ISSN 1819-7124, Neurochemical Journal, 2011, Vol. 5, No. 4, pp. 251–256. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © I.F. Belenichev, S.V. Pavlov, E.P. Sokolik, 2011, published in Neirokhimiya, 2011, Vol. 28, No. 4, pp. 287–293.

EXPERIMENTAL ARTICLES

Disturbance of HSP-70 Chaperone Activity Is a Possible Mechanism of Mitochondrial Dysfunction

I. F. Belenichev, S. V. Pavlov, and E. P. Sokolik[1]

Zaporozhskii State Medical University, Zaporozh'e, Ukraine

Received March 26, 2011

Abstract—We studied the involvement of HSP70 genes in the development of pathological changes in the brains of experimental animals during ischemia. We found neuro- and mitoprotective characteristics of heat shock protein HSP70 and HIF1b, which result from the enhancement of the synthesis of antioxidant enzymes, stabilization of molecules damaged by oxidation, and antiapoptotic and mitoprotective actions. The protective role of these proteins provides a new direction for the development of novel neuroprotective drugs that protect or modulate the genes that encode these proteins.

Keywords: mitochondrial dysfunction, heat shock proteins, chaperones, ischemia

DOI: 10.1134/S1819712411040040

INTRODUCTION

It is well known that cerebral ischemia induces the death of brain cells and is accompanied by the activation of the genes that provide the adaptation of cells and tissues to low oxygen [1].

Recently it has been found that the genes that encode the HIF-1 protein (hypoxia-inducible factor) and its subunit HIF-1b (120 kDa) are activated during ischemia. HIF-1 modulates the expression of the erythropoietin gene and also about 60 genes that encode proteins that are involved in processes, such as proliferation, apoptosis, angiogenesis, and stabilization of proteins during oxidative stress [2, 3]. Moreover, it has been found that heat shock proteins (HSPs) are involved in the stabilization of HIF-1b during cerebral ischemia, which is accompanied by intensification of the processes of free radical oxidation, shift in thiol-disulfide balance, development of nitrosative stress, and glutamate excitotoxicity [3,4]. HSPs are induced in cells in response to stress factors, such as heat shock, hypoxia, ischemia, metabolic disturbances, viral infection, and action of pharmacological agents. The genes that encode these proteins are activated, not only under stress conditions, but also during the main processes of vital activities of cells, viz., proliferation, differentiation, and apoptosis [5]. HSPs are involved in all the processes of the vital activities of tissues and organs. It seems that the majority of protective functions of HSPs depend on their chaperone activity. HSPs recognize damaged or recently synthesized polypeptides and repair their structure via ATP-dependent processing or destroy non-repairable proteins by proteosome machinery. It has been shown that the chaperone HSP90 can bind to the PAS domain of the B-factor and stabilize it. Another chaperone HSP70 recognizes a motif of the HIF-1b molecule, which is known as a domain for oxygen-dependent degradation (ODD) [6]. Note that the role of these interactions between proteins is not clear. It has been hypothesized that these interactions are necessary for the stabilization of HIF-1b under conditions of normoxia. Under conditions of hypoxia, at least one of the chaperones (HSP70) is displaced from the complex by the ARNT protein, which preserves the factor HIF-1b from proteolysis for 20–30 minutes of hypoxia. Thus, it is possible that HSP70 can increase the lifetime of factor HIF-1b before and after hypoxia and, thus, is necessary for the correct response of cells to hypoxic conditions [7].

Recent data suggest that HSPs can affect mitochondrial dysfunction that develops during ischemia due to biochemical changes in the brain tissue. However, information on the role of HSP is contradictory, so this problem requires further investigation [8–11].

The purpose of this work was to study the role of genes that encode HIF-1b and HSP-70 proteins in the formation of mitochondrial dysfunction and in the cell's response to brain ischemia.

