

Dynamics of Changes in the Concentration of Heat Shock Protein (HSP70) in the Cerebral Cortex and Hippocampus in Experimental Violation of Cerebral Circulation: The Ability to Regulate this Process through Positive Modulation of Thiol-Disulfide System

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Abstract

Objective: Ischemic brain damage is the main factor of pathogenesis of various CNS disorders, which are observed in clinical practice. Nowadays, it is established that the parts of the brain are differ in their sensitivity to ischemia. It

was noted that in the hippocampus and cerebellum, the proportion of necrotic neurons is significantly higher than in other cerebral structures. The goal of this research was estimation of possible pharmacological regulation of endogenous neuroprotective factor (HSP70 - heat shock protein) by the positive modulators of thiol-disulfide system in the neuronal cortex and hippocampus in the different periods after experimental cerebral vascular accident.

Methods: Experiments were carried out on outbred rats of both sexes weighing 180-200 g. Experimental animals were divided on six groups: pseudo operated animals, control group and four groups of animals, that were treated by Thiotriazoline, Angioline, Thiocetam or lipoic acid. Cerebral vascular accident was reproduced by bilateral common carotid arteries ligation. In the brain homogenate we defined the content of HSP70 and recovered glutathione at different periods of ischemia (day 1, day 4 and day 18).

Results: Modeling of cerebral vascular accident led to the different changes in the transcription of genes, which coded heat shock protein. We found decreasing of HSP70 content and recovered glutathione concentration in brain of animals with cerebral vascular accident. Such dynamics was observed during all period of observation until the day 18. Administration of thiol-disulfide system modulators to the experimental animals caused positive effect on HSP70 concentration in neurons of hippocampus and cortex. These could be explained by ability of these medicines to increase the content of recovered glutathione in the neuronal tissue. The most pronounced effect showed Angioline and Thiotriazoline.

Conclusions: Analysis of obtained results confirms the idea that ischemic neuronal damage is accompanied by changes in the functional state of the components of heat shock proteins and glutathione system. Injection of thiol-disulfide system modulators caused directly increasing of recovered glutathione content and indirectly led to the activation of expression of HSP70.

Keywords: Stroke, HSP70, recovered glutathione

Introduction

Ischemic brain damage is the main factor of pathogenesis of various CNS disorders, which are observed in clinical practice. Several studies on rats had shown that resistance of brain to ischemia associated with individual peculiarities of behavior in animals. But the mechanisms for such differences are still scarcely explored. Nowadays, it is established that the parts of the brain are differ in their sensitivity to ischemia. It was noted that in the hippocampus and cerebellum, the proportion of necrotic neurons is significantly higher than in other cerebral structures. Histochemical studies of localization of free radical oxidation (FRO) products in the brain of animals, which had cardiac arrest, showed that neurons of selectively vulnerable hippocampal areas are primal targets for FRO in the brain during reperfusion [1].

It was proven, that the most important role in the FRO play redox reaction, during which thiol groups are easily oxidized in disulfide groups, which then could newly regenerate by reductive cleavage. All these transformations create reversible thiol-disulfide system (TDS), which is very important in the regulation of redox balance in the cells and tissues of the body. [2]

Intermediates of thiol-disulfide system take place in the transport of NO, thereby enhancing its bioavailability, in addition, many thiols (glutathione, cysteine, methionine) can significantly limit the cytotoxicity of NO and its derivatives, increasing the chance of neurons to survive during ischemia [3].

Glutathione system, which consist from recovered glutathione (GSH) and its metabolic enzymes (glutathione peroxidase (GPx), glutathione transferase and glutathione reductase (GR)), is one of the leading antioxidant systems in the body and plays a key role in the tolerance of brain neurons to ischemia [4]. Glutathione directly or by the help of enzymatic reactions efficiently protects cells from free radicals and other reactive oxygen species such as hydroxyl radical, a lipid peroxy radicals, hydrogen peroxide and peroxynitrite. In addition glutathione is involved in the functioning of glutaredoxin-dependent system, which plays an important role in maintaining of the intracellular redox homeostasis [5].

In recent days, a number of researchers obtained evidence of protective function of heat shock protein 70 kDa (HSP70) in the neurons. Carried out immunohistochemical studies revealed the role of the HSP70 in the development of ischemic neuronal changes. It was found that a high initial level of immunoreactivity of neuronal populations to HSP70 had been an important factor of their resistance to ischemia [6]. At the same time, a number of experimental studies showed the regulatory role of the level of recovered glutathione in HSP70 expression mechanisms [3, 7].

The goal of this research was estimation of possible pharmacological regulation of endogenous neuroprotective factor (HSP70) by the positive modulators of thiol-disulfide system in the neuronal cortex and hippocampus in the different periods after experimental cerebral vascular accident (CVA).

Materials and Methods

Animals

Experiments were carried out on outbred rats of both sexes weighing 180-200 g. All animals were on standard food ration of vivarium, with natural alteration of day and night. Rats were received from nursery of «Institute of Pharmacology and Toxicology, Academy of Medical Sciences of Ukraine». All experimental procedures and operative interventions were done in accordance with WMA Statement on Animal Use in Biomedical Research.

