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Relations between nitric oxide synthase DNOS1, Hsp70 and apoptosis regulatory gene *grim* in *Drosophila melanogaster* after heat stress induction

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Aim. *To investigate correlation between nitric oxide synthase, heat shock protein Hsp70 and apoptosis regulatory gene grim in D. melanogaster.* ***Methods.*** *The heat stress (37 C for 1 hour) induction in third instar larvae of Oregon R strain and transgenic strains, containing additional copies of dNOS1 gene. RT-PCR and Western-blot analysis were used to study the expression of dNOS, Hsp70 and grim genes.* ***Results***. *It is demonstrated that additional copies of dNOS1 gene in transgenic strains are intensively expressed immediately after heat stress induction. It was revealed that in all Drosophila strains the level of Hsp70 gene expression and its protein synthesis increase with subsequent decline after 2–3 hours; whereas the level of expression of grim gene increases immediately after heat stress induction in transgenic strains and declines in wild type flies, while the level of Hsp70 expression remains high.* ***Conclusions***. *The increased level of Hsp70 has negative impact on the expression of grim, whereas additional NO synthesis neutralizes anti-apoptotic effects of Hsp70 and increases the expression level of grim. Thus, we assume the competitive relationships between anti-apoptotic functions of Hsp70 and pro-apoptotic effects of nitric oxide.*

Keywords: apoptosis, Drosophila, heat shock proteins, nitric oxide, grim.

Introduction. A large number of data evidence to the fact that apoptosis in fruit fly *Drosophila* has many common characteristics with apoptosis in mammals [1, 2]. Apoptotic signaling mechanisms render complex sequence of molecular events and a multitude of molecular components is required for realization of apoptotic cascades. Recent publications have demonstrated that different endogenous and exogenous factors and signaling molecules are either apoptosis inducers or its inhibitors, being able to play a dual role in particular conditions [3].

Nitric oxide (NO) is known for a wide spectrum of characteristics and a range of activities inside the cells, including physiological and pathological processes. NO is synthesized during the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) [4]. In *Drosophila NOS* gene encodes 10 different transcripts, which can produce at least seven proteins, including the only enzymatically active isoform – DNOS1. Other isoforms are more or less truncated, which results in the loss of functionally important NOS regions and subsequent loss of activity [5]. The data on NO role in apoptosis development are quite controversial. The balance between anti- and pro-apoptotic effects of nitric

oxide depends on many factors including the NO tissue concentration and its interaction with other components of apoptosis tools [6].

Many recent studies have shown that heat shock protein 70 (Hsp70) plays a crucial role in regulating the apoptotic cascade [7–9]. Hsp70 family is the most conserved, diverse and best characterized class of Hsps, which include constitutive and stress-inducible molecules. Hsp70 is believed to interact with a large number of cellular proteins and therefore is a vital component of cellular networks. Despite the fact that Hsp70 was assumed to be mostly anti-apoptotic, its precise role in well-orchestrated apoptosis machinery is not clearly defined [10, 11].

Realization of apoptosis program in *Drosophila melanogaster* requires activity of specific genes acting as integrators: *reaper* (*rpr*), *head involution defective* (*hid*), and *grim* [1, 2, 12]. It is believed that these genes are capable of inducing apoptosis independently and transcriptionally regulated by different death-inducing stimuli [13]. However, many aspects of their functions and protein-protein interactions with other components of apoptosis network, especially apoptosis regulatory functions of *grim*, are yet to be revealed.

Therefore, the aim of our study was to investigate relationship between nitric oxide synthase, heat shock protein Hsp70 and apoptosis regulatory gene *grim* in *D. melanogaster*.

Materials and methods. *Objects*. Experiments were carried out on *D. melanogaster* late third instar larvae, which were kept on standard nutrient medium at 25 °C. Wild strain *Oregon R* was used as a control. The following transgenic strains, containing additional copies of cDNAs from full-length DNOS1 transcript (additional NO production), were used in the experiments:

1. *HS dNOS1* – contains a part of *dNOS* gene under HS-promoter in X chromosome, which synthesizes functionally active DNOS1 protein. X chromosome is marked by mutations *y* and w^a;

2. *HS dNOS1 Flag* – contains Flag-vector with a part of *dNOS* gene under HS-promoter in X chromosome. *HS dNOS1 Flag* synthesizes functionally active DNOS1 protein. Markers of X chromosome: *y* and w^a;

Heat shock induction. The induction of heat shock was performed by placing larvae containing tubes into water bath heated to 37 C for 1 hour. Afterwards larvae were collected in the following time span: immediately after heat shock induction; 2–3 hours after heat shock and 5–6 hours after heat shock induction. Larvae which were not subjected to heat stress served as a control.

