https://doi.org/10.15360/1813-9779-2023-6-54-61

Influence of Stress Resistance on Myocardial Expression of the Pro-Autophagic Protein Beclin-1 After Cardiac Contusion in Experimental Setting

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For citation: *Evgenia I. Klyuchnikova, Olga V. Korpacheva, Sergey I. Mozgovoy, Alexander N. Zolotov, Alexey V. Kononov.* Influence of Stress resistance on Myocardial Expression of the Pro-Autophagic Protein Beclin-1 After Cardiac Contusion in Experimental Setting. *Obshchaya Reanimatologiya = General Reanimatology.* 2023; 19 (6): 54–61. https://doi.org/10.15360/1813-9779-2023-6-54-61 [In Russ. and Engl.]

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Summary

Objective. Evaluation of myocardial expression of the pro-autophagic protein Beclin-1 after cardiac contusion in experimental animals with different stress resistance.

Materials and methods. The study included 68 white mongrel male rats weighing 250–300 g. After ranking for extreme variants of stress resistance, moderately stress-resistant rats (*N*=36) were excluded from the study. The remaining animals were split into the control (*N*=16) and study (*N*=16) groups, each group composed of 8 high stress resistant and 8 low stress resistant rats. In the study group, 24 hours after inflicted cardiac contusion, 5×5 mm myocardial tissue specimens were sampled from the intraventricular septum, anterior walls of the left and right ventricles, histological sections were made, and a reaction with primary polyclonal Anti-Beclin-1 antibodies was performed. Beclin-1 expression was evaluated under the microscope.

Results. Immunohistochemical evaluation revealed a statistically significant increase in Beclin-1 protein expression (*P*=0.0002) in the cytoplasm of cardiomyocytes in the study group vs the control group, regardless of animals' baseline stress resistance. However, expression of Beclin-1 protein in the myocardium of highly stress-resistant rats (*Me*=4.3; *LQ*=4.0; *HQ*=4.3) was significantly higher versus low-resistant animals (*Me*=3.6; *LQ*=3.3; *HQ*=3.6) (*P*=0.0009).

Conclusion. Increased expression of Beclin-1 protein in the post-traumatic period of experimental cardiac contusion indicates autophagic flux activation. Intensity of autophagy varied depending on the animal's stress resistance.

Keywords: cardiac contusion, autophagy, Beclin-1, stress resistance

Conflict of interest. The authors declare no conflict of interest.

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Introduction

The main pathogenetic factors of the post-traumatic period of cardiac contusion are circulatory hypoxia and disruption of energy-dependent processes [1, 2], together with mechanical damage leading to accumulation of damaged organelles, unfolded proteins, lactate, Ca^{2+} ions, as well as lack of substrate to maintain the necessary level of homeostasis and initiate reparative processes [3–5]. In models of myocardial ischemic injury, these factors are described as triggers capable of increasing the intensity of autophagy, a type of programmed cell death, which involves the degradation of damaged organelles which are further used by the cell to restore metabolism and form new fully functional structures [6]. In addition, the stress response related to the general adaptation syndrome plays an important role in the pathogenesis of blunt cardiac trauma. Previous studies have identified patterns of systemic and tissue hormonal and metabolic shifts in the post-traumatic period of experimental cardiac contusion that are characteristic of the stress response but vary in severity in animals with different levels of stress resistance [7]. Another study [8] demonstrated the dependence of hematopoietic organ functions on the activity of the sympathetic and hypothalamic-pituitary-adrenal systems in dogs with different stress sensitivity.

Based on this assumption, it is hypothesized that autophagy as a stress-associated response of myocardial tissue may occur during the post-traumatic period of cardiac contusion and its severity may vary depending on the level of stress resistance in the organism.

Aim: To evaluate the expression of the autophagy protein Beclin-1 in myocardium after experimental cardiac contusion, taking into account different levels of stress resistance.

Materials and Methods

Sixty-eight male white albino rats, weighing 250–300 g, were used in the study in accordance

with the rules for conducting research and animal care (Order of the Ministry of Health of the Russian Federation dated 01.04.2016 No. 199n «On approval of the rules of good laboratory practice»), with free access to combined food and water. The study was approved by the local ethics committee of Omsk State Medical University of the Ministry of Health of Russia. Zoletil 100 (tiletamine, zolazepam) at a dose of 30 mg/kg intraperitoneally was used as an anesthetic for all invasive stages of the experiment.

