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PECULIARITIES OF REACTION OF HIF-1α PROTEIN OF THE HIPPOCAMPUS NEURONS IN RATS WITH EXPERIMENTAL DIABETES MELLITUS IN THE DYNAMICS OF ISCHEMIC-REPERFUSION DAMAGE OF THE BRAIN

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Abstract

Introduction. The role of the transcriptional factor Hif-1α in pathogenesis of hypoxic damages and diabetes mellitus (DM) is proved, although molecular mechanisms underlying the basis of this factor dysfunction in association with DM with ischemic-reperfusion damage of the brain remain unknown.

Objective. The objective of this investigation was to study the content of Hif-1α protein in the hippocampus neurons of rats with experimental DM in the dynamics of ischemic-reperfusion damage of the brain.

Results. In rats without DM 20 minute ischemia with one hour reperfusion increases the content of Hif-1α protein in all the fields of the hippocampus. On the 12th day of ischemic-reperfusion period in the hippocampus CA2-CA4 fields the values of certain examined indices of the activity of Hif-1α transcriptional factor continue to increase, and in CA1field they normalize or approach to the values of animals in the control group. In rats with DM during early post-ischemic period there are no changes of Hif-1α protein content in CA1 field, in CA2 field there are signs of its reduced activity, in CA3 field they are limited by the reaction of one index, in CA4 field they are of a similar character with those of the control rats under experimental conditions. On the 12th day of ischemic-reperfusion period in CA1

field all the indices of activity of Hif-1α transcriptional factor increase exceeding corresponding indices by absolute values in animals of the control group under the same experimental conditions, in CA2 and CA3 fields changes of the examined parameters are limited as compared to the same ones in animals from the control group, in CA4 field values that were increased in the control group decrease.

Conclusions. Diabetes mellitus restricts reaction of Hif-1α protein on ischemia-reperfusion inn the neurons of CA1-CA3 fields in early ischemic-reperfusion period and in the neurons of CA2-CA4 fields – on the 12th day of observation.

Key words: hippocampus, diabetes mellitus, brain ischemia-reperfusion, Hif-1α protein.

Contradiction between oxygen supply and its requirement is generally known to be the initiator of a cascade of biochemical and molecular events resulting in destruction of neurons in the brain during its ischemic-reperfusion damage. In this situation protective cellular mechanisms act in the cerebral tissue, and induction of different transcriptional factors in particular, including very important Hif-1α factor induced by hypoxia – a transcriptional regulator of oxygen homeostasis and a key factor of adaptive response formation [1, 2]. Hif-1α is a powerful regulator of various target genes increasing erythropoiesis, stimulating angiogenesis through the activation of the vascular endothelial growth factor (VEGF), ensuring adequate metabolism of glucose and its transport into the neurons, promoting maintenance of mitochondrial structure and survival of cells [3, 4].

Although, not all researchers so definitely evaluate the role of Hif-1α. Experimental evidence is obtained concerning not only neuroprotective but neurotoxic effects of Hif-1α as well. The latter are realized through the increased activity of p53 gene product – p53 protein and other factors of apoptosis activation [5]. In addition, Hif-1α participates in death of cells by means of necrosis interacting with calcium and calpain; it can intensify brain edema increasing permeability of the hematoencehalic barrier [6, 7, 8, 9]. Protective effects of Hif-1α are considered to be realized mainly in case of mild hypoxia, and neurotoxic – in case of severe one.

In addition to hypoxia hyperglycemia is a powerful regulator of Hif-1α activity [8]. In their turn hypoxia and hyperglycemia are the main factors determining chronic complications of diabetes. Scientific studies in recent years are indicative of the fact that Hif-1α destabilization transduced by hyperglycemia is manifested by the loss of cellular response to hypoxia in case of diabetic complications, that in its turn has a negative effect on adaptation

of cells and tissues to low oxygen content [10, 11]. Since the mechanisms of Hif-1α stabilization by hypoxia are well studied, destabilization and decreased activity of this factor under conditions of hyperglycemia remain disputable. One of the recently detected mechanisms of destabilization and functional repression of Hif-1α with DM is methylgyoxal effect accumulated under conditions of high glucose levels and resulted in quick proteasome-dependent degradation of Hif-1α under conditions of hypoxia [10]. Insignificant hyperglycemia activates Hif-1α signalization in certain specific types of cells, although a high glucose level inhibits it [12].

As we can see the role of Hif-1α transcriptional factor in pathogenesis of hypoxic damages and DM is proved, although the mechanisms of its activation and destabilization are still studied. However molecular mechanisms underlying dysfunction of this factor in association with DM with ischemic-reperfusion damage of the brain remain unknown.

