DOI 10.1007/s10517-020-04994-4

GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

Heat Shock Protein HSP60 in Left Ventricular Cardiomyocytes of Hypertensive Rats with and without Insulin-Dependent Diabetes Mellitus

M. L. Blagonravov[1], A. P. Sklifasovskaya[1], A. Yu. Korshunova[1], M. M. Azova[2], and A. O. Kurlaeva[1]

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 170, No. 7, pp. 15-20, July, 2020
Original article submitted May 14, 2020

In cardiomyocytes, high molecular ATP-dependent HSP70 and HSP90 play an important role in protecting the myocardium from abnormal proteins that appear, in particular, due to activation of oxidative stress. Molecular chaperone HSP60 is of particular importance for cardiomyocytes as it is responsible for assembly of mitochondrial matrix proteins. We studied the peculiarities of expression of HSP60 in left ventricular cardiomyocytes in hypertension, insulin-dependent diabetes mellitus, and their combination. The experiment was performed on 38-week-old male Wistar-Kyoto and SHR (spontaneously hypertensive) rats aged 38-57 weeks. Insulin-dependent diabetes mellitus was modeled by a single parenteral administration of 65 mg/kg streptozotocin. Expression of HSP60 in left ventricular cardiomyocytes was evaluated by immunohistochemical methods. It was found that hypertension, diabetes mellitus, and their combination are associated with a significant decrease in the content of HSP60 in left ventricular cardiomyocytes in comparison with the control. This finding can be considered as a pathogenetic mechanism of myocardial damage induced by hypertension and diabetes mellitus.

Key Words: *heat shock proteins; HSP60; myocardium; hypertension; diabetes mellitus*

Heat shock proteins (HSPs) are actively studied due to their ability to exhibit cytoprotective properties under various types of cell stress. They are involved in many intracellular processes such as protein folding, assembly of multimolecular complexes, and maintenance of enzyme conformation. Their production is enhanced in response to a wide range of stress factors including oxidative and thermal stress, ischemia, functional overload, *etc*. [12].

[1]V. A. Frolov Department of General Pathology and Pathological Physiology, [2]Department of Biology and General Genetics, Institute of Medicine, Peoples' Friendship University of Russia (RUDN University), Moscow, Russia. ***Address for correspondence:*** blagonravovm@mail.ru. M. L. Blagonravov

The protective properties of HSPs in muscle cells, including cardiomyocytes, play a special role in the maintenance of their activity. Contractile activity of the myocardium requires constant control of the quality of the sarcomere protein composition including their synthesis, conformation, refolding, and utilization in proteasomes or processing to molecular peptides. Pathogenesis of myocardial damage of various genesis at the cellular and subcellular levels is mediated by the impairment of composition and functional properties of cardiomyocyte proteins. In particular, there is an enhancement of oxidative stress, post-translational modifications, and a decrease in the stability of proteins contributing to their incorrect coagulation. These

damaged proteins exhibit toxic properties towards cardiomyocytes which occurs, in particular, during the development of heart failure. Molecular chaperones, such as HSP70 and HSP90 and co-chaperones CHIP BAG-3, play a key role in protecting the myocardium from these processes [11].

It has been shown in a number of studies that HSPs act as intracellular chaperones protecting the genetic material of the cell and preventing initiation of apoptosis by the mitochondrial pathway, as well as necrotic cell death [13]. A special term "Stress Observation System" (SOS) was introduced for the mechanism of perception of the extracellular pool of HSPs which is supposedly a form of cellular communication under stress conditions [2]. However, the mechanisms of the release of HSPs with different molecular weights activating alternative communication pathways under pathological conditions remain poorly understood.