Experimental animals were divided on six groups (10 animals in each): I group – pseudo operated animals; II group – animals with experimental CVA (control group), III group – animals with experimental CVA, which were treated by Thiotriazoline («Arterium», Ukraine) in dosage 50 mg/kg, IV group – animals with experimental CVA, which were treated by Angioline («Pharmatrone», Ukraine)

in dosage 50 mg/kg, V group – animals with experimental CVA, which were treated by Thiocetam («Galichpharm», Ukraine) in dosage 125 mg/kg, VI group – animals with experimental CVA, which were treated by lipoic acid («Marbiopharm», Russian Federation) in dosage 50 mg/kg.

Stroke model

For experiment we used a model of global incomplete cerebral ischemia, that is the most adequate in terms of clinical implications of ischemic stroke. This model was reproduced by bilateral common carotid arteries ligation that was performed under ethaminal sodium anesthesia (40 mg/ kg), with implication of surgical approach by means of separation of carotid arteries and single-step silk deligation.

Medicines were injected intraperitoneally one time per day during 18 days of observation, beginning the first day after operation. We injected saline solution in the same volume to the animals of the I and II groups.

Animals were taken out from the experiment under etaminal sodium anesthesia (40 mg / kg) [8]

Biochemical studies

For biochemical analysis we used fragments of the sensorimotor areas of the cortex and hippocampus of the brain. Cytosolic fraction was isolated by differential centrifugation (15,000 g) at + 4 °C using 0.15 M phosphate buffer, pH 7.8. The content of oxidized and recovered glutathione was determined fluorometric [9].

HSP 70 content

Concentration of HSP 70 in the brain homogenate was determined by Western blotting. The proteins were separated in 10% polyacrylamide gel electrophoresis. Separation of protein fractions was performed by electrophoresis at a voltage of 100 V (for gel sealing) until the sample reached the gel interface. Then, with a voltage of 200 V until the sample reached the end of the gel.

The proteins were transferred from polyacrylamide gel to the nitrocellulose membrane by electroelution during 45 minutes. After transfer the membrane was placed in blocking buffer containing 1% solution of bovine serum albumin (SIGMA, USA, cat. A2153) for 20 h. Then membrane was washed for 5 minutes by 0.1 M phosphate buffer, placed in a solution of a primary antibodies against HSP 70 (1: 500), (Santa Cruz Biotechnology, cat. sc-24) and incubated for 2 hours at room temperature. Then membrane was washed for four times by 0.1 M phosphate buffer, placed in a solution of secondary antibodies (1:1000) (SIGMA, USA, Cat. №051M4885) and incubated for 2 hours. For visualization membrane was processed with a solution, which contained: 1 tablet of 3-amino-9-ethylcarbazole (Sigma, USA, cat. a6926), 2.5 ml of DMF, 47.5 ml of 0.05 M acetate buffer (pH = 5.0) and 25 µl of 30 % H₂O₂. Then membrane was washed with distilled water for several times and dried between sheets of filter paper under a

stream of cold air. Detection of HSP 70 was performed with use of densitometry in the program Adobe Photoshop [9].

Statistical analysis

Results of the experiment were processed using STATISTICA® 6.0 licensed software for Windows (Stat Soft Inc NAXXR712D833214FANS). Individual statistical procedures and algorithms were implemented in the form of specially written macros. Results were represented as sample mean \pm standard error of the mean. Significant differences between trial groups were evaluated by means of Student t-test. For all types of analysis, the differences were deemed to be statistically significant at $p \leq 0.05$ [10, 11].

Results and Their Discussion

Modeling of cerebral vascular accident (CVA) by bilateral occlusion of common carotid arteries led to the different changes in the transcription of genes, which coded heat shock protein. Thus, on the day 1 of experiment we observed decreasing of HSP70 content in the neurons of hippocampus in comparison with pseudo operated animals (table 1). Decreasing of HSP70 progressed during next observation until the day 18.

It should be noted, that neurons of studied areas of brain differed not only by HSP70 content, but also by recovered glutathione concentration. In 24 hours after cerebral ischemia modeling we registered more pronounced decreasing of glutathione concentration in hippocampus (on 37.4 %), then in neurons of cortex (on 19.4 %). This is consistent with literature data on vulnerability of hippocampus to oxidative stress. Such pronounced decreasing of recovered glutathione content explained depression of HSP70.

Neurons of cortex were characterized by medium severe reactions of free radical oxidation. In condition of CVA, we observed increasing of HSP70 content in the cortical neurons. In our opinion, this was caused by chaperone activity of HSP70, that aimed at the intensification of reserve-adaptive capabilities in conditions of ischemia. At the later stage of ischemia (day 18) the same dynamics was observed. These could be connected with the development of oxidative and nitrosative stresses and disruption of compensatory possibilities of the organism. Furthermore, overproduction of reactive oxygen species caused oxidative modification of HSP70 and decreased the activity of the expression of their genes. All these led to disruption of functional activity of HSP70 and limited their protective properties in the conditions of ischemia. In the same time, accumulation of oxidized form of glutathione caused direct inhibitory influence on the expression of heat shock proteins and led to the cell death.

