DOI: 10.2956/v.04.02.2020.escbm07

THE CONCENTRATION OF HSP_{70} IN THE CYTOSOL AND MITOCHONDRIA OF THE BRAIN IN ANIMALS WITH EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS AND AFTER THE TIME COURSE OF INTRANASALLY ADMINISTERED N-PHENYLACETYL-L-PROLYLGLYCINE ETHYL ESTER (NOOPEPT)

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Multiple sclerosis (MS) is a complex disease of the central nervous system, presumably is autoimmune in nature, with a chronic course, which leads to damage to neurons and axons, as well as inflammatory demyelination. For recurrent forms of multiple sclerosis, some immunomodulatory and immunosuppressive therapeutic agents can be used that can have some success in achieving remission and preventing relapse, but most drugs for relapsing MS are not effective in treating progressive disease, and, unfortunately, are not without significant disadvantages. Therapy with monoclonal antibodies, affecting such mechanisms as blocking the interaction of a4-integrin or lysis of cells carrying certain markers, is very effective. But, unfortunately, other serious pathologies such as progressive multifocal leukoencephalopathy, or various immune disorders can develop after treatment with monoclonal antibodies [1, 2, 3].

Recently, data about the regulatory influence of HSP_{70} on mitochondrial dysfunction that develops in ischemic brain damage have appeared. A number of studies have demonstrated that HSP_{70} *in vitro* is capable of preventing the aggregation of oxidatively damaged citrate synthase, glutathione S-transferase, glutathione reductase, superoxide dismutase, lactate dehydrogenase, malate dehydrogenase, and regulating thiol-disulfide equilibrium. In addition, one of the main functions of HSP_{70} is the induction, as well as the extension of the lifespan of the stable form of HIF-1a, which launches subsequent adaptive responses in the cell. It has been established that HSP_{70} "prolongs" the action of HIF-1a, and also independently maintains the expression of NAD-MDH-mx, thereby maintaining the activity of the compensatory mechanism of ATP production – the malate-aspartate shuttle mechanism for a long time [4, 5, 6].

The most widely accepted animal model of MS is experimental autoimmune encephalomyelitis (EAE) that has clinical manifestations and pathophysiological mechanisms similar to MS [7, 8, 9,10].

The purpose of the study was to investigate the specific activity of a nasal dosage form with ethyl ester of n-phenylacetyl-l-prolylglycine on the expression of HSP_{70} in the rat brain.

Materials and methods. The experiments were carried out on 100 white outbred rats weighing 190-220 g, collected from the breeding center of institute of pharmacology and toxicology of the academy of medical sciences of ukraine. The duration of quarantine (acclimatization period) for all animals was 14 days.

During the quarantine, each animal was examined daily (behavior and general condition. Twice a day the animals were observed in cages (morbidity and mortality). Before the start of the study, animals meeting the criteria for inclusion in the experiment were distributed into groups using a randomization method. Animals that did not meet the criteria were excluded from the study during quarantine. The animal cages were placed in separate rooms. Light cycle: a 12-hour light/ 12-hour dark cycle. The air temperature was maintained within 19-25°C with relative humidity of 50-70 %. Air temperature and humidity were recorded daily. The ventilation mode was applied providing in each room a ventilation rate of about 15 air changes per hour. The experimental animals were kept on the same rations under normal vivarium conditions. Animals were housed in standard cages – 5 rats per cage. Diet included feed grain, bread, root crops (beets, carrots) [11].

Experimental autoimmune encephalomyelitis (EAE) was developed after a single subcutaneous inoculation of an encephalitogenic mixture (EGM) in Freund's complete adjuvant (FCA) based on 100 mg of homologous spinal cord homogenate; 0.2 ml of FCA (the content of killed mycobacteria 5mg/ml) and 0.2ml of physiological saline per animal.

There were five groups of animals in the study:

1) intact (100 rats);

2) control – untreated animals with EAE, received saline solution;

3) animals with EAE that received basic medical treatment – methylprednisolone (MP) 3.4 mg/kg intraperitoneally slowly in physiological saline no more than 1/10 of the CBV (20 rats);

4) animals with EAE treated with MP + nasal gel with n-phenylacetyl-l-prolylglycine at a dose of 10 mg/kg (20 rats);

5) animals with EAE treated with MP + Citicoline (Ceraxon, Ferrer Internacional S.A., Spain) D003U1 series, 500 mg/kg, intragastrically(20 rats).

The medications were administered 2 days after EAE induction: methyl prednisolone for 7 days, and nasal gel with n-phenylacetyl-l-prolylglycine and citicoline for 14 days (latent phase + clinical phase until the end of the disease peak). Control and intact animals received intraperitoneal and intragastric physiological saline in similar volumes during the entire course of treatment.

On the 18th day of the experiment, after pharmacological and physiological studies, the animals were withdrawn from the experiment under thiopetal sodium anesthesia (40 mg/kg).