The induction of HSPs caused by metabolic disorders activates antioxidative intracellular processes reducing cell acidosis and inhibits generation of inflammatory mediators [9]. At the same time, it was found that the body response to heat stress is impaired in patients with type 2 diabetes mellitus. In particular, the expression of HSP70 is reduced in patients with diabetes mellitus [7]. In contrast, increased production of HSPs including HSP60, HSP70, and HSP72 was noted both in the adipose tissue and blood cells in obese people without diabetes mellitus [14]. Moreover, induction of HSP72 promotes lipid accumulation in the liver and adipose tissue, suppresses inflammatory signals, and increases insulin sensitivity. In diabetes mellitus, insulin resistance leads to a decrease in the content of active HSPs in insulin-dependent tissues, presumably via glycation of HSP70, which impairs refolding of intracellular proteins [6].

HSP60, a molecular chaperone, is essential for the assembly of proteins imported into the mitochondrial matrix [3]. An inducible cardiospecific model of *Hsp60* knockout was created. Deletion of the *Hsp60* gene in cardiomyocytes of adult mice changes activity of the mitochondrial complex, mitochondrial membrane potential, and ROS production, which ultimately leads to the development of dilated cardiomyopathy and heart failure [4]. There is an assumption that in case of diabetes mellitus, HSP60 also enters the extracellular space, which leads to activation of proinflammatory cytokines and aggravation of metabolic disorders in the tissue on the whole [5].

Despite ample data on the involvement of HSPs in cell responses to pathological factors in various diseases, there is no clear idea of their role in the pathogenesis of myocardial damage caused by chronic overload and metabolic disorders.

The objective of this work was to study the features of HSP60 expression in the left ventricular (LV) cardiomyocytes in case of arterial hypertension (AH), insulin-dependent diabetes mellitus (DM), and a combination of these pathologies.

MATERIALS AND METHODS

The study was performed on 25 male rats of Wistar-Kyoto (WKY, normotensive) and SHR (spontaneously hypertensive) strains with body weight 290-400 g. All manipulations with the animals were carried out in accordance with the Order No. 755 of the Ministry of Health of the USSR (August 12, 1977) and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986).

The rats were distributed into 5 groups (each group included 5 animals). Group 1 (control) included intact 38-week-old WKY rats; group 2 — 38-week-old SHR rats; group 3 — 57-week-old SHR rats; group 4 — 38-week-old WKY rats with insulin-dependent DM; group 5 — 38-week-old SHR rats with insulin-dependent DM.

WKY rats and SHR rats were obtained from the Pushchino Nursery for Laboratory Animals (Branch of M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences). In 5 SHR rats of the same animal batch at the age of 38 weeks, BP was measured by the method of telemetric monitoring using DSI equipment. In all the animals, systolic and diastolic BP were above 190 and 140 mm Hg, respectively.

Insulin-dependent DM in animals of groups 4 and 5 was modeled by single intraperitoneal administration of streptozotocin (Alfa Aesar) in a dose of 65 mg/kg body weight. Streptozotocin for injections was dissolved in citrate buffer at 4°C immediately before administration. In 3 days, the blood was taken from the caudal view and glucose concentration was measured using an AccuChek Active glucometer (Roche Diabetes Care GmbH). The animals with glycemia >16 mmol/liter were selected for further experiments. The duration of DM development was 30 days from the moment of verification of hyperglycemia. Blood glucose concentration was also measured on day 30 of the experiment. The level of glycemia in the group of WKY rats with DM was 17.92±0.8 mmol/liter on day 3 and 28.8±2.28 mmol/liter on day 30; in the group of hypertensive SHR rats with DM, glycemia was 19.44±1.9 mmol/liter on day 3 and 30.92±1.72 mmol/liter on day 30. On day 25 of the experiment, the levels of glucose and ketone bodies in the urine were measured in these animals. In all the animals, glucosuria (>112 mmol/liter) and ketonuria (0.5-4.0 mmol/liter) were observed.