Administration of TDS modulators to the experimental animals caused positive effect on HSP70 concentration in neurons of hippocampus and cortex. These could be explained by ability of these medicines to increase the content of recovered glutathione in the neuronal tissue.

Angioline and Thiotriazoline showed the most pronounced effect. Thus, usage of Angioline stimulated activation of HSP70 expression on 16,8% in 24 hours after CVA modeling, on the day 4 HSP70 content increased on 24.4 %, on the day 18 – on 52.6 % respectively (table 1).

Our earlier studies showed the ability of TDS modulators to increase nitric oxide bioavailability and limit toxic effects of its derivatives on neurons in condition of ischemia. [7]. NO activated synthesis of chaperone proteins. NO-dependent activation of HSP70 could be an important mechanism of cell protection during ischemia, in the same time, heat shock proteins could decrease hyperexpression of inducible NO-synthase (iNOS) by the inhibition of iNOS transcription factor (NFkB) [3]. Meaning of these influences all these effects limit the overproduction of nitric oxide and its cytotoxic effects.

Table 1. Content HSP70 and level of glutathione in the different areas of brain of rats with CVA on the background of TDS modulators usage (M±m)

Group of animals	HSP-70, u/g of protein		Recovered glutathione, mcmol/g of protein	
	Cortex	Hippocampus	Cortex	Hippocampus
Group I	15,2 ± 1,35	14,0 ± 0,95	3,6 ± 0,17	3,2 ± 0,23
Group II, day 1	32,8 ± 2,64*	11,6 ± 1,04	2,9 ± 0,21*	2,0 ± 0,15*
Group III, day 1	35,4 ± 2,47*	12,3 ± 1,26	3,1 ± 0,35	2,4 ± 0,24
Group IV, day 1	38,3 ± 1,84*	13,6 ± 1,14	3,3 ± 0,28	3,0 ± 0,19 [#]
Group V, day 1	34,7 ± 2,51*	12,5 ± 1,09 [#]	3,0 ± 0,26	2,5 ± 0,21
Group VI, day 1	33,2 ± 2,32*	10,9 ± 1,17	2,9 ± 0,37	2,1 ± 0,18*
Group II, day 4	27,4 ± 1,84*	7,4 ± 0,64*	0,6 ± 0,08*	0,33 ± 0,05*
Group III, day 4	31,3 ± 2,11*	12,5 ± 0,63	1,8 ± 0,22* [#]	1,3 ± 0,21* [#]
Group IV, day 4	34,1 ± 2,25*	18,1 ± 1,25* [#]	2,1 ± 0,34* [#]	1,8 ± 0,27* [#]
Group V, day 4	30,5 ± 2,63*	15,3 ± 1,11 [#]	1,9 ± 0,21* [#]	1,5 ± 0,18* [#]
Group VI, day 4	28,6 ± 2,58*	10,6 ± 1,58	1,7 ± 0,41*	0,86 ± 0,06* [#]
Group II, day 18	18,5 ± 1,22	5,8 ± 0,35*	0,4 ± 0,11*	0,17 ± 0,04*
Group III, day 18	26,9 ± 1,47* [#]	11,5 ± 1,1 [#]	1,7 ± 0,19* [#]	1,4 ± 0,15* [#]
Group IV, day 18	28,3 ± 1,39* [#]	23,4 ± 2,00* [#]	2,1 ± 0,20* [#]	1,9 ± 1,4* [#]

Table 1. (Continued): Content HSP70 and level of glutathione in the different areas of brain of rats with CVA on the background of TDS modulators usage (M±m)

Group V, day 18	26,4 ± 1,33*#	14,8 ± 1,11#	1,9 ± 0,18*#	1,6 ± 0,24*#
Group VI, day 18	24,8 ± 1,74*	8,7 ± 0,63*#	1,5 ± 0,24*#	0,95 ± 0,12#

Remark: * - $p \leq 0,05$ in comparison with pseudo operated animals;

- $p \leq 0,05$ in comparison with animals with CVA

Decreasing of recovered glutathione and accumulation of its oxidized intermediates caused formation of oxidative and nitrosative stresses in the neuronal tissues and inhibition of HSP70 expression. Analysis of obtained results confirms the idea that ischemic neuronal damage is accompanied by changes in the functional state of the components of heat shock proteins and glutathione system, that is necessary not only for antioxidant protection, but also for the folding of protein molecules.

Thus, we found the relationship of expression of chaperone proteins with molecular weight of 70 kDa with glutathione system violation. Injection of thiol-disulfide system modulators caused directly increasing of recovered glutathione content and indirectly led to the activation of expression of HSP70. Identified mechanism may be one of the manifestations of the neuroprotective effect of the studied drugs.

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