RNA extraction from larvae. Extraction of total mRNA was performed using the RNA STAT-60 reagent (Tel-Test Inc., USA) in accordance with manufacturer's instructions. RNA concentration was measured spectrophotometrically by determination of optical density at 260 and 280 nm.

cDNA synthesis. Reverse transcription was performed using the reverse transcription set (Sileks, Russian Federation) according to manufacturer's instructions. Reaction was performed in 25 l of reaction mixture, containing 2 g of total RNA.

PCR analysis. Reaction was performed in 20 l of reaction mixture, 0.3 M of each primer and 0.5 l of cDNA. PCR conditions were as follows: for *hsp70* gene (s 5'-CTG CGA GTC GTT GAA GTA CG-3' and as 5'-TCG GTA TTG ATC TGG GAA CC 3'): 94 C – 2 min 30 s, and further 35 cycles: 94 C – 45 s, 56 C – 45 s, 72 C – 1 min. Final extinction was conducted at 72 C for 10 min; for *dNOS1* gene (s 5'-TTG TTG TGG CCT CCA CCT TT-3' and as 5'-CAA TCC ATG CTC GGA AGA CTC-3'): 94 C – 2 min, and further 40 cycles: 94 C – 15 s, 60 C – 1 min. Final extinction was conducted at 60 C for 30 s; for *grim* gene (s 5'-ATG AGG ACG ACG TTA CC-3' and as 5'-TTC TTG TTG CTG CGG TTG-3'): 95 C – 30 s, 53 C – 30 s, 72 C – 40 s. Final extinction was conducted at 72 C for 7 min. Amplification of -actin gene fragment (s 5'-cgt cga caa tgg atc tgg aa-3' and as 5'-cga cca tca cac cct gat ga-3') was used as internal control. PCR products were visualized in 2 % agarose gel electrophoresis.

Western-blot-analysis. 20 g of protein extract were separated in 8 % SDS-PAGE gel electrophoresis at 100 V and 4 C. Then proteins were transferred to PVDF membrane (Immobilon-P, Millipore Co., USA) for 1.5 hour at 200 mA current at 4 C. Primary antibody against Hsp70 (SantaCruz, USA) was used in 1:4000 dilution in the blocking buffer. Data of Western-blot-analysis were analyzed with ImageJ software.

Results and discussion. Transgenic *Drosophila* strains with additional copies of *NOS* gene (*HS dNOS1*

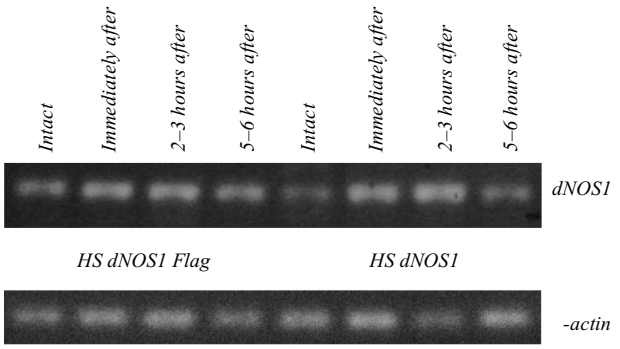

Fig. 1. *dNOS* mRNAs expression in intact and heat shock (37 C) treated larvae of dNOS1 transgenic strains (RT-PCR)

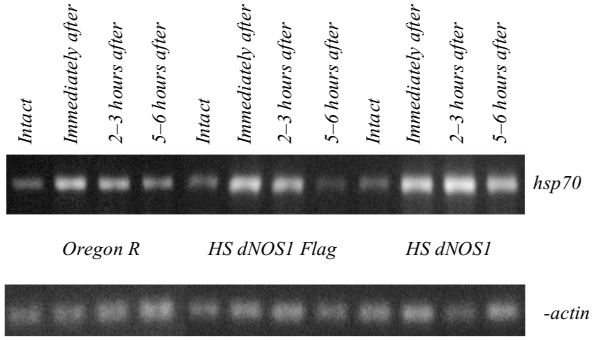

Fig. 2. *hsp70* mRNAs expression in intact and heat shock (37 C) treated larvae of wild (*Oregon R*) and dNOS1-transgenic strains (RT-PCR)