Under general anesthesia, thoracotomy and extirpation of the heart were performed. The samples of the LV myocardium were fixed in 4% neutral paraformaldehyde for 72 h. The material was processed and embedded in paraffin by the standard technique. Histological sections (5 μ) were sliced on a Slidt 2003 microtome and mounted on poly-L-lysine-coated glass slides (for the immunihistochemical study) and on ordinary glass slides (for the morphological analysis). Sections for the immunohistochemical study were deparaffinized with xylene and processed in descending concentrations of ethanol. To evaluate HSP60 expression in cardiomyocytes, a reaction was performed with primary rabbit anti-HSP60 polyclonal antibodies (Sigma-Aldrich). The results of the immunohistochemical reaction were visualized with a Rabbit specific HRP/DAB (ABC) Detection IHC Kit (Abcam). The slides were counterstained with Mayer's hematoxylin. Brown color of cardiomyocyte cytoplasm indicated positive reaction. Light microscopy in 30 fields of view in each section of the myocardium at ×400 was performed using a Nikon Eclipse E-400 microscope fitted with Watec 221S camera.

Quantitative analysis of positively stained cardiomyocytes was carried out using Avtandilov's grid: the ratio of equally distant points occupied by positively stained cardiomyocyte cytoplasm to the total number of points occupied by the cytoplasm was calculated. For morphological analysis, the sections of the myocardium were stained with hematoxylin and eosin. The contents of myofibrils, cardiomyocyte nuclei, foci of destruction and infiltration, volume of extracellular space were assessed under a light microscope at ×400 in 30 fields of view.

The data were processed using Statistica 6.0 software (StatSoft, Inc.). For each parameter, the mean and standard error of the mean were calculated. Significance of differences was evaluated using Mann—Whitney *U* test (the difference between the mean values at $p \leq 0.05$ was considered significant).

RESULTS

Immunihistochemical study. In all the experimental groups, the content of HSP60 in the cardiomyocyte cytoplasm was significantly lower than in the control (Fig. 1). In cases of longer duration of AH (57-week-old SHR rats) and combination of AH and DM, the decrease in HSP60 expression was less pronounced than in case of shorter duration of AH (38-week-old SHR rats) and isolated DM.

Qualitative analysis of LV myocardial sections after immunohistochemical reaction for HSP60 revealed the following picture. In the control group, positive staining of the cytoplasm was seen in a considerable

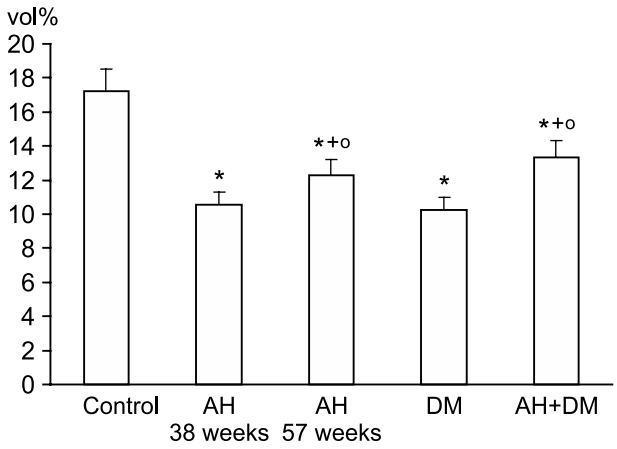

Fig. 1. Content of HSP60 in LV cardiomyocytes in AH (38- and 57-week-old SHR rats), insulin-dependent DM (38-week-old WKY rats with DM), and combination of AH and DM (38-week-old SHR rats with DM). $p \leq 0.05$ in comparison with *control, $^{+}$AH, 38 weeks, ^{o}DM.

number of cardiomyocytes. High staining density was observed mainly in the inner layer of the myocardium, while in layers adjacent to the epicardium and endocardium and in areas around blood vessels, the number of cells with a positive reaction for HSP60 slightly decreased and staining intensity also tended to decrease. In cardiomyocytes visualized in longitudinal section, positive staining of the cytoplasm was observed at a significant length.