The concentration HSP_{70} in the cytoplasmic or myochondrial fractions of organs (brain) was determined by Western blot analysis. Proteins were separated on a 10% polyacrylamide gel (PAG). Separation of protein fractions was carried out by electrophoresis method at a voltage of 100 V (to compact the gel), when the samples reached the interface between the gels - at a voltage of 200 V, until the samples reached the end of the gel.

Proteins from the gel were transferred to a nitrocellulose membrane at a voltage of 100 V and a current of 0.35 A for 1 h. After transfer, the membrane was placed in a blocking buffer containing 1% bovine serum albumin solution (SIGMA, USA, cat. No. A2153) for 20 hs. The membrane washed on a shaker in a solution of 0.1 M phosphate buffer (pH 7.4) for 5 min was placed in a solution of primary antibodies against HSP_{70} (1: 500), (Santa Cruz Biotechnology) and incubated for 2 hs at room temperature. It was washed on a shaker 4 times for 5 min in 0.1 M phosphate buffer (pH 7.4). The membrane was placed in a solution of secondary antibodies (1:1000) (biotinylated anti-mouse IgG, SIGMA, USA, cat. No. 051M4885), and incubated for 2 hs. It was washed on a shaker 4 times for 5min in a solution of 0.1M phosphate buffer. The membrane was placed in a solution of ExtrAvidin-peroxidase (SIGMA, USA, cat. No. 051M4885) in 1% bovine serum albumin solution (1:1000). It was incubated for 1 h and washed.

To help visualize the membrane, it was treated with AEC solution: 1 tablet of 3-amino-9-ethylcarbazole (Sigma, USA, cat. No. a6926) dissolved in 2.5 ml of DMFA containing 47.5 ml of 0.05M acetate buffer pH 5.0, and 25µl of 30% H_2O_2. The membrane was incubated in the substrate mixture for 5-10 min. A red insoluble precipitate characterizes the antigen-antibody complex in the blot. The membrane was washed in distilled water several times. The strips were dried between sheets of filter paper under the flow of cold air. HSP_{70} was detected using densitometry in the Adobe Photoshop program.

The research results were processed using the statistical package of the licensed program "STATISTICA® for Windows 6.0" (StatSoft Inc., No. AXXR712D833214FAN5), as well as "SPSS 16.0", "Microsoft Excel 2016". Certain statistical procedures and algorithms were implemented as specially written macros in the corresponding programs. For all types of analysis, the differences were considered statistically significant at $p < 0.05$.

Results. The neuroprotective effect of n-phenylacetyl-l-prolylglycine is associated with an increase in the expression of mRNA for HIF-1 and HSP_{70}. We carried out additional studies to identify the influence of medications on the concentration of HSP_{70} in the brain of animals with EAE.

Recently, data about the protective role of heat shock proteins in cerebral ischemia, accompanied by an intensification of free radical oxidation processes, a shift in thiol-disulfide equilibrium, the development of nitrosative stress, and glutamate excitotoxicity. We have discovered a decrease in the levels of heat shock protein HSP_{70} in the brain of experimental animals with EAE.

Table 1

Influence of medications on neurological status and the levels of HSP_{70} in the brain of animals with EAE on the 18th day of the experiment

Group of animals	HSP_{70}, c.u./g	
	Mitochondrial fraction	Cytosolic fraction
Intact	7,7 ±0,16	16,4 ±0,21
EAE (contol)	1,8 ± 0,10	5,3 ± 0,32
EAE + Methylprednisolone (MP)	1,9 ± 0,31	5,5 ± 0,24
EAE + MP + Citicoline	2,7 ± 0,10*2	8,7 ± 0,32*2
EAE + MP + n-phenylacetyl-l-prolylglycine	4,0 ± 0,14*12	11,2 ± 0,52*12

Note: * – p≤0.05 in relation to the control ;
1 – p≤0.05 in relation to Citicoline
2 – p≤0.05 in relation to Methylprednisolone

Thus, in the group of untreated animals, there was a decrease in the levels of HSP_{70} in the cytosolic and mitochondrial fractions of the brain homogenate by 76.6% and 65.2 %, respectively, on the 18th day of the experiment, compared with similar indicators in the intact group. The course administration of Methylprednisolone to animals with EAE has not led to any significant increase in HSP_{70} in the cytosol and mitochondria of the rat brain.

The introduction of Citicoline with Methylprednisolone has led to an increase in HSP_{70} levels in the mitochondria and cytosol of neurons of the brain in rats with EAE by 50% and 64%, respectively.

The course administration of n-phenylacetyl-l-prolylglycine with Methylprednisolone contributed to the normalization of the levels of this indicator, which revealed its protective properties and the ability to prevent inhibition of the HSP_{70} gene expression in neurons. After the course of administration of n-phenylacetyl-l-prolylglycine, the concentration of the HSP_{70} protein in the cytosol homogenate of the brain increased by 111 %, and in mitochondria by 122 % compared with the control.