The objective of this investigation is to study the indices of activity of Hif-1α transcriptional factor of the hippocampus field neurons of rats with experimental DM in the dynamics of ischemic-reperfusion damage of the brain.

Materials and methods. The study was conducted under conditions of modeling bilateral carotid ischemia by means of 20 minute clipping of both general carotid arteries with reperfusion of a various duration in rats with and without DM. DM was modeled by means of intraperitoneal injection of streptozotocin («Sigma», USA, 60 mg / kg) in albino nonlinear male rats two months of age [13]. Duration of diabetes was 4 months which is sufficient to form diabetic encephalopathy in rats [13]. Early consequences of ischemic-reperfusion damages of the hippocampus were studied after one-hour reperfusion, and remote ones – on the 12^{th} day of post-ischemic period.

Ischemia modeling and euthanasia of animals were carried out under calipsol narcosis (75 mg/kg intraperitoneally). Availability of DM was verified by detection of glucose level in the blood (by means of glucose-oxidase method) and examination of morphological condition of the pancreas; experimental groups were formed with rats with the level of glycemia equal or higher than 10 mmol/L.

The brain was removed as soon as possible under conditions of low temperature, according to coordinates of the stereotactic atlas [14] the areas were isolated containing the hippocampus CA1, CA2, CA3 and CA4 fields and placed into 10% Buen solution for their 24-hour fixation. After appropriate histological treatment the samples were saturated into paraffin blocks.

Hif-1α protein was identified by means of immunofluorescent method. Rehydrated

histological sections were incubated during 18 hours in a moist camera at 4° C with primary murine monoclonal antibodies to Hif-1α of a rat (mouse IgG1 isotype, «Santa Cruz», USA) in ratio 1:1000. The excess of primary antibodies was washed in 0,1 M phosphate buffer, sections were incubated during 60 minutes at 37° C with secondary antibodies (rabbit antibodies to the complete molecule of IgG of a mouse conjugated from fluorescein by means of isothiocyanate («Santa Cruz», USA) in the ratio 1:64. After that the sections were washed by 0,1 M phosphate buffer and placed into the mixture of glycerin and phosphate buffer (9:1) for further luminescence microscopy. The concentration of Hif-1α protein, its specific content and square of Hif-1α-immunoreactive material were determined. Histilogical sections were studied by means of the fluorescent microscope AXIOSKOP. The images were entered into the computer system of the digital analysis VIDAS-386 («Kontron Elektronik», Germany) [15].

The data obtained were statistically processed in the applied programs "Statistica 6.0" and "SPSS 13" with the use of parametric Student t-criterion. Critical significance level while checking statistical hypotheses was equal 0,05.

Results and discussion

The results of the study are presented in Tables 1 and 2. 20-minute carotid ischemia with one hour reperfusion in CA1 field was found to cause the increase of concentration and specific content of Hif-1α protein in 2,1 and 1,9 times respectively. In CA2 field under conditions of this intervention the square of Hif-1α-immunoreactive material (IRM) increased in 1,6 times, concentration and specific content of Hif-1α protein – in 1,5 and 1,8 times. The reaction of cells in CA3 field was similar, where the square of Hif-1α-IRM, concentration and specific content of Hif-1α protein increased in 1,7, 1,3 and 2,3 times respectively. In CA4 field of rats from this experimental group the increase of concentration and specific content of Hif-1α protein in 1,8 and 1,7 times was found.

Analysis of the data obtained is indicative of the fact that in early ischemic-reperfusion period the activity of Hif-1α transcriptional factor intensifies in the hippocampus fields (considering changes of the product of Hif-1α protein activity).

On the 12th day after modeling 20-minute carotid ischemia in CA1 field concentration of Hif-1α protein remains increased (on 20 %) as compared to the values in the control group, although it reliably decreased as compared to the early period in 1,8 times. Specific content of Hif-1α protein was also 1,8 times reduced as compared to the previous term and got the values equal to those of the control group.