In the group of 38-week-old SHR rats, the numerical density of positively stained cardiomyocytes was significantly reduced in comparison with the control group. A mosaic type of single cells staining was characteristic. A relatively higher staining density was seen in the middle layer of the myocardium. Towards the epicardium and endocardium, the number of positively stained cardiomyocytes decreased markedly. Around the blood vessels, only local areas with weakly positive staining were revealed.

In the group of 57-week-old SHR rats, the numerical density of cardiomyocytes with positive reaction for HSP60 was also reduced in comparison with the control group. Staining of the cytoplasm of some single cardiomyocytes was predominantly observed. However, cardiomyocytes with a positive immunohistochemical reaction were abundant than in 38-week-old hypertensive rats. Positively stained cardiomyocytes were mainly detected in the middle layer of the myocardium and in the layer adjacent to the endocardium. Towards the epicardium, local staining was concentrated around blood vessels. Intensification of cardiomyocyte staining was observed in the LV region bordering the interventricular septum.

In WKY rats DM, the number of positively stained cardiomyocytes was lower than in the control

group. The numerical density of cells with a positive immunihistochemical reaction tended to increase near the interventricular septum. On the other hand, there was no considerable difference in the number and distribution of stained cells between the layers of the LV myocardium.

In 38-week-old SHR rats with DM, similar to other experimental groups, the number of cardiomyocytes with positive reaction for HSP60 was reduced, but staining was more intensive than in 57-week-old SHR rats and in WKY rats with DM. A tendency to a more diffuse distribution of staining compared to other groups was noted. Higher number of cells with positive reaction were seen in the myocardium layer adjacent to the endocardium. Near the epicardium, positive staining was mainly concentrated around microvessels. Intensification of staining near the interventricular septum was also noted.

Morphological study. In the control group, muscle fibers were clearly contoured, and were densely packed parallel to each other. Tissue, cellular, and intracellular structures were clearly discernible. Collagen was in small quantities, located between myofibrils in the form of thin layers and around microvessels. Destruction foci were extremely rare and small. In the group of 38-week-old SHR rats, myofibrils were hypertrophied with local disorganization foci. The intercellular space and stromal elements did not quantitatively differ from the control. The number of microvessels tended to increase. In the group of 57-week-old SHR rats, considerable hypertrophy of myofibrils was observed, the number disorganization foci significantly increased. The number of stromal elements slightly increased in comparison with the control, the area of intercellular space did not change. The tendency to an increase in the number of microvessels persisted. In WKY rats with DM, the muscle fibers were arranged parallel to each other, the amount of collagen between myofibrils and around blood vessels significantly increased. Hypertrophy of myofibrils and thickening of the wall of microvessels were noted. In the group of 38-week-old SHR rats with a combination of AH and DM, myofibrils were hypertrophied with foci of spatial disorientation and ruptures. Cardiomyocyte nuclei were less numerous. The number of stromal elements increased markedly in comparison with the control, the area of intercellular space considerably decreased. The number of microvessels with a thickened wall increased in comparison with the control.

The results of the study indicate a decrease in the intensity of HSP60 expression in the LV cardiomyocytes in comparison with the control under conditions of both hemodynamic overload of the LV due to AH and metabolic disorders caused by DM. Moreover, the combination of AH and DM was not accompanied by a synergistic negative effect on this process. On the contrary, inhibition of HSP60 production was slightly less pronounced in this case. Inhibition of HSP60 synthesis is most likely associated with energy deficiency developing under conditions of LV overload or insulin-dependent DM. It was shown that expression of ATP-dependent high-molecular-weight HSP70 and HSP90 in the brain, myocardium, liver, and skeletal muscles is significantly reduced under the effect of hypoxia [8].

HSP60 is of particular importance for cells with contractile activity: it is responsible for assembly of mitochondrial matrix proteins. Reduced production of HSP60 can lead to impairment of the functional state of mitochondria. In particular, it was found that HSP60 deficit in cells is accompanied by enhanced ROS generation and, as a consequence, the development of mitochondrial dysfunction [1]. The maintenance of HSP60 at the physiological level in skeletal muscle cells prevents intensification of oxidative stress due to inhibition of superoxide dismutase in insulin-dependent DM [10].