To create control (C) and experimental (E) groups, we performed an assessment of the animals' stress resistance using a modified ranking method that included open field and Porsolt forced swim tests [9]. Previous studies evaluating the concentration of corticosterone, glucose, triglycerides, lactate in blood plasma, as well as the levels of restored glutathione and total antioxidant capacity in myocardium, indicated that the modified ranking method for rat stress resistance allows the selection of subjects with the most pronounced differences in systemic and tissue stress response markers [7].

Animals with average stress resistance (*N*=36) were excluded from the study because extreme variants of stress resistance were used as selection criteria.

In each group, animals with low (L) and high (H) stress resistance were included. Thus, two subgroups of the control group (CH and CL) and two subgroups of the experimental group (EH and EL) were formed, with 8 animals in each subgroup.

Cardiac contusion was simulated in the experimental group using an original device that mimics the impact of the frontal surface of the chest against the steering column in a collision of a moving car with an obstacle [10].

Rat heart was harvested 24 hours after the trauma simulation and heart sections were prepared. To identify the damaged areas, the sections were stained with a solution of nitroblue tetrazolium and then placed in a 10% formalin solution for 30 minutes to enhance the contrast of the staining. The damaged myocardial areas were gray-white in color, whereas the undamaged myocardium was blue-black (Fig. 1). Myocardial fragments measuring 5×5 mm were harvested from the areas of greatest trauma, including the interventricular septum and the anterior walls of the left and right ventricles. The specimens were processed for microscopy using standard techniques and embedded in paraffin. Histologic sections of 5 micrometers thickness were prepared using an Epredia HM 340E microtome (Epredia, UK) and placed on adhesive-coated slides. The resulting samples were deparaffinized with xylene and treated with descending concentrations of alcohol.

To evaluate the expression of the autophagy protein Beclin-1, a reaction was performed using primary rabbit polyclonal anti-Beclin-1 antibody

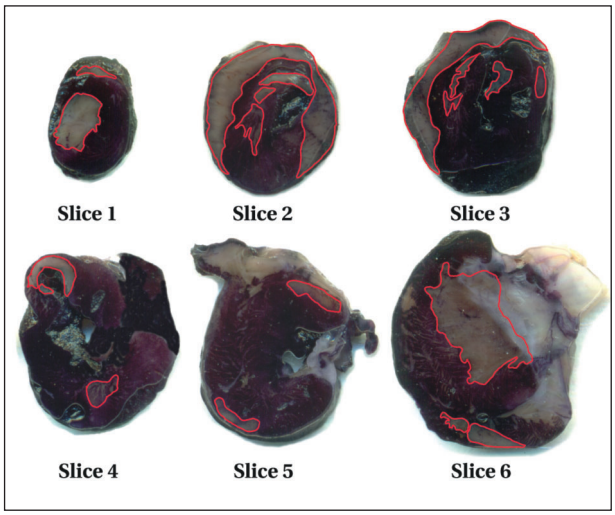

Fig. 1. Macroscopic visualization of myocardial injury foci with nitroblue tetrazolium solution.
Note. The areas of injury are outlined with a red line.

(HUABIO, China), No. R1509-1, diluted 1:100. The results of the immunohistochemical study were visualized using the «Universal Two-Step Detection System PrimeVision»: Mouse/Rabbit IgG Antibodies — HRP/DAB» kit (PrimeBioMed, Russia). Slides were counterstained with hematoxylin. The immunohistochemical reaction was considered positive when brown staining appeared in the cytoplasm of cardiomyocytes. Light microscopy was performed with an Axioskop 40 microscope (Zeiss, Germany) at 400× magnification in 10 fields of view for each slide. Images of each field of view were captured using an Axiocam 503 color camera (Zeiss, Germany) and ZenBlue graphics software for further evaluation of immunohistochemical study results.

Myocardial image analysis was performed using a semi-quantitative method. The scoring system considered two parameters: the intensity of immunohistochemical staining on a four-point scale (0, no staining; 1, weak staining intensity; 2, moderate staining intensity; 3, strong staining), and the staining area expressed in points corresponding to the percentage of stained cardiomyocytes among all cardiomyocytes in the field of view (0–20% — 1 point, 20–40% — 2 points, 40–60% — 3 points, 60–80% — 4 points, 80–100% — 5 points). The final result was the sum of the intensity and area points for each individual field of view, followed by calculation of the arithmetic mean for all 10 fields of view and calculation of the expression index for each animal. The values of the indicators obtained from animals in different groups and subgroups were then compared.