Table 1

Effect of ischemia-reperfusion on the reaction of Hif-1α protein in the neurons of the hippocampus CA1-CA2 fields of control animals and animals with diabetes mellitus (M ± m)

Group of observation	Concentration of Hif-1α protein ($E_{IФ}$)	IRM square per 10 000 mcm^2	Specific content of Hif-1α protein ($E_{IФ}$)
CA1 field			
Control	0,0103±0,0003	11,412±0,953	0,110±0,026
Ischemia-reperfusion 20 min/1 hour	0,0212±0,0005*	10,871±0,828	0,207±0,028*
Ischemia-reperfusion 12 days	0,0120±0,0008*^	10,706±1,41	0,114±0,010^
Diabetes	0,0259±0,0025*	13,552±1,120	0,251±0,027*
Diabetes and ischemia-reperfusion 20 min/ 1hour	0,0290±0,0023	10,918±1,069	0,267±0,028
Diabetes and ischemia-reperfusion 12 days	0,0451±0,0017#&	22,028±2,066#&	0,491±0,044#&
CA2 field			
Control	0,0076±0,0002	7,566±0,709	0,064±0,007
Ischemia-reperfusion 20 min/1 hour	0,0116±0,0007*	11,905±1,091*	0,116±0,016*
Ischemia-reperfusion 12 days	0,0086±0,0004*^	16,724±1,314*^	0,136±0,024*
Diabetes	0,0112±0,0005*	11,071±1,043*	0,118±0,017*
Diabetes and ischemia-reperfusion 20 min / 1 hour	0,0138±0,0009#	7,552±0,694#	0,103±0,017
Diabetes and ischemia-reperfusion 12 days	0,0199±0,0023#&	11,489±1,312&	0,160±0,018&

Note: here and in the following Table: difference probability as compared to: * - control; ^ - ischemia-reperfusion (20 min / 1hour) in the control animals; # - diabetes; & – ischemia-reperfusion (20 min / 1 hour) in animals with diabetes.

In CA2 field in the late ischemic-reperfusion period all the three examined indices remained increased as compared to those of the control group: the square of Hif-1α-IRM – in 2,2 times, concentration and specific content of Hif-1α protein – on 13 % and 2,1 times respectively. Although their dynamics differed: as compared to the early post-ischemic period the concentration of Hif-1α protein became 1,3 reduced, its specific content did not change, and the square of Hif-1α-IRM – became 1,4 times increased. In CA3 field on the 12th day of observation all the examined indices increased the control ones: the square of Hif-1α-IRM – twice, concentration and specific content of Hif-1α protein – in1,8 and 3,9 times. Although two latter indices were 1,4 and 1,7 times increased respectively as compared to the early post-ischemic period, and the square of Hif-1α-IRM remained on the level of the previous term.

Table 2

Effect of ischemia-reperfusion on the reaction of Hif-1α protein in the neurons of the hippocampus CA3-CA4 fields of control animals and animals with diabetes mellitus (M ± m)

Group of observation	Concentration of Hif-1α protein ($E_{IФ}$)	IRM square per 10 000 mcm^2	Specific content of Hif-1α protein ($E_{IФ}$)
CA3 field			
Control	0,0072±0,0003	9,254±0,893	0,058±0,007
Ischemia-reperfusion 20 min/ 1hour	0,0093±0,0003*	15,507±1,776*	0,133±0,020*
Ischemia-reperfusion 1 days	0,0132±0,0012*^	18,649±1,459*p	0,227±0,038*^
Diabetes	0,0116±0,0005*	12,676±1,155*	0,142±0,023*
Ischemia-reperfusion 20 min/ 1hour	0,0189±0,0013#	13,869±1,861	0,167±0,022
Diabetes and ischemia-reperfusion 12 days	0,0159±0,0021#	20,746±2,248#&	0,204±0,034
CA4 field			
Control	0,0068±0,0001	7,563±1,014	0,052±0,007
Ischemia-reperfusion 20 min/ 1hour	0,0124±0,0006*	7,118±0,879	0,087±0,013*
Ischemia-reperfusion 12 days	0,0136±0,0011*	12,158±1,148*^	0,125±0,016*^
Diabetes	0,0229±0,0021*	10,796±1,067*	0,151±0,021*
Diabetes and ischemia-reperfusion 20 min/ 1hour	0,0299±0,0023#	12,733±1,542	0,252±0,027#
Diabetes and ischemia-reperfusion 12 days	0,0988±0,0092#&	6,776±1,327#&	0,177±0,025&

In CA4 field as compared to the indices of animals from the control group the square of Hif-1α-IRM, concentration and specific content of Hif-1α protein were in 1,6, 2,0, 2,4 times higher. The increasing dynamics was observed as compared to the indices in the early period of the experiment concerning the square of Hif-1α-IRM and specific content of Hif-1α protein in 1,7 and 1,4 times.