MATERIALS AND METHODS

The experiments were performed in two stages. At the first stage of in vitro experiments, we studied the effects of glutamate, 2,4-dinitrochlorobenzene (selective inhibitor of glutathione-S-transferase), and DNIC (dinitrosyl iron complex and cystein, which is a donor of nitric oxide) on a neuronal suspension. These

[1] Corresponding author; address: pr. Mayakovskogo 26, Zaporozh'e, 69035 Ukraine; e-mail: sokolikep@gmail.com.

substances in vitro cause pathological changes in brain tissue, such as glutamate excitotoxicity, nitrosative stress, and a shift in the thiol–disulfide balance. At the second stage of our experiments, we modeled cerebral ischemia and studied the genomic cellular response.

The in vitro experiments were performed with cortical neurons extracted from the cortex of 4-week-old Wistar rats. The extraction of neuronal and neuroglia-enriched fractions was performed in two stages. At the first stage, the brain tissue was disintegrated to prepare a cell suspension. The second stage included differential ultracentrifugation in the density gradient of sucrose and ficoll. To obtain neurons and neuroglia, rats were decapitated and their brains were removed. The cortex was separated from the white matter, minced and transferred into a solution containing polyvinylpyrrolidone (PVP, 7.5%), bovine serum albumin (BSA, 1%) and $CaCl_2$ (10 mM). The suspension that was thus obtained was filtered through three separators using a low pressure to decrease neuronal loss. The cell suspension was sequentially filtered through free separators and layered on a gradient of sucrose (from 1 M to 1.75 M). Centrifugation was performed at 60000 *g* in a VAC-25 refrigerating centrifuge. As a result of the centrifugation, we obtained two layers and dense precipitate. The upper layer included the residues of myelin sheaths; the other layer included glial and neuronal cells. The precipitate contained 90% of the neuronal somas. The second layer was then additionally purified by a second filtration and ultracentrifugation. The extracted neurons were washed from sucrose and albumin with a cold physiological solution [12]. The obtained suspension was divided into three portions: an intact portion, a portion treated with glutamate (100 μM), and a portion treated with chlorodinitrobenzene (80 μM) and DNIC (250 μM). We collected samples at the 15th, 30th, and 60th minutes and measured concentrations of HSP70 and HIF1b in the samples by immunoblotting. To prepare protein samples, we collected cells, separated them from the substrate in a mixture of trypsin and versene (1 : 1), washed them three times in 10 ml of cold PBS, and centrifuged at 200 *g* for 5 min. The cell precipitate was lyzed by 100 μl of a buffer that contained 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 2 mM EDTA and 1 mM PMSF (Sigma, United States). The extracts were centrifuged at 8000 *g* for 10 min and then we collected supernatant and measured the concentration of total protein in it using a method described by Bradford (Bradford, 1976). Electrophoretic separation of proteins was performed using a method described by Laemmli (Laemmli, 1970). A nitrocellulose membrane with proteins transferred from the gel was incubated for 1 hour with monoclonal antibodies to HSP70 and HIF1b and with secondary antibodies against murine immunoglobulin (IgG) labeled with horseradish peroxidase (Sigma, United States) [13, 14].

The concentrations of the HIF and HSP-proteins in the brain homogenate were measured using Western-blot analysis. Proteins were separated in a 10% polyacrylamide gel (PAAG). The transfer of proteins from PAAG to a nitrocellulose membrane was performed by electroelution for 45 min. The Western blots were preincubated for 1 hour in a TBST solution containing 5% skim milk. The Western blots were then incubated for 1 hour with primary monoclonal antibodies (Santa Cruz Biotechnology) against HIF and HSP a dilution of 1 : 1000. After washing, the blots were incubated for 1 hour with secondary antibodies conjugated with horseradish peroxidase (dilution 1 : 2000). We detected HIF and HSP using densitometry and Adobe Photoshop software.

To detect nitrosative stress in suspension, we measured the accumulation of nitrotyrosine. Quantitative measurements of proteins containing nitrotyrosine were performed using an ELISA Nitrotyrisine kit, which is a kit for the solid-phase enzyme-linked immunosorbent assay of nitrotyrosine, based on the "sandwich" principle [15].