Cardiac injury modeling and sample preparation were performed in the laboratory of the Department of Pathophysiology, and immunohistochemical analysis was performed in the laboratory

of the Department of Pathological Anatomy of Omsk State Medical University.

Normality of the distribution of quantitative variables was tested using the Shapiro–Wilk test. Data were analyzed using descriptive statistics and sample comparisons (Mann–Whitney *U* test). A significance level of 0.05 was used. The data were processed using IBM SPSS Statistics 23 software. The results are presented as median *(Me)* and interquartile range *(UQ–LQ)*.

Results

Macroscopic changes in the myocardium after staining with nitroblue tetrazolium solution consisted of gray-white staining in the damaged areas and blue-black staining in the undamaged areas. Microscopic examination of the myocardial injury zone after experimental cardiac contusion revealed edema and widening of spaces between cardiomyocytes, irregular structure of intercalated discs, focal loss of cross-striations with the appearance of hyper-eosinophilic areas, wave-like deformation of cardiomyocytes, and the beginning of fragmentation of single cardiomyocytes.

Immunohistochemical analysis revealed a significant increase (*P*=0.0002) in the expression of the autophagy protein Beclin-1 in the cytoplasm of cardiomyocytes in the experimental group compared to that in the control group (Fig. 2). A significantly higher level (*P*=0.0009) of Beclin-1 expression (*Me*=4.3; *LQ*=4.0; *UQ*=4.3) was observed in the myocardium of stress-resistant traumatized rats (subgroup EH) than in stress-sensitive rats from subgroup EL (*Me*=3.6; *LQ*=3.3; *UQ*=3.6). No differences were observed between the subgroups in the control group (*Me*=3.0; *LQ*=3.0; *UQ*=3.0).

Qualitative assessment of Beclin-1 expression showed that in the control group, positive reactions were either absent or detected in small amounts as irregular inclusions of low-intensity brown color in the cytoplasm of cardiomyocytes. No differences were observed between stress-resistant and stress-sensitive animals in the control group (CH and CL subgroups) (Fig. 3, *a*, *b*).

In high stress resistance rats subjected to blunt cardiac injury, we observed uneven diffuse moderate or high intensity staining of the cytoplasm (Fig. 3, *c*). In the subgroup of low stress resistant traumatized rats (EL), single foci of cardiomyocytes with moderate or low intensity cytoplasmic staining (Fig. 3, *d*) were found. In traumatized animals, regardless of stress resistance (subgroups EH and EL), there was a tendency for increased staining intensity and number of positively stained cardiomyocytes towards the epicardium in the areas of traumatic injury.

Discussion

Beclin-1 is a major subunit of the class III phosphatidylinositol 3-kinase complex (PI3K class III

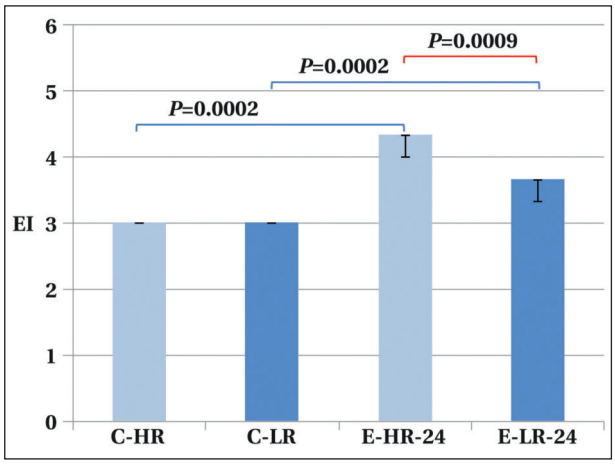

Fig. 2. Expression of the autophagy protein Beclin-1 in the cytoplasm of cardiomyocytes in the area of cardiac injury 24 hours after cardiac contusion modeling.
Note. EI (expression index) is the index of expression in absolute units, which represents the result of dividing the sum of staining intensity scores and staining area scores by the number of fields of view; CH — control group, subgroup with high stress resistance; CL — control group, subgroup with low stress resistance; EH — experimental group, subgroup with high stress resistance; EL — experimental group, subgroup with low stress resistance.