The total analysis of the results obtained enables to conclude that activation of Hif-1α transcriptional factor in the early ischemic-reperfusion period is peculiar for all the examined areas of the hippocampus. On the 12th day of the experiment in all the fields except CA1 field the activity of indices continues to increase, and in CA1 filed it decreases. It is indicative of the facts that till the 12th day of the experiment adaptive mechanisms in CA2-CA4 fields remain intense, and in CA1 field they are inhibited, which in general coincides with the existing opinion concerning the greatest susceptibility of this field to harmful factors.

In rats with four month DM the signs of activation of Hif-1α transcriptional factor were found in all the fields. In CA1 field it was manifested by higher than those of the control values of the concentration and specific content of Hif-1α protein (in 2,5 and 2,3 times), in CA2, CA3, CA4 fields – by the values of the square of Hif-1α-IRM, concentration and specific content of Hif-1α protein (in 1,5, 1,5, 1,8 times – in CA2 field; 1,4, 1,6, 2,4 times – in CA3 field; 1,4, 3,4, 2,9 times – in CA4 field). These results enable to suggest that in the mentioned term of DM formation there are signs of angiopathy creating hypoxic conditions in the examined portions of the brain. Quantitative differences of the increased values of the indices examined are indicative of different susceptibility of the hippocampus fields to the formation of diabetic encephalopathy.

In early ischemic-reperfusion period concerning the indices with diabetes non-complicated by disorders of the cerebral circulation any reliable changes of the examined indices in the hippocampus CA1 filed with DM were not found, and therefore during this period of observation contrary to the animals without diabetes adaptive mechanisms do not work. In CA2 field during this period of the experiment the concentration of Hif-1α protein became 23% higher with simultaneous 1,5 times decrease of the square of Hif-1α-IRM; in CA3 field the concentration of Hif-1α protein became 1,6 times higher; and in CA4 field – the concentration and specific content of Hif-1α protein – in 1,3 and 1,7 times. Comparison of changes of the indices studied after 20 minute ischemia-reperfusion in animals without diabetes and with it demonstrates more restricted reaction of the product of Hif-1α transcriptional factor in animals of the last group.

On the 12^{th} day of modeling carotid ischemia as compared to the indices with diabetes non-complicated by cerebral circulation disorders the square of Hif-1α-IRM, concentration and specific content of protein became1,6, 17, 1,96 times higher in the hippocampus CA1 field. Considering the previous term these indices were in 2,0, 1,6, 1,8 times higher. In CA2 field concerning the indices with diabetes the concentration of Hif-1α protein remained increased (in 1,6 times). It should be noted that this index increased as compared to the early period of observation (in 1,4 times). The square of Hif-1α-IRM and specific content of Hif-1α protein during this period were similar to the values of animals with diabetes without cerebral circulatory disorders, and concerning the early term they were in 1,5 and 1,6 times higher. Concerning CA3 field on the 12^{th} day of the post-ischemic period the concentration of Hif-1α protein and the square of Hif-1α-IRM were in 1,4 and 1,6 times higher as compared to the values with diabetes. The last index was in 1,5 times higher than that in the early post-ischemic period. In CA4 field the concentration of Hif-1α protein was considerably higher

than that in the animals with DM and during the early post-ischemic period (in 4,3 and 3,3 times). Considering in 1,6 and 1,9 times reduction of the square of Hif-1α-IRM it might be suggested that concentration of this protein increased due to reduction of the square. Specific content of Hif-1α protein returned to the values of animals with DM and it was lower than that in the previous term in 1,4 times.

Conclusions. 1. In rats without DM 20 minute ischemia with one hour reperfusion increases the content of Hif-1α protein in all the fields of the hippocampus. On the 12^{th} day of ischemic-reperfusion period in the hippocampus CA2-CA4 fields the values of certain examined indices of the activity of Hif-1α transcriptional factor continue to increase, and in CA1field they normalize or approach to the values of animals in the control group.

2. In rats with four month diabetes mellitus higher than those of the control values of Hif-1α activity are found in all the examined hippocampus fields.

3. In rats with DM during early post-ischemic period there are no changes of Hif-1α protein content in CA1 field, in CA2 field there are signs of its reduced activity, in CA3 field they are limited by the reaction of one index, in CA4 field they are of a similar character with those of the control rats under experimental conditions. On the 12^{th} day of ischemic-reperfusion period in CA1 field all the indices of activity of Hif-1α transcriptional factor increase exceeding corresponding indices by absolute values in animals of the control group under the same experimental conditions, in CA2 and CA3 fields changes of the examined parameters are limited as compared to the same ones in animals from the control group, in CA4 field values that were increased in the control group decrease.

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