Samples and standards were incubated in a microplate covered with antibodies that bind to nitrotyrosine. During incubation nitrotyrosine is bound to a solid phase-linked antibody. Biotinylated secondary antibody (tracer) is added into the wells. If there is nitrotyrosine in the sample then the tracer-antibody binds to the nitrotyrosine. The intensity of coloring is proportional to the amount of nitrotyrosine in the sample. The concentration of nitrotyrosine in the samples was measured simultaneously with the standards and was determined using a standard curve.

The modeling of cerebral ischemia was performed with *Meriones uniculatus* that weighed 70–90 g. These animals are very often used to model stroke due to a weakly developed system of collateral blood circulation. The stroke was evoked by irreversible unilateral ligation of the carotid artery [16].

On the 4th and 12th days of the experiment, extracted ischemic parencephalon was washed with a cold (4°C) 0.9% KCl solution, minced, and homogenized in ten volumes of a medium containing 250 mM sucrose, 20 mM tris HCl-buffer, and 1 mM EDTA (pH 7.4). To extract mitochondria, homogenate was centrifuged for 7 min at 700 *g*, (4°C). After this, the supernatant was centrifuged for 15 min at 11000 *g* (4°C). Mitochondria were suspended in a small volume of the extraction medium without EDTA and were stored on ice. Mitochondrial swelling due to the pore opening was detected using spectrophotometric analysis at 540 nm (A_{540}). The pore opening was induced by addition of an excess of glutamate and calcium to mitochondrial suspension [12, 17].

The expression of HSP70 and HIF1b genes was performed using immunoblotting as described above. The results of the experiments were analyzed using "STATISTIKA for Windows 6.0" software (StatSoft Inc.), SPSS 16.0, and Microsoft Excel 2003. Some

statistical procedures and algorithms were programmed as special macros in the respective software.

The differences were considered as significant at $p < 0.05$.

RESULTS AND DISCUSSION

Studies in vitro have shown that addition of glutamate (100 μM), dinitrochlorobenzene (DNCB)(80 μM), and DNIC (250 μM) to incubation medium alters the expression of HSP70 and HIF1b; however, these data were quite contradictory. In suspensions, the addition of glutamate and DNCB caused a continuous increase in the concentration of HSP70 and HIF1b, which achieved their maximum at the 30th minute of incubation. Later, at the 60th minute, the concentration of HSP70 decreased on the average by 33% as compared to the 15th minute, whereas HIF1b was more resistant and the decrease in its activity was on the average 18% (Fig. 1a, 1b).

Addition of DNIC to the suspension induced the accumulation of HSP70 and HIF1b, which at the 15th minute was less intense than in the suspensions treated with glutamate and DNCB. At the 60th minute, the decrease in HSP70, as compared to the 15th minute, was 66% and HIF1b, 50% (Fig. 1c).

The increase in the expression of HSP70 and HIF1b by the 15th minute in suspensions may be accounted for by chaperone function of HSP70 under conditions of damaging action of different toxic agents. We believe that heat shock proteins work as molecular chaperones and prevent the aggregation of damaged proteins in the cell. It has been shown in a number of studies that HSP70 prevents aggregation in vitro of oxidized citrate kinase, glutathione-S-transferase, superoxide dismutase, lactate dehydrogenase, and malate dehydrogenase [18, 19].

In addition, one of the main functions of HSP70 is induction and an increase in the lifetime of the stable form of HIF1b, which triggers further adaptive responses of the cell. HIF1b, in turn, forms an active dimer with the HIF-1 subunit and turns into a transcription factor, which triggers the transcription of the genetic response to hypoxia. In addition, it was mentioned above that HIF1b is an induction factor for synthesis of some enzymes of antioxidant defense. This explains the longer accumulation of HIF1b in suspension; moreover, HIF1b is more stable against oxidative stress [20].