C1), which triggers autophagy by producing phosphatidylinositol 3-phosphate (PtdIns3P) and recruiting the DFCP1 and WIPI genes, which are responsible for the formation of the insulating membrane and its separation from the endoplasmic reticulum (Fig. 4). In addition, Beclin-1 is a member of PI3K class III C2, whose main effects are realized at the stage of autophagosome and lysosome fusion to ensure the degradation of intracellular substrates [12–14]. The observed increase in Beclin-1 protein expression in animals of the experimental group compared to the control group indicates the activation of autophagic flux in the post-traumatic period of experimental cardiac contusion.

Autophagy activation after blunt cardiac injury is associated with oxidative stress in cardiomyocytes, organelle damage, accumulation of reactive oxygen species (ROS) and Ca^{2+} ions, inadequate synthesis of adenosine triphosphate (ATP) and, as a consequence, increasing energy deficit [15–17]. Mitochondrial stress leads to increased production of ROS in the respiratory chain. ROS are the primary triggers of autophagic flux and can directly inactivate the mTOR complex (Fig. 4), thereby initiating autophagy processes [18, 19]. The decrease in the levels of reduced glutathione and total antioxidant capacity in traumatized rats with low stress resistance (EL subgroup) compared to highly resistant animals (EH subgroup) confirms a more intense oxidative stress and subsequent accumulation of ROS in cardiomyocytes. According to this logic, the intensity of autophagy in the damaged myocardium should also be significantly greater in the case of higher ROS in the low stress resistant animals. However,

