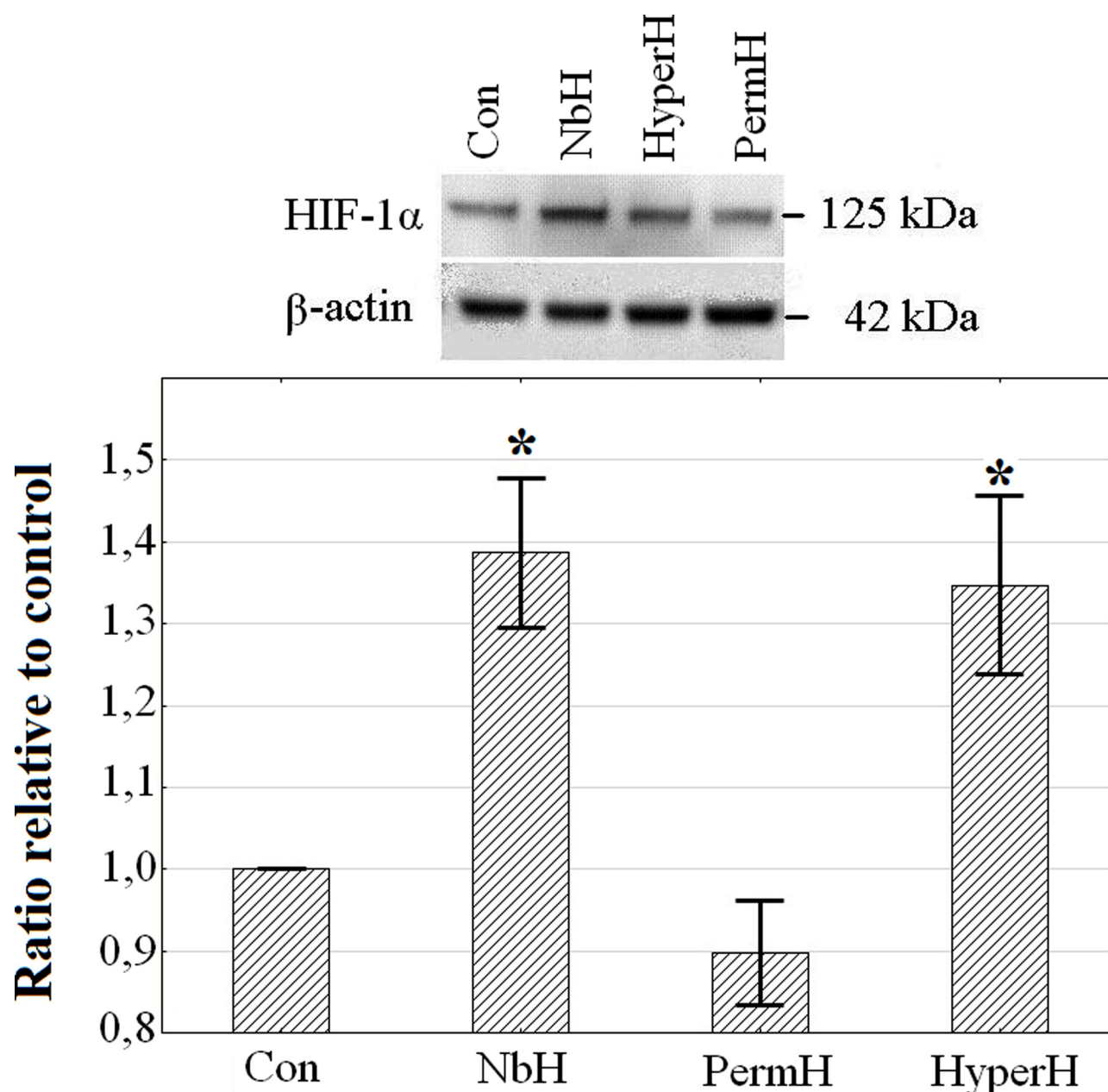


Fig. 1. The content of HIF-1 α in the hippocampus of rats after 7-fold respiratory exposure to hypoxia and/or hypercapnia. Top panels show HIF-1 α protein bands and corresponding β -actin bands representative for each experimental group. Bottom panels include levels of HIF-1 α protein expression measured by densitometry using samples obtained from 24 h after last exposure. Data are presented as mean $M \pm \sigma$. Control = control group ($n = 10$); NbH = normobaric hypoxia ($n = 10$); PermH = permissive hypercapnia ($n = 10$); HyperH = hypercapnic hypoxia ($n = 10$). * - differences compared to control ($p < 0.01$).

a vibrating microtome (the thickness of the frontal sections was 60 μ m). Sections were individually transferred to 24-well culture plates for storage or analysis.