Thus, we showed that in the presence of toxic agents HSP70 enhances the expression of HIF1b, which plays a major role in the cell's response to hypoxia. Hence, it is possible to hypothesize that HSP70 participates in the cellular response to hypoxic stress at the level of regulation of HIF1b stability. We believe that the two-stage protection of the cell is an evolutionarily developed mechanism and is necessary for the amplification of transduction signal in response to damaging agents.

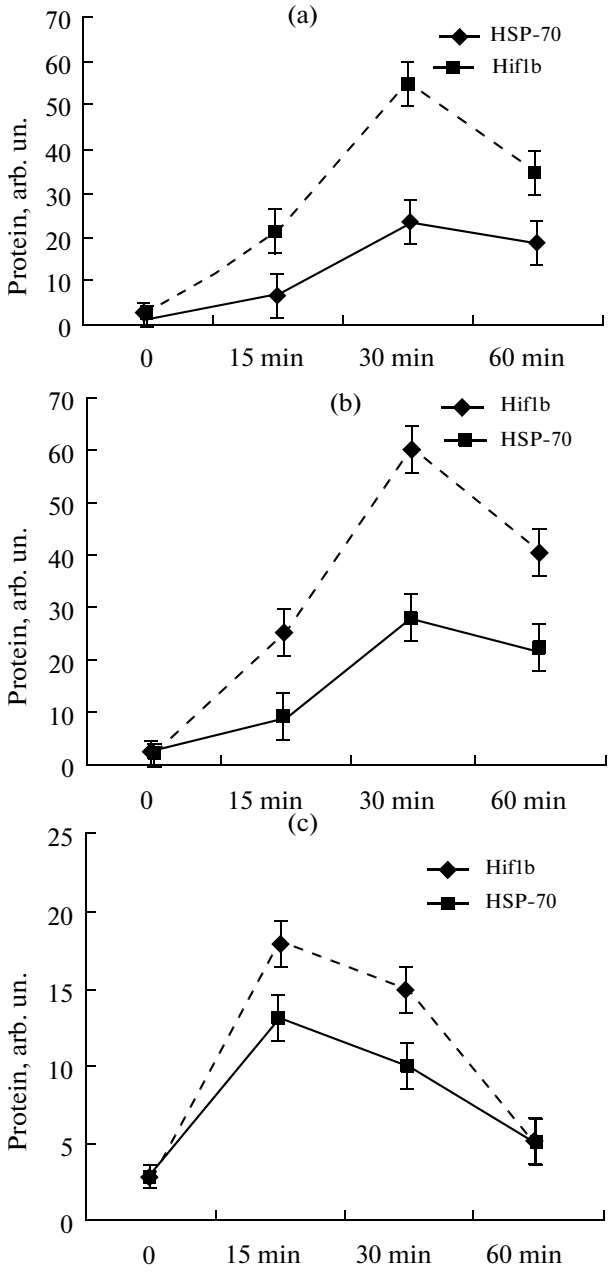

Fig. 1. The dynamics of HSP70 and HIF1b expression in neuronal suspension in the presence of glutamate (a), DNCB (b), and DNIC (c).

A different character of expression of the proteins studied in suspensions after the addition of glutamate, DNCB, and DNIC may be accounted for by the fact that DNIC is the more toxic molecule because NO and, especially products of its transformation, such as peroxinitrite ($ONOO^-$), nitrosonium ion (NO^+), nitroxyl (NO^-), and dinitrogen trioxide (N_2O_3), are the main factors that cause nitrosative stress, which results in the direct interaction of NO with metals (heme iron of hemoglobin, myoglobin, iron-containing enzymes and non-heme iron of iron-sulfur pro-

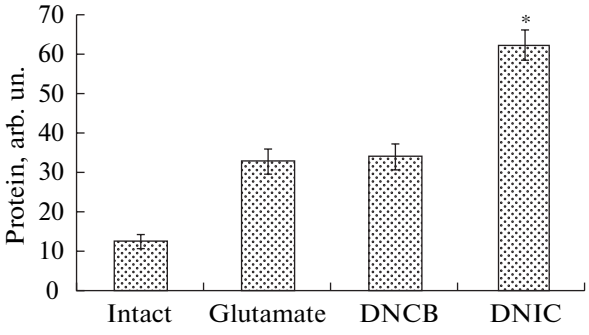

Fig. 2. Concentration of nitrotyrosine in suspension of neurons after addition of glutamate, DNCB, and DNIC.