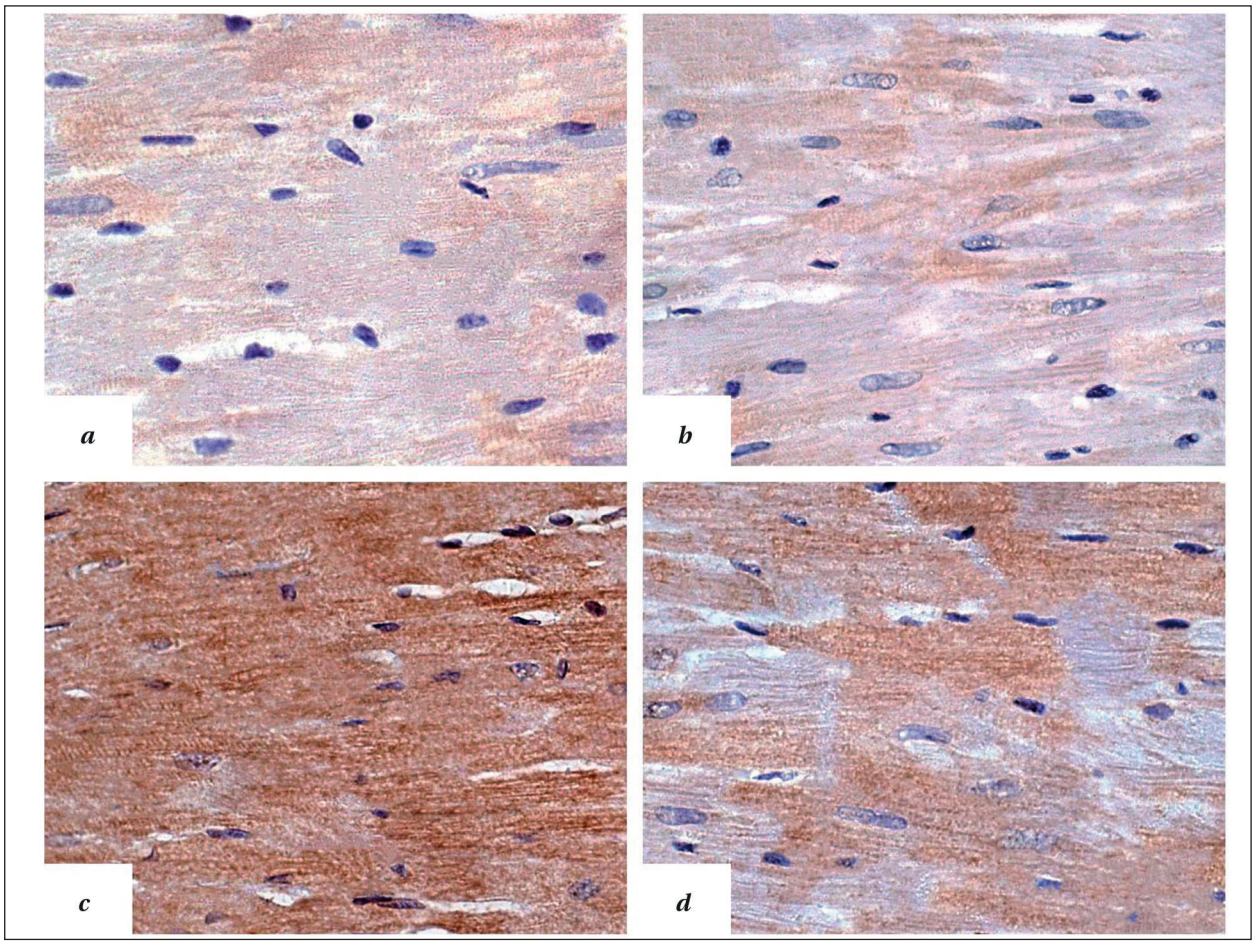

Fig. 3. Beclin-1 protein expression in rat left ventricular cardiomyocytes. Longitudinal section of the myocardium.
Note. Immunohistochemical staining, ×400. *a* — control group, subgroup with high stress resistance (CH); *b* — control group, subgroup with low stress resistance (CL); *a, b* — weak cytoplasmic expression in single cardiomyocytes; *c* — experimental group, subgroup with high stress resistance (EH); cytoplasmic expression of high and moderate diffuse intensity; *d* — experimental group, subgroup with low stress resistance (EL); single cardiomyocyte foci with cytoplasmic expression of moderate intensity.

the immunohistochemical data reveal that the expression of Beclin-1 in low stress resistant animals from the experimental group was less pronounced than in rats with high stress resistance.

This may be explained by the fact that the consequences of autophagy may differ significantly under different conditions (varying models and severity of injury). For example, in relatively mild trauma, autophagy plays an exclusively protective and adaptive role [20], whereas in more severe trauma, hyperactivation of the autophagic flux or its incompleteness, in particular excessive production and accumulation of autophagosomes without their further fusion with lysosomes, can lead to cell death [21, 22]. Conversely, moderate levels of ROS in the cell cause an increase in autophagy, which promotes cell repair and survival (Fig. 4). However, accumulation of ROS above a certain level can lead to increased phosphorylation of the proapoptotic protein Bcl-2 [23], as well as permanent activation of the JNK pathway, which mediates cell death through mitochondrial pathways, enhancing apoptosis and inhibiting autophagy [16]. Probably, due to the excessive concentration of oxygen metabolites in cardiomyocytes and the low initial stress resistance of the animals, causing an excessive level of stress-related system tension, the traumatic impact first triggered autophagy and then, due to its inefficiency, switched the cell death program to the apoptosis pathway.

Damage to the mitochondria also leads to a decrease in ATP synthesis and a disturbance in the cellular energy balance. Adenosine monophosphate-activated protein kinase (AMPK) acts as a sensor that monitors the ATP/AMP ratio and is able to induce autophagy by inactivating the mTOR complex in response to insufficient ATP levels in the cell (Fig. 4). As an adaptive mechanism, autophagy provides the cell with energy by degrading damaged organelles and abnormal proteins. However, when the equilibrium achieved by the activation of this backup mechanism is disturbed, autophagy is inhibited and apoptotic cell death is triggered with the participation of Bax/Bak proteins (members of the Bcl-2 family necessary for the permeabilization of the outer mitochondrial membrane) or by direct activation of