2.5. Western blot

After isolation of the hippocampus from the brain of rats and selective extraction of cytoplasmic and nuclear proteins using the Mammalian Nuclear and Cytoplasmic Protein Extraction Kit (SERVA, Cat. No. 39243.01), the nuclear protein extract was stored until the day of the analysis at -80°C . Prior to electrophoresis, the samples were thawed at room temperature and equivalently diluted with Tris/Glycine/SDS with addition of mercaptoethanol, and then heated for 5 minutes at 95°C on a solid-state thermostat.

Protein electrophoresis was carried out in a commercially available

8% TG PRiME acrylamide gel (SERVA, Cat. No. 43260.01). To measure the total protein content in the samples and determine the volume for application, the Lowry micromethod was used on a CM2203 spectrofluorometer (Solar, Belarus). The electrophoresis procedure was carried out in a miniVE Amersham Biosciences chamber (GE Healthcare, USA) for 90 minutes at 20 mA and 230 V. To transfer proteins from the gel to the PVDF membranes, a set of Semi-Dry Blotting Buffers was used (SERVA, Cat. No. 42559.01). Semi-dry blotting was carried out on a TE77 Amersham system (GE Healthcare, USA) at 65 mA for 60 min.

Blocking of non-specific antigens on the membrane was carried out with a BlueBlock PF solution (SERVA, Cat. No. 42591.02) for 60 minutes. After that, incubation was performed overnight at $+4^{\circ}\text{C}$ with primary anti-HIF-1 α antibodies (Thermo Fisher, Cat. No. SA5-10211) and β -actin (Thermo Fisher, Cat. No. MA5-15739) in 1:1,000 dilution followed by incubation with secondary HRP-conjugated antibodies (1:

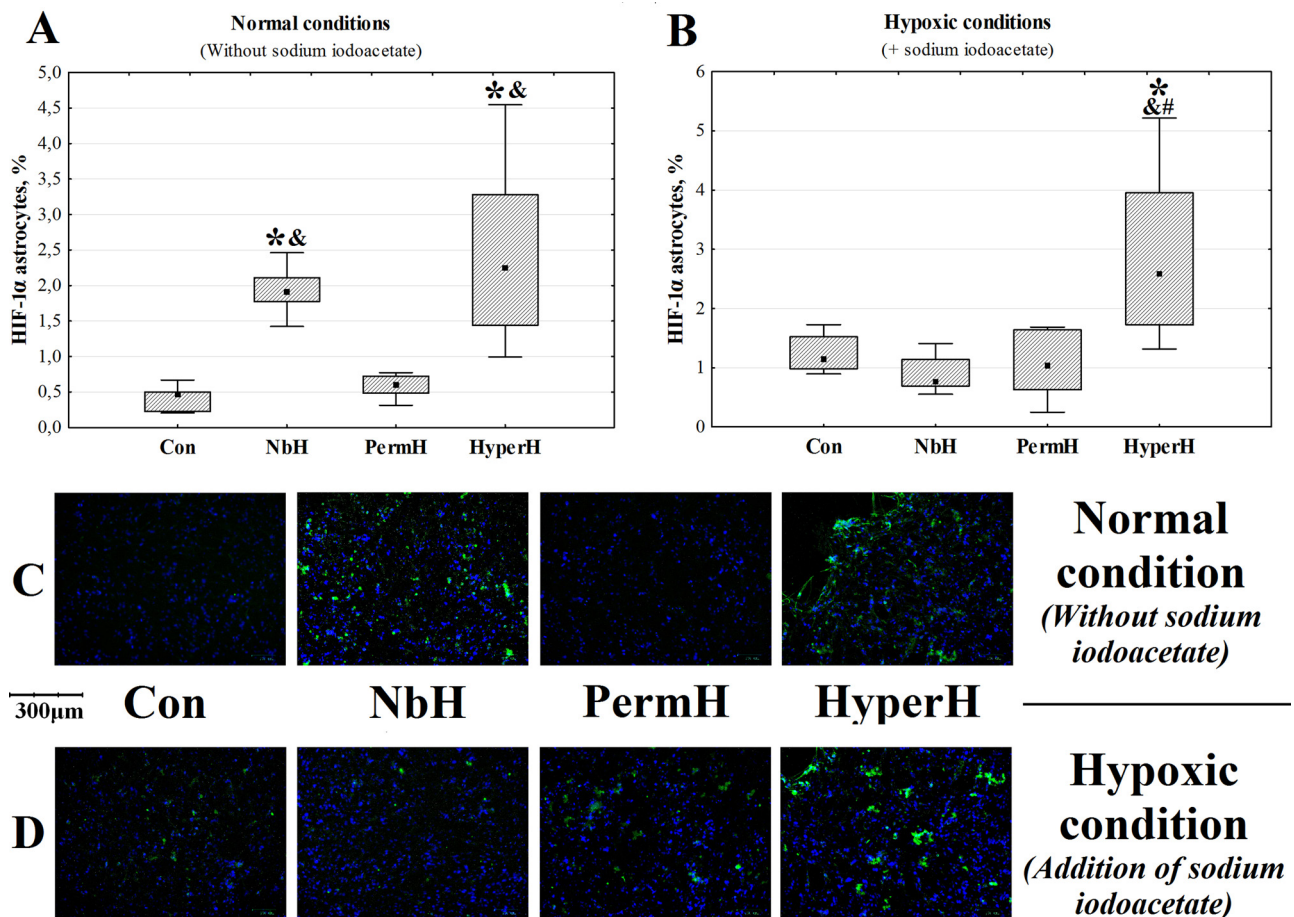


Fig. 2. The content of HIF-1 α in astrocytes isolated from rat progenitor cells followed by respiratory exposure *in vivo*: A and B are the percentage of marker-expressing cells in relation to the total number under normal and hypoxic conditions; Data are presented as Me (25; 75%). * - differences compared to control ($p < 0.001$); # - differences compared to the NbH group ($p < 0.001$); & - differences compared to the PermH group ($p < 0.001$). C and D - expression of HIF-1 α in astrocytes under normal and hypoxic conditions. Magnification x175. Blue color - DAPI; green - primary antibodies to HIF-1 α associated with a secondary antibody conjugated to AlexaFluor488. Control = control group ($n = 5$); NbH = normobaric hypoxia ($n = 5$); PermH = permissive hypercapnia ($n = 5$); HyperH = hypercapnic hypoxia ($n = 5$);

10,000 dilution) (Thermo Fisher, Cat. No. A27-025) for 2 hours at room temperature.

Chemiluminescent detection was performed using the SERVALight Eos kit (SERVA, Cat. No. 42585.02) and Amersham Imager 600 UV gel-documenting system (GE Healthcare, USA) in semi-automatic mode followed by densitometry using ImageJ 1.41 (Scion Inc., USA).

2.6. Obtaining the initial neurosphere culture and the primary culture of neurons and astrocytes

The hippocampus was isolated from the brain and placed in phosphate-buffered saline supplemented with 2% glucose. The resulting tissue fragments were transferred to a freshly prepared 2% PBS solution followed by removal of the supernatant formed after passive precipitation. After the precipitation, the tissue was resuspended in 1 ml of NeuroCult Proliferation Kit medium (Mouse & Rat) (StemCell, Canada) and triturated to obtain a homogeneous cell suspension. Further, the supernatant obtained as a result of precipitation was centrifuged at 150 g for 5 minutes.

Progenitor cells isolated from the brain (with a density of 6-12 million viable cells / ml) were seeded in T-25 cm² culture vials in 10 ml of NeuroCult Proliferation Kit (Mouse & Rat) culture medium (StemCell, Canada). Subsequently, the cells were incubated in a CO₂ incubator with 5% CO₂ at 37 °C.

2.7. Differentiation of neurospheres into astrocytes

After 24 hours of incubation, we observed the formation of neurospheres (a conglomerate of cells proliferating as spheroids) freely floating in the thickness of the medium. Neurospheres were used for differentiation into astrocytes after 4-5 days of culture.

Differentiation of the neurospheres was carried out by adding differentiation factors to the culture medium. The commercially available media used for the test included Astrocyte Medium kit (Cat. No. 1821) media manufactured by ScienCell (USA).

After the differentiation of the neurospheres in the differentiation medium was completed and the necessary cell mass was accumulated to continue the experiments, the cells of the astrocyte culture were transferred to 24-well plates for immunocytochemical assessment under the conditions of normal oxygen level and after exposure to chemical hypoxia.

2.8. In Vitro Chemical Hypoxia Model

Astrocytes were incubated with sodium iodoacetate for 30 minutes at 37 °C in a CO₂ incubator. The final concentration of sodium iodoacetate in the medium was 50 μ M (Gutmann et al., 2002). At the end of the incubation, the cells were washed and the culture medium was completely replaced, after which the cells were cultured under standard conditions of the CO₂ incubator for 24 hours before the start of the