Taken together, these data indicate the activation of the HSP_{70} gene expression in neurons under the action of an intranasal gel with n-phenylacetyl-l-prolylglycine. Since the protein belongs to the family of heat shock proteins and has chaperone activity, an increase in its levels has a significant regulatory role for control of the activity of regulatory proteins, cell activity and prevention of neuron apoptosis and necrosis development in neurodegenerative diseases.

Proteins of the HSP_{70} family are induced in cells of all living organisms in response to the action of numerous stress factors such as heat shock, hypoxia, ischemia, metabolic disorders, viral infection, and exposure to pharmacological agents. The genes of these proteins are activated not only under stress conditions, but also during the main processes of cellular life, proliferation, differentiation, and apoptosis. HSPs take part in all vital processes of tissues and organs.

The expression heat shock proteins, a large family of molecular chaperones, can be rapidly upregulated in cells of all living organisms in response to the action of numerous stress factors such as heat shock, hypoxia, ischemia, metabolic disorders, viral infection, and exposure to pharmacological agents. The genes of these proteins are activated not only under stress conditions, but also during the main processes of cellular life, proliferation, differentiation, and apoptosis. HSP_{70} proteins take part in all vital processes of tissues and organs. There is growing evidence that HSPs are able to promote or inhibit neurodevelopment through specific pathways regulating cell differentiation, cell migration, or angiogenesis. Apparently, most of the protective functions of HSP_{70} are associated with chaperone activity, i.e. with their ability to recognize damaged or newly synthesized polypeptides, correct their structure in an ATP-mediated manner, or remove unneeded, damaged or non-amenable proteins through the proteasome apparatus.

HSP_{70} deficiency is one of the reasons for mitochondrial dysfunction development with all the ensuing consequences for the vital activity of the cell. HSP_{70} deficiency in neurons against the background of deprivation of the glutathione link of the thiol-disulfide system, in our opinion, is associated with the overproduction of reactive oxygen species and cytotoxic forms of nitric oxide, leading not only to the modification (reversible and irreversible) of macromolecules, including HSP_{70} itself, but also to a decrease in expression activity of genes encoding the synthesis of the latter.

In addition, the neuroprotective effects of HSP_{70} under EAE conditions may be accounted for by its antiapoptotic and mitoprotective effects. Currently, three major pathways for the influence of the heat shock proteins on apoptosis are known. First, they can affect the functioning and transmission of a signal from the FasApo1 receptor inside the cell; second, they can affect the release of cytochrome C from mitochondria; and, third, these proteins can influence the formation of apoptosomes and activation of the caspase cascade.

A number of studies have shown the role of nitric oxide derivatives in suppressing gene activity and reducing the levels of various transcription factors. Apparently, an excess of such forms of nitric oxide, as peroxynitrite and nitrosonium ions, first nitrolyze thiol redox dependent parts of these genes, then, with an increase in concentration may oxidize them. The appearance of peroxinitrite results in nitrosylation of guanine and breakage of DNA chains [6]. Thus, taken together, our data suggest that HSP_{70} proteins are inevitable concomitants of pathobiochemical reactions that occur during autoimmune brain damage in EAE and play a protective role due to enhancement of the synthesis of antioxidant enzymes, the stabilization of oxidized macromolecules, and direct antiapoptotic and mitoprotective actions. This role of the proteins in the cellular responses in autoimmune encephalomyelitis suggests that it is possible to develop new neuroprotective drugs that can modulate/protect the genes that code for HSP_{70} proteins [7].

Most of the protective functions of HSP_{70} are associated with chaperone activity, i.e., with their ability to recognize damaged or newly synthesized polypeptides and correct their structure in an ATP-mediated manner or remove non-amenable proteins through the proteasome apparatus. By stabilizing oxidatively damaged macromolecules, HSP_{70} is able to prevent the opening of the mitochondrial pore, thereby blocking the release of cytochrome C from mitochondria, thereby exhibiting a direct antiapoptotic effect. Besides, the antiapoptotic effect of heat shock proteins is noted to be mediated by their ability to activate the expression of the antiapoptotic protein Bcl-2 [6,7].

Conclusions.

1. In a model of experimental multiple sclerosis, studies have been conducted to investigate the effect of n-phenylacetyl-l-prolylglycine nasal gel on HSP_{70} expression in the brain.
2. It was revealed that after the course administration of n-phenylacetyl-l-prolylglycine, the concentration of the HSP_{70} protein in the cytosol of the brain homoge-

nate of animals increased by 111 %, and in mitochondria – by 122 % in relation to the control. The results obtained suggest about the activation of the HSP_{70} gene expression in neurons after an intranasal gel with n-phenylacetyl-l-prolylglycine administration.

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Key word: HSP 70, experimental autoimmune encephalomyelitis, N-phenylacetyl-L-prolylglycine ethyl ester