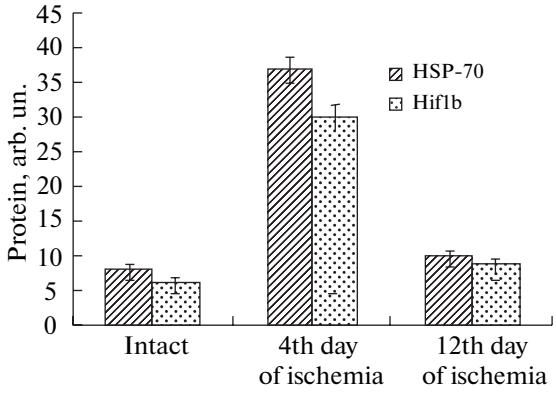

Fig. 3. Expression of HSP70 and HIF1b genes on the 4th and 12th days of modeling of brain ischemia.

teins and DNA and copper and zinc in the active centers of enzymes), as well as the indirect interaction of NO^+ (S-, N-, and O-nitrosation), thiol, phenol, hydroxyl, and amino groups of proteins and DNA [21–23]. A considerable increase in nitrotyrosine, the main marker of oxidative damage of protein molecules, in a suspension of neurons treated with DNIC, as compared to cells treated with glutamate and DNCB, may be accounted for by the weak expression of HSP70 and HIF1b (Fig. 2).

The second stage of our experiments was analysis of the expression of HSP70 and HIF1b in a model of ischemic damage of the brain on the 4th and 12th days of ischemia.

On the 4th day of the experiment, we found a considerable increase in the concentration of HSP70 and HIF1b, which, as we believe, is related to their chaperone activity under conditions of developing oxidative stress and is aimed at the intensification of the reserve and adaptive capacities during acute period of ischemia (Fig. 3).

It is known that depending on the concentration of reactive oxygen species, oxidative stress finally results in either necrosis or apoptosis. A high level of reactive oxygen species induces strong damage of proteins, lipids, and nucleic acids, which leads to necrosis. A moderate oxidative stress induces programmed cell death, i.e., apoptosis. Due to their positive influence on the synthesis of antioxidant enzymes, charepone activity, and stabilization of actin filaments, HSP70 and HIF1b prevent the development of necrosis. The neuroprotective role of HSP70 under ischemic conditions has been shown in several studies [14, 18, 20]. For example, Papadopoulos et al. showed that the expression of HSP70 protects brain astrocytes from death induced by oxygen deprivation. In addition, it has been demonstrated that during severe anoxia purified HSP70 can increase the viability of neurons involved in glutamatergic synaptic transmission in the olfactory cortex of the rat brain [24]. Nevertheless, it is still unclear which mechanisms underlie the protective effect of HSP70. Taking into account the data on the capacity of HSP70 to enhance the viability of neurons under hypoxic conditions and the interaction of HSP70 and HIF1b, which plays a major role in the cellular response to hypoxia, it is possible to hypothesize that HSP70 is involved in the regulation of signal pathways of the cellular response to hypoxic stress at the level of regulation of HIF1b stability. The increase in HSP70 expression on the 4th day of experimental ischemia confirms the importance of the chaperone HSP70 for stabilization of the response of cells to ischemia; it seems that this mechanism underlies the protective activity of the protein.