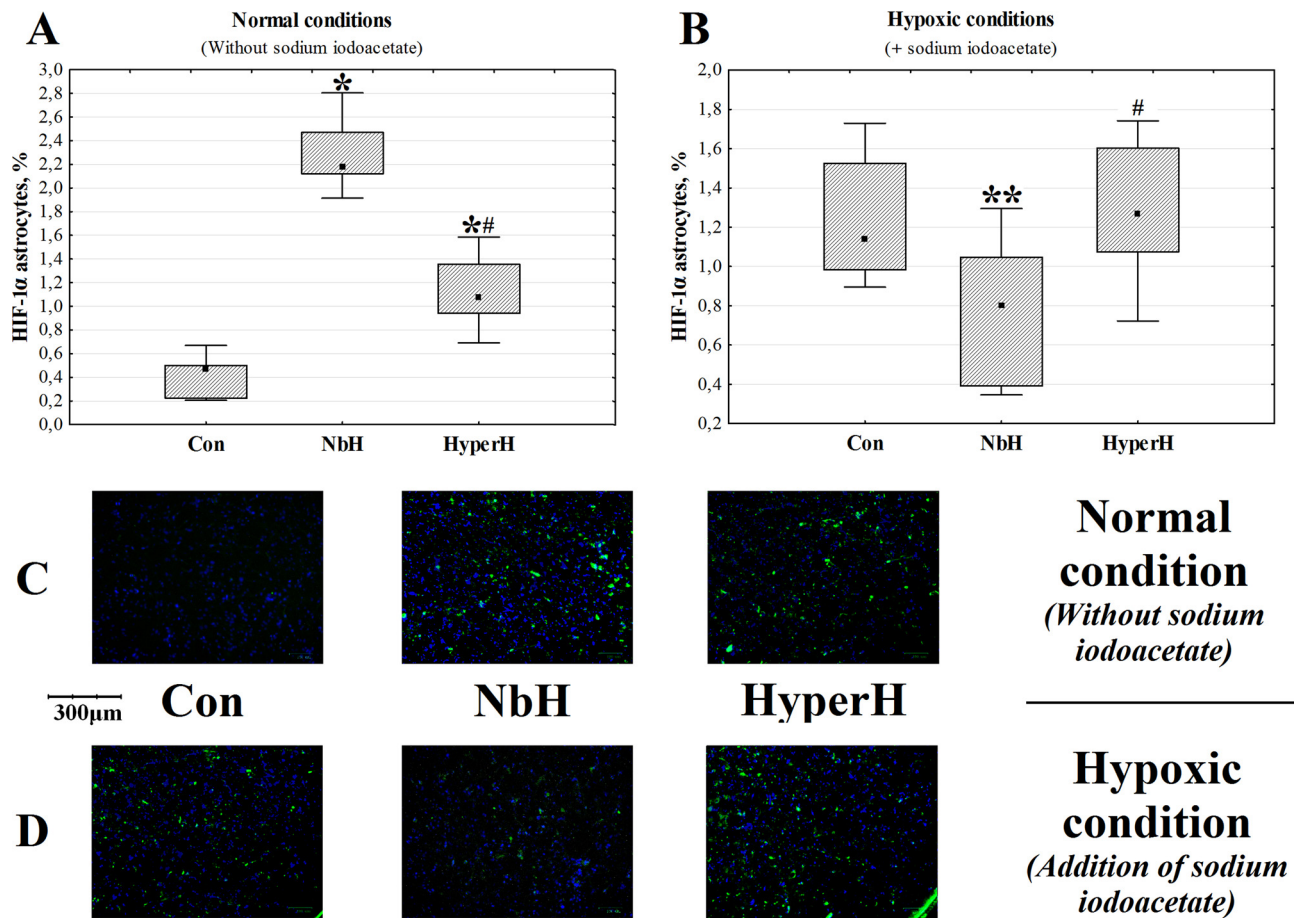


Fig. 3. The content of HIF-1 α in astrocytes exposed to hypoxia and hypercapnia *in vitro*: A and B are the percentage of marker-expressing cells in relation to the total number under normal and hypoxic conditions; Data are presented as Me (25; 75%). * - differences compared to control ($p < 0.001$); ** - differences compared to control ($p < 0.01$); # - differences compared to the NbH group ($p < 0.001$). C and D - expression of HIF-1 α in astrocytes under normal and hypoxic conditions. Magnification $\times 175$. Blue color - DAPI; green - primary antibodies to HIF-1 α associated with a secondary antibody conjugated to AlexaFluor488. Control = control group ($n = 5$); PermH = permissive hypercapnia ($n = 5$); HyperH = hypercapnic hypoxia ($n = 5$);

immunocytochemical study.

2.9. *In vitro* modeling of hypoxia and hypercapnia

Modeling of hypoxic and hypercapnic-hypoxic effects in a culture of astrocytes isolated from progenitor cells of rats of the control group was carried out in a special HypoxyLab chamber (Oxford Optronix Ltd, UK), which automatically maintained a given composition of ambient air ($PO_2 \approx 35$ mm Hg; $PCO_2 \approx 50$ mm Hg; the rest was N_2) at 37°C and a humidity level of 80% for 24 hours.