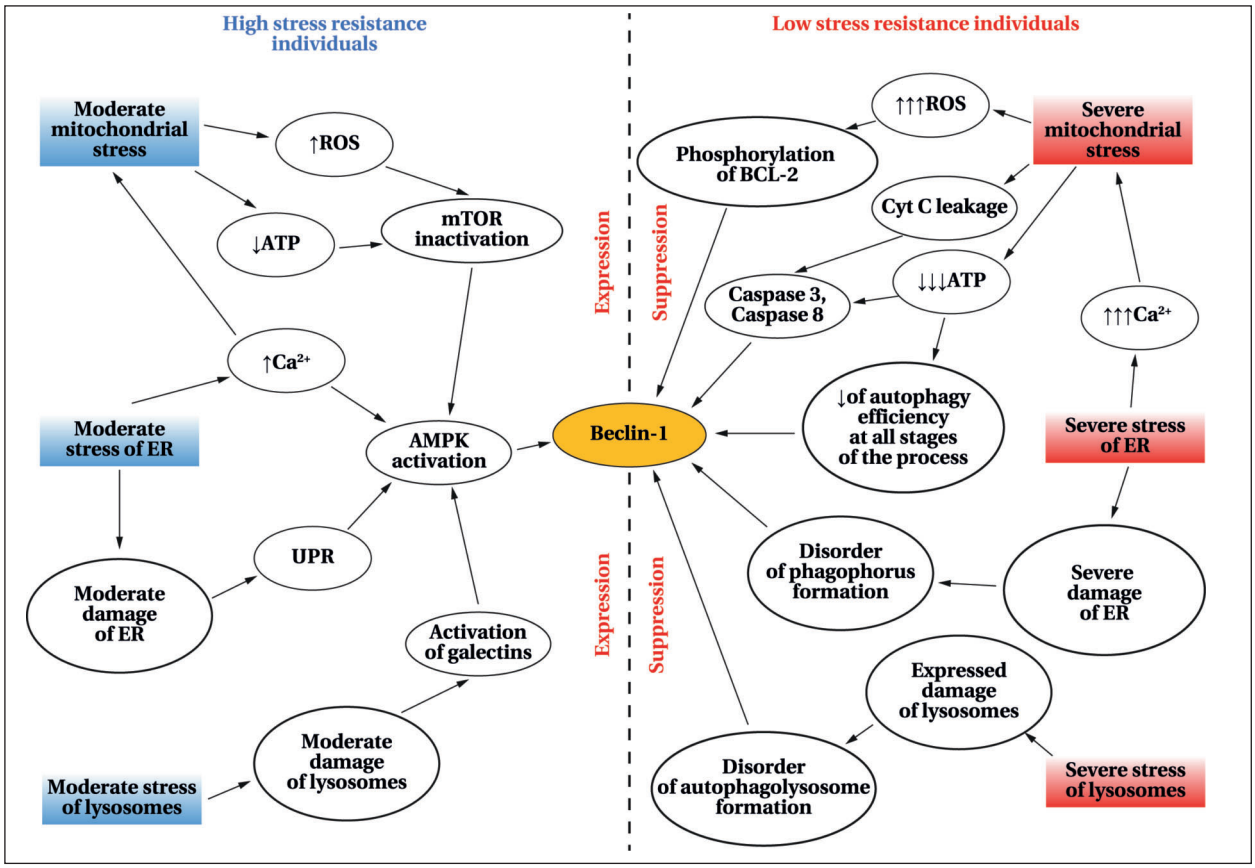

Fig. 4. Expression of the autophagy protein Beclin-1 in the myocardial injury zone after heart contusion modeling under different stress resistance levels. Authors' illustration.

Note. ER — endoplasmic reticulum; ROS — reactive oxygen species; ATP — adenosine triphosphate; AMPK — adenosine monophosphate-activated protein kinase; UPR — unfolded protein response; cyt C — cytochrome C; mTOR — mammalian target of rapamycin.

caspases due to the inability to compensate for the cellular energy deficit. In addition, the autophagy process itself consumes a large amount of ATP at all stages, from initiation to autophagolysosome formation, so a significant energy deficit can directly affect any stage of autophagy [24].

The increase in blood lactate concentration after experimental cardiac injury [7] reflects the accumulation of products of anaerobic glycolysis and indirectly indicates the development of cellular energy depletion. Higher lactate levels in traumatized animals with low stress resistance suggest that the ATP content in their cardiomyocytes was also significantly lower than in those with high stress resistance. Under these conditions, the initiation of autophagy was probably impossible or the progression of autophagic reactions was halted at some stage.

The stress induced by blunt cardiac trauma on cardiomyocytes leads to the accumulation of misfolded proteins and the release of Ca^{2+} ions from the endoplasmic reticulum (ER), the primary storage site of intracellular calcium. Both factors can trigger the process of autophagy. In the first case, initiation occurs via the unfolded protein response (UPR) and activation of ER transmembrane proteins that indirectly affect the AMPK and mTOR complexes (Fig. 4). However, in situations of significant structural damage to the ER, the process of autophagy can be disrupted or halted at the stage of phagophore formation [25].