In addition, the neuroprotective effects of HSP70 under ischemic conditions may be accounted for by its antiapoptotic and mitoprotective effects. Currently, three major pathways for the influence of the heat shock protein 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. HSP27 blocks apoptosis induced by activation of the Fas/Apo1 receptor. After binding with ligand, the receptor interacts with adaptor proteins, such as the FAD protein [25, 26]. This adaptor protein binds inactive procaspase 8 and promotes its activation upon the binding of a receptor with ligand. Caspase 8 activates caspases 3, 6, and 7 and, hence, triggers proteolysis of target proteins, which, finally, leads to apoptosis. In addition, caspase 8 can activate Bid, which induces the release of cytochrome *C* from mitochondria. It is not clear where HSP27 acts in this complex chain of reactions. An alternative pathway of apoptosis triggering Fas/Apo1 includes the Daxx protein. The mechanism of action of this protein is not known very well. In the norm, Daxx is located in the nucleus, where it is bound with certain proteins, but it can translocate to the cytoplasm and play the role of an adaptor protein that is responsible for the initiation of

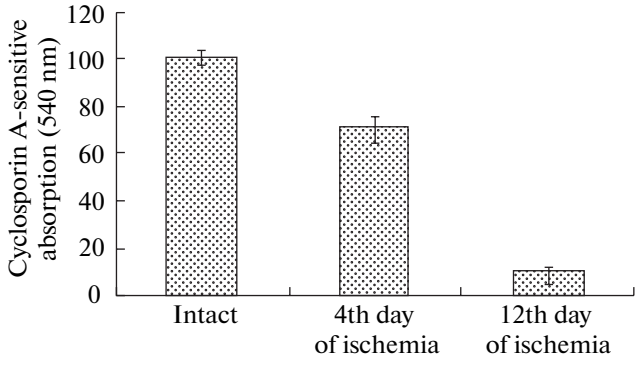

Fig. 4. Opening of mitochondrial pore on the 4th and 12th days of experimental brain ischemia.

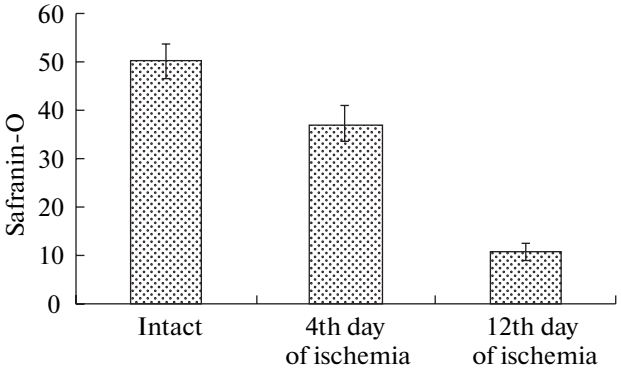

Fig. 5. Changes in the membrane potential of brain mitochondria on the 4th and 12th days of ischemia.

the cascade of JNK kinases via the activation of Fas/Apo. It has been hypothesized that HSP70 can translocate to the nucleus, where it interacts with Daxx and prevents its release in the cytoplasm and the activation of the receptor. We mentioned above that HSP70 may be involved in the regulation of apoptosis, not only at the level of Fas/Apo1 receptor, but also at the level of several intracellular target proteins [27]. It has been shown that HSP70 prevents apoptosis induced by mitochondria and the authors proposed various mechanisms for the actions of heat shock proteins. It is known that the collapse of membrane potential caused by brain ischemia results in the release of cytochrome *C* from the mitochondria. In the cytoplasm, cytochrome *C* binds to Apaf1, deoxyATP, and procaspase 9 and forms a so-called apoptosome. The formation of an apoptosome is accompanied by autocatalytic activation of procaspase 9 and its transition into the active form of caspase 9. This enzyme activates procaspase 3 and the following caspases involved in apoptosis. HSP70 inhibits apoptosis at the stage between cytochrome *C* release and cleavage of procaspase 9 in the apoptosome. According to recent data, HSP70 can interact with cytochrome *C* [28]. The question of which portion of released cytochrome *C* binds to HSP70 is still open. It has been shown in a number of studies that HSP70 binds only with a small portion of the cytochrome *C* that is released from mitochondria and, hence, cannot play a considerable role in the formation of the apoptosome [18, 29]. According to the data of these authors, HSP27 prevents Bax-induced collapse of membrane potential but HSP27 does not interact with Bax protein. The authors hypothesized that in the mitochondrial pathway of apoptosis, HSP70 acts at earlier stages of this complex process and prevents disruption of the structure of actin filaments. This hypothesis was confirmed by our results. On the 4th day of the experiment, a considerable increase in HSP70 and HIF1b expression was not accompanied by mitochondrial dysfunction (opening of the mitochondrial pore, maintenance of mitochondrial potential). However, on the 12th day of the experiment these indices were considerably changed, viz., a decrease in the mitochondrial potential by more than 60% and opening of the mitochondrial pore. Note that these changes occurred during a decrease in the activity of HSP70 and HIF1b.