2.10. Immunocytochemical study

HIF-1 α expression was determined using the technique of double indirect immunocytochemical staining of astrocyte culture cells on 24-well plates. Before the analysis, the cells were prefixed in 4% paraformaldehyde in a culture medium (1:1 dilution) for 5 minutes. Subsequent cell fixation was carried out in 4% paraformaldehyde for 15 minutes.

After fixation, permeabilization with 0.1% Triton X-100 was performed (for 10 minutes at room temperature). Nonspecific activity was blocked by incubation with 10% BSA (bovine serum albumin) in PBS at 37°C for 30 minutes. After the blocking procedure, 200 μl of the primary antibody solution was added to the preparations followed by their incubation in a wet chamber for 2 hours at 37°C . Then, 200 μl of secondary antibodies were applied to the preparations followed by incubation in a wet chamber for 60 minutes at $+37^\circ\text{C}$. To contrast the

nuclei, a DAPI solution was applied to the preparations. Monoclonal mouse HIF-1 α antibodies in 1: 500 dilution (Thermo Fisher, Cat. No. SA5-10211) were used as primary antibodies. Polyclonal goat anti-mouse antibodies conjugated with Alexa Fluor488 in a 1: 2,000 dilution (Thermo Fisher, Cat. No. A-11001) were used as secondary antibodies. Some drugs were exposed to all stages of the immunofluorescence study, but without the addition of a primary or secondary antibody (negative control).

Fluorescence microscopy was performed on a ZOE cell analysis system (Bio-Rad, USA) with the calculation of the number of immunopositive cells expressing target antigens relative to the total number of cells. Counting was performed in no less than 5 fields of view per 1,000 cells in the sample. Microphotographs were processed using ImageJ 1.41 (Scion Inc., USA).

2.11. Immunohistochemical study

Immunohistochemical studies were carried out on free-floating sections according to the protocol. A blocking BSA solution was added to the wells for 60 minutes with shaking in an amount of 300 μl and then washing twice (500 μl of Wash solution + Triton-X100 $\times$ 2). Next, a solution of primary antibodies (300 μl) was applied to the sections with incubation on a shaker overnight at a temperature of $+4^\circ\text{C}$. Then, after washing three times, a solution of secondary antibodies was incubated with shaking in the dark for 2 hours at room temperature. For immunohistochemical studies, primary and secondary antibodies were used in similar dilutions as indicated in the description of