Thus, the decrease in HSP60 production can be considered as a mechanism of pathogenesis of alteration of the LV myocardium caused by AH and (or) DM.

REFERENCES

1. Cömert C, Fernandez-Guerra P, Bross P. A cell model for HSP60 deficiencies: modeling different levels of chaperonopathies leading to oxidative stress and mitochondrial dysfunction. Methods Mol. Biol. 2019;1873:225-239.
2. De Maio A. Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: a form of communication during injury, infection, and cell damage. It is never known how far a controversial finding will go! Dedicated to Ferruccio Ritossa. Cell Stress Chaperones. 2011;16(3):235-249.
3. Deocaris CC, Kaul SC, Wadhwa R. On the brotherhood of the mitochondrial chaperones mortalin and heat shock protein 60. Cell Stress Chaperones. 2006;11(2):116-128.
4. Fan F, Duan Y, Yang F, Trexler C, Wang H, Huang L, Li Y, Tang H, Wang G, Fang X, Liu J, Jia N, Chen J, Ouyang K. Deletion of heat shock protein 60 in adult mouse cardiomyocytes perturbs mitochondrial protein homeostasis and causes heart failure. Cell Death Differ. 2020;27(2):587-600.
5. Juwono J, Martinus RD. Does Hsp60 provide a link between mitochondrial stress and inflammation in diabetes mellitus? J. Diabetes Res. 2016;2016:8017571. doi: 10.1155/2016/8017571
6. Krause M, Heck TG, Bittencourt A, Scomazzon SP, Newsholme P, Curi R, Homem de Bittencourt PI Jr. The chaperone balance hypothesis: the importance of the extracellular to intracellular HSP70 ratio to inflammation-driven type 2 diabetes, the effect of exercise, and the implications for clinical management. Mediators Inflamm. 2015;2015:2049205. doi: 10.1155/2015/249205

7. Nakhjavani M, Morteza A, Khajeali L, Esteghamati A, Khalilzadeh O, Asgarani F, Outeiro TF. Increased serum HSP70 levels are associated with the duration of diabetes. Cell Stress Chaperones. 2010;15(6):959-964.
8. Nguyen VC, Deck CA, Pamenter ME. Naked mole-rats reduce the expression of ATP-dependent but not ATP-independent heat shock proteins in acute hypoxia. J. Exp. Biol. 2019;222(Pt 22):jeb211243. doi: 10.1242/jeb.211243
9. Noble EG, Shen GX. Impact of exercise and metabolic disorders on heat shock proteins and vascular inflammation. Autoimmune Dis. 2012;2012:836519. doi:10.1155/2012/836519
10. Nonaka K, Une S, Komatsu M, Yamaji R, Akiyama J. Heat stress prevents the decrease in succinate dehydrogenase activity in the extensor digitorum longus of streptozotocin-induced diabetic rats. Physiol. Res. 2018;67(1):117-126.
11. Ranek MJ, Stachowski MJ, Kirk JA, Willis MS. The role of heat shock proteins and co-chaperones in heart failure. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 2018;373:20160530. doi: 10.1098/rstb.2016.0530
12. Rodríguez-Iturbe B, Johnson RJ. Heat shock proteins and cardiovascular disease. Physiol. Int. 2018;105(1):19-37.
13. Takayama S, Reed JC, Homma S. Heat-shock proteins as regulators of apoptosis. Oncogene. 2003;22(56):9041-9047.
14. Tiss A, Khadir A, Abubaker J, Abu-Farha M, Al-Khairi I, Cherian P, John J, Kavalakatt S, Warsame S, Al-Ghimlas F, Elkum N, Behbehani K, Dermime S, Dehbi M. Immunohistochemical profiling of the heat shock response in obese nondiabetic subjects revealed impaired expression of heat shock proteins in the adipose tissue. Lipids Health Dis. 2014;13:106. doi: 10.1186/1476-511X-13-106