In turn, an increase in the concentration of Ca^{2+} ions in the cytoplasm leads to the activation of the AMPK complex by calcium/calmodulin-dependent protein kinase (CaMKK), resulting in the inhibition of mTOR and the initiation of autophagic processes [17]. However, due to the significant accumulation of calcium in the cell, there is a corresponding increase in the unidirectional transport of Ca^{2+} across the mitochondrial membrane, which, like the endoplasmic reticulum, serves as a storage site. The increased calcium content in the mitochondria leads to a significant activation of the electron transport chain to generate more ATP, but these processes are accompanied by leakage of free electrons, resulting in the formation of reactive oxygen species (Fig. 4). In addition, excessive calcium uptake by mitochondria can cause their dysfunction and leakage of cytochrome C, which contributes to the activation of the caspase cascade and the implementation of the programmed cell death pathway [25].

When lysosomes are damaged, Ca^{2+} ions may also leak out, as calcium is used in the regulation of

autophagy to facilitate the fusion of autophagosomal and lysosomal membranes. However, Ca^{2+} release from lysosomes also contributes to elevated cytoplasmic calcium levels, which can trigger autophagic repair and overload the mitochondrial respiratory chain, leading to apoptosis [25]. In addition, structural defects of lysosomes cause activation of proteins of the galectin family, which are sensors of lysosomal damage and regulate the processes of autophagy aimed at repairing lysosomes directly (Fig. 4). However, when they are severely damaged or lysophagy is ineffective, structurally and functionally defective lysosomes inhibit autophagy at the fusion and degradation stages, leading to the accumulation of autophagosomes in the cell and autophagic cell death, called «autosis». In addition, significant damage to the lysosomal membrane can lead to the release of cathepsins into the cytoplasm and cell death [26].

In addition, the expression of Beclin-1 can be influenced by various apoptotic factors, such as Bcl-2, caspase-3, caspase-8, which are activated by excessive damage to cardiomyocyte organelles, accumulation of reactive oxygen species, Ca^{2+}, and severe ATP deficiency (Fig. 4). The pro-apoptotic protein Bcl-2 can inhibit autophagy by binding to Beclin-1 in the BH3 domain, leading to the dissociation of Beclin-1 and Vps-34 and the release of Beclin-1 from the active complexes PI3K class IIIC1 and PI3K class IIIC2 [13]. During the initiation of apoptotic processes, activated caspase-3 and caspase-8 can cleave the Beclin-1 protein into fragments that lack autophagic activity [13, 27, 28]. Furthermore, the cleavage products of Beclin-1 can bind to the mitochondrial membrane, leading to the release of pro-apoptotic factors that accelerate the apoptotic process. Other autophagic factors such as Vps-34, Atg5, LC3-II, AMBRA can also be targeted by apoptotic proteases, resulting in the inhibition of autophagy [29].

Thus, upregulation of the autophagy protein Beclin-1 in the posttraumatic period of experimental cardiac contusion in highly stress-resistant rats (subgroup EH) compared with low-resistant animals (subgroup EL) may be associated with optimal implementation of the stress-related response cascade and a lower degree of structural damage to cardiomyocytes. The protective effect of autophagy by eliminating defective cellular structures may only exist up to a certain degree of cardiomyocyte damage, which was observed in highly stress-resistant rats. Exceeding this degree of damage can lead to inhibition of autophagy, as seen in animals with low stress resistance, and the initiation of apoptotic processes. The mortality rate, which was 0% in highly stress-resistant animals (OV subgroup) and 25% in low stress-resistant animals (OH subgroup), can be considered as a parameter indirectly representing the severity of cardiomyocyte damage in experimental cardiac contusion and the resulting hemodynamically significant decrease in myocardial contractility.

Conclusion

In the post-traumatic period of experimental cardiac contusion, factors that activate autophagy appear at the site of a myocardial injury, as evidenced by increased expression of the autophagy protein Beclin-1. The extent of autophagy varies depending on the stress resistance of the organism, with significantly higher protein expression levels observed in highly stress-resistant animals compared to those with lower stress resistance.

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Received 01.06.2023
Accepted 21.09.2023