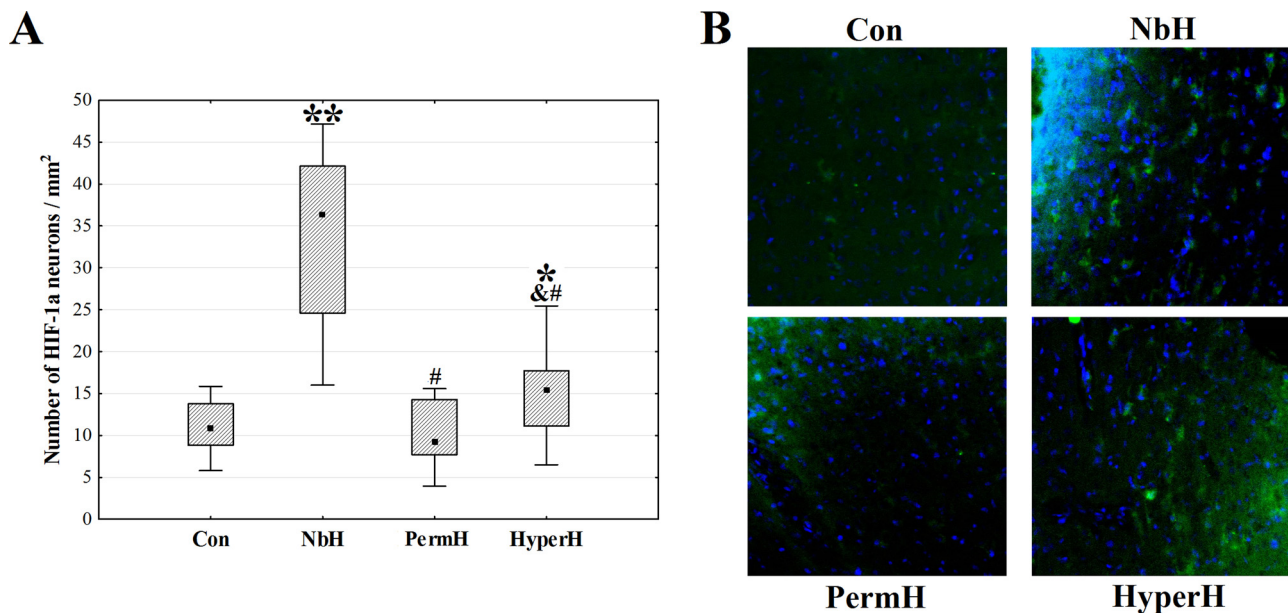


Fig. 4. The content of HIF-1 α in the peri-infarct area of the cerebral cortex (A). Data are presented as Me (25; 75%). ** - differences compared to control ($p < 0.001$); * - differences compared to control ($p < 0.01$); # - differences compared to the NbH group ($p < 0.001$); & - differences compared to the PermH group ($p < 0.05$). Microphotographs of a peri-infarct area of the cerebral cortex (B). Magnification x250. Confocal microscopy. Blue color - DAPI; green - primary antibodies to HIF-1 α associated with a secondary antibody conjugated to AlexaFluor488. Control = control group ($n = 10$); NbH = normobaric hypoxia ($n = 10$); PermH = permissive hypercapnia ($n = 10$); HyperH = hypercapnic hypoxia ($n = 10$);

the immunocytochemical study protocol. Some drugs were exposed to all stages of the immunofluorescence study, but without the addition of a primary or secondary antibody (negative control).

After incubation and washing three times, the sections were transferred onto glass slides followed by addition DAPI Fluoromount and covering with cover glasses. Counting the number of immunopositive cells expressing target antigens in the peri-infarct zone was performed in at least five fields of view using a FV10i-W confocal microscope (Olympus, Japan).

2.12. Statistical analysis

The total sample size and the sample size of each group was calculated based on the results of our previous studies on a similar model using the quantitative scale method (Dell et al., 2002). Statistical analysis was performed using SPSS 11.5 software (SPSS Inc, Chicago, IL). The Shapiro-Wilk test was used for normality testing.

Comparisons of normally distributed data were performed using Student's t-test. Comparisons of non-normally distributed data were performed using non-parametric methods. Variables in independent groups were compared using the Mann-Whitney test.

Normally distributed quantitative data are presented as mean (M) $\pm$ standard deviation (s); non-normally distributed data are shown in the form of the median (Me), the lower quartile (25%) and the upper quartile (75%). The differences were considered significant if the level of significance (p-value) was less than 0.05. Data are presented as the median $\pm$ 25/75 percentiles.

3. Results

3.1. Western blot

The content of HIF-1 α in the rat hippocampus after 7-fold respiratory exposure to hypoxia and/or hypercapnia (Fig. 1) was higher by 31% compared to the control in the NbH group ($p < 0.01$) and by 35% in the HyperH group ($p < 0.01$). Moreover, PermH did not have a significant effect on the HIF-1 α content in hippocampal cells.

3.2. HIF-1 α expression in astrocytes in vitro

In vitro HIF-1 α expression in astrocytes isolated from rat hippocampus followed by 15-fold respiratory exposure to hypoxia and/or hypercapnia was different in the control and experimental groups both under normal conditions of cell growth (Fig. 2: panels A and C) and in the model of chemical hypoxia (Fig. 2: panels B and D). HIF-1 α content increased by the factor of 4 compared to the control group in the NbH group (physiological conditions - Fig. 2A) and by the factor of 4.9 in the HyperH group ($p < 0.001$). Moreover, after chemical hypoxia modeling (Fig. 2B), an increase in the HIF-1 α expression with respect to the control and other experimental groups was observed only in the HyperH group ($p < 0.001$).

HIF-1 α expression in astrocytes isolated from rat hippocampus and exposed to the NbH and the HyperH for 24 hours differed between the control and experimental groups both under the conditions of normal cell growth (Fig. 3: panels A and C) and in the chemical hypoxia model (Fig. 3: panels B and D). Under physiological conditions (Fig. 3A), HIF-1 α content increased by the factor of 4.6 in the NbH group and by the factor of 2.3 in the HyperH group compared to the control group ($p < 0.001$). That is, the effect of hypercapnia in the HyperH group halved the level of HIF-1 α compared to the NbH group ($p < 0.001$). Moreover, after the addition of sodium iodoacetate (Fig. 3B) in the HyperH group, HIF-1 α expression level did not change compared to the control, however, this parameter was significantly decreased in the NbH group ($p < 0.001$).

3.3. HIF-1 α expression in the peri-infarct area

All the animals in the present research received typical photochemical infarcts affecting all layers of the sensorimotor cortex. In the infarction zone, fibrous alterations and microvessels with thrombi were distinguished in all sections from the region of ischemic injury, which is typical of the photochemical thrombosis method (Pevsner et al., 2001; Barth and Mody, 2011). The morphological feature was similar across all experimental groups, whereas differences were observed in the size of the cerebral infarction zone.