Similar dynamic changes may be due to disruption of the adaptive capacities of the body on the 12th day and the development of nitrosative stress, which is reflected by an increase in the amount of nitrotyrosine in the brain by more than 75%, as compared to animals on the 4th day (Table 1).

Similar changes were associated with hyperproduction of ROS and cytotoxic forms of nitric oxide, which led not only to modification (reversible or irreversible) of macromolecules, including HSP70 and HIF1b, but also to a decrease in the expression of genes that encode these molecules. A number of works have shown the role of nitric oxide derivatives in the suppression of gene activity and a decrease in the level of different transcription factors. In our previous studies, we showed that an excess of nitric oxide forms, such as peroxinitrite and the nitrosonium ion, initially nitrolyze the thiol redox-dependent parts of these genes and, with an increase in the concentration, oxidize them [17, 28]. The appearance of peroxinitrite results in nitrosylation of guanine and breakage of DNA chains. There is one more effect of NO that is related to damage to the genome: its derivatives and superoxide radical inhibit the enzymes that are responsible for DNA reparation. Depending on the source (different NO donors), NO may affect alkyl-

Dynamics of changes in nitrotyrosine level in homogenate of left ischemic hemisphere of the brain at different stages of experiment

Experimental series	Nitrotyrosine, arb. un./g protein
Intact	8.7 ± 1.43
4th day of ischemia	20.8 ± 1.75
12th day of ischemia	84.2 ± 2.7

transferase and formamidopyrimidine-DNA-glycosylase and ligase. NO increases PARP activity in Betz cells and ADP-ribosylation during global ischemia, presumably, due to DNA breaks, but this results in necrosis due to the depletion of NAD and ATP [22].

Thus, taken together, our data suggest that HSP70 and HIF1b are inevitable concomittants of pathobiochemical reactions that occur during ischemic brain damage 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 ischemia suggests that it is possible to develop new neuroprotective drugs that can modulate/protect the genes that code for HSP70 and HIF1b.