The level of HIF-1 α expression in the peri-infarct area of the cerebral cortex (Fig. 4) in rats from the NbH group was 3.3 times that parameter in the control group ($p < 0.0001$) and 40% higher in the HyperH group compared to the control ($p < 0.01$). The differences between the PermH group and the control group were not statistically significant. However, the level of HIF-1 α in the HyperH group was higher than in the PermH group ($p < 0.05$), but lower in comparison with the NbH group ($p < 0.001$).

4. Discussion

The major findings from this study include the following: 1) Hypercapnia alone did not elicit any change in the HIF-1 α levels; 2) combined hypoxia and hypercapnia resulted in the increased expression of HIF-1 α in hippocampal tissues, in vitro astrocytes and neurons in the peri-infarct region of the cerebral cortex; 3) In the chemical hypoxia model, the expression of HIF-1 α in astrocytes was increased in the group of animals exposed to combined hypercapnia and hypoxia when compared to animals exposed to hypoxia alone suggesting potentiating effect of hypercapnia; 4) Similarly, in the chemical hypoxia model, the expression of HIF-1 α was increased in the culture of astrocytes exposed in vitro to combined hypercapnia and hypoxia when compared to astrocytes exposed to hypoxia alone, suggesting potentiating effect of hypercapnia; 5) In contrast, HIF-1 α expression in the peri-infarct area of the cerebral cortex was diminished in animals exposed to combined hypercapnia and hypoxia when compared to animals exposed to hypoxia alone.

According to our results (see Section 2.1), the presence of hypercapnia does not affect HIF-1 α concentrations in the hippocampal tissues following exposure to intermittent hypoxia. Moreover, the measured level of HIF-1 α after exposure to normobaric hypoxia is comparable with the results of Gu et al., 2008, who found a 1.5-fold increase in the relative level of protein expression of this factor 24 hours after the completion of 5-fold exposure to hypobaric hypoxia (2 hours a day). It should be noted that the hippocampus was not exposed to damaging ischemia/hypoxia, and high levels of HIF-1 α should only be considered as an adaptive response of the cells to the hypoxic stimulus (Marti et al., 2000).

Given the data obtained in one of our previous studies, which showed increased HIF-1 α concentrations in the brain tissue not only as a result of oxygen deficiency, but also following exposure to increased CO₂ concentrations in the presence of normal oxygen level (Kulikov et al., 2015), it is not possible to rule out potential effects of hypercapnia on HIF-1 α level in other brain structures (e.g. cortex or hypothalamus) or its ability to exert protective and/or potentiating effect at earlier stage.

Experimental data obtained using cell models in vitro have demonstrated a number of important facts. For example, HIF-1 α content in astrocyte cultures was increased if the rats had been exposed to intermittent hypoxia. HIF-1 α concentration in astrocytes did not depend on the presence of hypercapnia. These effects confirm the lack of a direct effect of CO₂ on the mechanism of HIF-1 α accumulation in the brain cells under the conditions of moderate oxygen deficiency. Furthermore, it provides evidence that intermittent hypoxia affects the epigenetic regulation of HIF-1 α level in neurons at the gene levels thus maintaining its high level even in the culture. This might be related both with the classical mechanism of prolyl-hydroxylase inhibition (Huang et al. 1998; Lando et al., 2003) in astrocytes and an alternative signaling pathway of HIF-1 α activation via MAPK and PI3K pathways (Lukyanova, 2011) under the conditions of progenitor cells differentiation.

At the same time, the analysis of astrocyte cultures that had been exposed to hypercapnia and/or hypoxia only in vitro for 24 hours demonstrated a clear correlation between the levels of HIF-1 α and partial pressure of CO₂. Thus, an increase in CO₂ concentrations from 5.0 to 7.5% accompanied by oxygen deficiency caused a half-fold decrease in

HIF-1 α levels. This may be due to the lack of factors at the level of the body or the organs (Iyer et al., 1998) or higher tolerance to hypoxia in astrocytes (Kwasiborski, 2012), which has developed as a result of a potentiating effect of hypercapnia as evidenced by the results of a test with addition of sodium iodoacetate.

As a model of acute hypoxia in vitro, we chose the method of adding sodium iodoacetate to the culture of astrocytes (Gutmann et al., 2002). This experimental method seems appropriate since it was tested on an in vitro model of nerve cells and is similar in pathogenesis to acute hypoxia/ischemia, which allows us to interpret our results as the possible potential for neuroprotection. The assessment of HIF-1 α level in astrocytes in an in vitro model of damaging hypoxia induced by addition of sodium iodoacetate demonstrated that hypercapnia had a potentiating effect when accompanied by hypoxia. This was observed both in astrocyte cultures prepared following in vivo exposure to hypercapnic hypoxia and astrocytes following similar exposure in vitro.

Pronounced decrease in HIF-1 α levels under the conditions of chemical hypoxia following exposure to hypoxia alone is probably due to the deficiency of its accumulation (Kallio et al., 1999) with subsequent decrease in tolerance to hypoxia (Maxwell et al., 1999).

These facts allow to suggest that hypercapnia (when accompanied by hypoxia) modifies the signal mechanism of HIF-1 α activation in case of exposure to damaging hypoxia/ischemia. This mechanism is probably aimed at stimulation of the adaptive cell potential and increase in the nerve tissue tolerance to ischemia/hypoxia by protection from excessive accumulation of HIF-1 α in response to moderate hypoxia (Zhong et al. 1999).

A model of focal ischemia-induced damage of the cerebral cortex with subsequent assessment of HIF-1 α level in the cells of the peri-infarct zone demonstrated a similar tendency showing that hypercapnia did not affect the transcription factor levels following isolated exposure and reduced its expression when was accompanied by hypoxia. This fact (considered in the context of larger potential of hypercapnic hypoxia in respect of ischemic tolerance development) may also be considered as a mechanism of prevention of excessive HIF-1 α activation.

It is not completely clear which signal mechanism is involved in the effects of CO₂ on HIF-1 α accumulation/activation. However, it can be assumed that such mechanisms might be related to prolyl hydroxylase inhibition and HIF-1 α accumulation in acidosis (Lando et al., 2003); MAPK and PI3K signaling pathways activation in the proliferating microglia cells with increased HIF-1 α production (Lukyanova, 2011), or modification of the activity of chaperone-mediated lysosome autophagy (Ferreira et al., 2015; Hubbi et al., 2014).

High levels of HIF-1 α are known to induce apoptosis impairment in cells (Yeom et al., 2016; Li et al. 2017) as well as an imbalance of cell energy homeostasis (Knyazev et al., 2019; Nagao et al. 2019), which is often associated with various oncogenic conditions (Vaupe and Mayer, 2007; Pezzuto and Carico, 2018). Therefore, HIF-1 α levels above the upper limit may be considered an unfavorable factor in the pathogenesis of acute ischemic injury.

It is important to note that some authors came to opposite conclusions regarding the effects of hypercapnia on the mechanism of HIF-1 α activation. Thus, Selfridge et al. (2016) showed in their experimental study that hypercapnia suppressed the HIF protein stability and expression of the target gene both in vivo and in vitro, which was associated with a direct decrease in intracellular pH level. Raeis et al. (2010) demonstrated the absence of correlation between the increased HIF-1 α expression and CO₂ level following a 24 -h exposure of cardiomyocytes to hypercapnic hypoxia in vitro.

Despite the fact that some of the results obtained in the current study also indicate the inhibitory effect of the hypercapnic component on the HIF-1 α level, the data obtained in the experiment with chemical hypoxia modelling show the protective potential of carbon dioxide when combined with the effect of intermittent hypoxia. Moreover, it should be noted that in these studies, HIF protein suppression was detected using a model with high level of hypercapnia (FetCO₂ = 10%)

under the conditions of long exposure duration (4–6 hours in vivo and 24 hours in vitro), which could cause a maladaptive effect. At the same time, data on cardiomyocytes cannot be fully extrapolated to neurons due to a great difference in the mechanism of neuro- and cardioprotection (Maslov et al. 2009).

The effect of the combined effects of moderate doses of hypoxia and hypercapnia found in our study can be viewed promisingly from the prospective of the hormesis paradigm (Leak et al. 2018). The main reason for this is the fact that both the lack of oxygen and carbon dioxide as well as their excess in the body causes serious damage. Therefore, the positive effects, demonstrated for the intermittent effects of mild doses of hypoxia and hypercapnia, could be achieved only with a certain therapeutic range of gas concentrations, duration of sessions, and frequency of courses. In addition, it is important to emphasize that intermittent hypercapnia and hypoxia have already been considered as hormetic stimulus (Pruimboom and Muskiet, 2018).

5. Conclusions

Thus, we can conclude that hypercapnia does not increase the level of transcription factor HIF-1 α . However, the combined effects of hypercapnia and hypoxia in in-vitro simulations of acute chemical hypoxia stimulate the accumulation of HIF-1 α . This can be considered an important component of the neuroprotective potential of hypercapnic hypoxia, due to the process of carbonic potentiation of the positive effects of intermittent hypoxia and prevention of excessive activation of HIF-1 α factor.

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Ethics approval

Animal experiments were approved by the Bioethical Commission of the local Ethics Committee of the Krasnoyarsk State Medical University. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (EU Directive 2010/63/EU for animal experiments).

Declarations of Competing Interest

None.

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