REFERENCES

1. Iadecola, C., *Cerebral Ischemia*, New Jersey: Humana Press, 1999, pp. 3–33.
2. Margulis, B.A. and Guzhova, I.V., *Tsitologiya*, 2000, vol. 4, no. 42, pp. 323–328.
3. Mokrushin, A.A., Pavlinova, L.I., Guzhova, I.V., and Margulis, B.A., *Dokl. Akad. Nauk*, 2004, vol. 394, no. 3, pp. 419–422 [*Dokl.* (Engl. Transl.), vol. 394, no. 3, pp. 118–120].
4. Berchner-Pfannschmidt, U., Wotzlaw, C., Merten, E., Acker, H., and Fandrey, J., *Biol. Chem.*, 2004, vol. 385, pp. 231–237.
5. Dhar-Mascareno, M. and Cacramo, J.M., *Free Radic. Biol. Med*, 2005, vol. 38, no. 10, pp. 1548–1554.
6. Dery, M.A., Michaud, M.D., and Richard, D.E., *Int. J. Biochem. Cell Biol.*, 2005, vol. 37, pp. 535–540.
7. Huang, L.E., Gu, J., Schau, M., and Bunn, H.F., *Proc. Nat. Acad. Sci. U.S.A.*, 1998, vol. 95, pp. 7987–7996.
8. Lyu, B.N., *Usp. Sovr. Biologii*, 2001, vol. 121, no. 5, pp. 488–501.
9. Carmody, R.J. and Cotter, T.G., *Redox Rep.*, 2001, no. 6, pp. 77–90.
10. Katschinski, D.M., Le, L., Schindler, S.G., Thomas, T., Voss, A.K., and Wenger, R., *Cell Physiol. Biochem.*, 2004, vol. 14, pp. 351–360.
11. Suzuki, K., Sawa, Y., Kaneda, Y., Ichikawa, H., Shirakura, R., and Matsuda, H., *J. Mol. Cell Cardiol.*, 1998, vol. 30, pp. 1129–1136.
12. Prokhorova, M.I., *Sovremennye metody biokhimicheskikh issledovanii (lipidnyi i energeticheskii obmen)* (Modern Methods of Biochemical Research (Lipid and Energy Turnover)), Leningrad: Leningr. Gos. Univ., 1982.
13. Bradford, M., *Anal. Boichem.*, 1976, vol. 72, pp. 248–254.
14. Brown, I.R., *J. Neurosci. Res.*, 1990, vol. 27, pp. 247–255.
15. Halliwell, B. and Gutteridje, J.M.C., *Free Radicals in Biology and Medicine*, Oxford: Press, 1999.
16. Stefanov, O.V., *Doklinichni Doslidzhennya Likarskikh Zasobiv (Metodichni Rekomendatsi). Kiev: "Aviatsena",* 2002.
17. Belenichev, I.F., Kolesnik, Yu.M., Pavlov, S.V., Abramov, A.V., and Bukhtiyarova, N.V., *Mezhdunar. Nevrolog. Zhurn*, 2008, no. 4.
18. Morimoto, R.I., Kline, M.P., Bimston, D.N., and Cotto, J.J., *Essays Biochem.*, 1997, vol. 32, pp. 17–29.
19. Nowak, T.S.., Osborne, O.C., and Suga, S., *Prog. Brain Res.*, 1993, vol. 96, pp. 195–208.
20. Plumier, J.C., Krueger, A.M., Currie, R.W., Kontoyiannis, D., Kollias, G., and Pagoulatos, G.N., *Cell Stress Chaperones*, 1997, no. 2, pp. 162–167.
21. Solov'ev, A.I. and Stefanov, A.V., *Sovrem. Problemy Toksikologii*, 1998, no. 1, pp. 35–38.
22. Dimatteo, M.A., Loweth, A.C., and Thomas, S., *Apoptosis*, 1997, no. 2, pp. 164–169.
23. Rivier, C., *Neuroimmunomodulation*, 1998, no. 5, pp. 203–213.
24. Papadopoulos, M.C., Sun, X.Y., Cao, J., Mivechi, N.F., and Giffard, R.G., *Neuroreport,* 1996, vol. 7, pp. 429–432.
25. Kehrer, J.P., *Teratology*, 2000, vol. 62, pp. 235–246.
26. Suzuki, K., Sawa, Y., Kagisaki, K., Taketani, S., Ichikawa, H., Kaneda, Y., and Matsuda, H., *Basic Res. Cardiol.*, 2000, vol. 95, pp. 397–403.
27. Zhou, J., Schmid, T., Franc, R., and Bruno, R., *J. Biol. Chem.*, 2004, vol. 279, pp. 13506–13513.
28. Belenichev, I.F., Chernii, V.I., Kolesnik, Yu.M., Pavlov, S.V., et al., *Ratsional'naya neiroprotektsiya* (Rational Neuroprotection), Donetsk: Izd. Dom Zaslavskii, 2009.
29. Yenari, M.A., Giffard, R.G., Sapolsky, R.M., and Steinberg, G.K., *Mol. Med. Today*, 1999, vol. 5, pp. 525–531.

SPELL: 1. ok