and *HS dNOS1 Flag*) were used to investigate the relationship between nitric oxide and apoptosis in response to heat shock exposure as well as to study the mechanisms of regulation of full-length DNOS1 protein activity [14]. As soon as the additional copies of *dNOS1* gene were placed under the heat shock promoter we induced heat shock to «switch on» *dNOS* gene. In order to monitor the expression of additional copies of *dNOS* gene in dynamics (different time points after heat shock induction), we performed reverse transcriptase PCR using the specific primers. We demonstrated (Fig. 1) that immediately after heat shock induction the level of expression of additional copies of *dNOS1* gene in *HS dNOS1* and *HS dNOS1 Flag* (nitric oxide donors of *Drosophila* strains increases dramatically and remains on the same level for 2–3 hours, then the level of expression of the gene decreases, which is noticeable at 5–6 hours time point after stress induction. RT-PCR data confirmed the activity of additional copies of *dNOS1* gene in the given fly strains and demonstrated gene expression dynamics at different time points.

Many studies demonstrated that heat shock proteins, especially Hsp70, are capable of inhibiting stress-induced apoptosis and acting as anti-apoptotical factors, however, exact pathways of this protective mechanism are yet to be determined.

To define *hsp70* gene expression in third instar, *Drosophila* larvae were subjected to heat stress, as described above. The data obtained demonstrate that drastic increase of *hsp70* gene expression immediately after heat shock induction is observed in wild type strain *Oregon R,* and in transgenic strains *HS dNOS1 Flag* and *HS dNOS1* with subsequent evident decrease only 5–6 hours after heat stress (Fig. 2).

Results of RT-PCR analysis were proved by Western-blot analysis. As it is demonstrated in Fig. 3, immediately after heat stress the level of Hsp70 increases significantly in control group *Oregon R* and transgenic strains, containing additional copies of *dNOS1* gene, which results in generation of full length functional transcript. Within 2–3 hours after heat induction the level of expression of the given protein starts decreasing in the experimental strains *HS dNOS1 Flag* and *HS dNOS1*, and only after 5–6 hours it reduces in the control strain.

Very low *hsp70* expression was found in intact animals, though it was undetectable on protein level. It is notable that intact groups of all studied strains do not display Hsp70 expression which may be explained by very insignificant amounts of the protein in samples that could not be detected. According to Schmitt et al., contrary to Hsp90, which is abundantly expressed in cells in constitutive manner, the expression of Hsp70 and Hsp27 is stimulated by different stress types, and under normal conditions the expression of these proteins in unstressed cells either does not occur at all or occurs at a very low level [15].

Results obtained by Lakhotia and Prasanth demonstrated that «regulation of synthesis and turnover of Hsp70 and Hsp64 during and after HS (heat stress shock) in different cell types of *Drosophila* is complex, involving transcriptional, translational, and posttranslational controls». Thus, the authors demonstrated that in larval malpighian tubule the *hsp70* genes are quickly transcribed, however, the protein synthesis requires

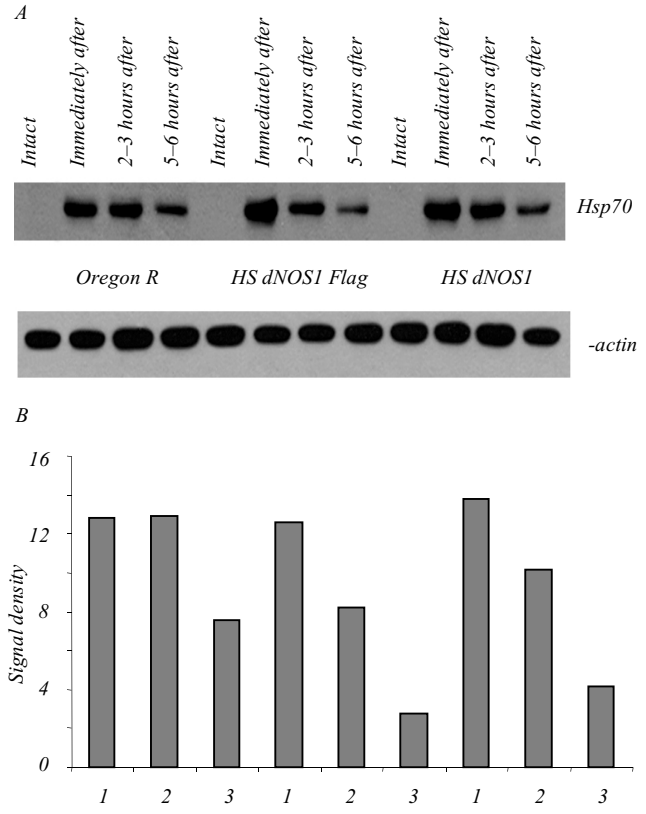

Fig. 3. Semi-quantitative analysis of Hsp70 protein content in intact and heat shock (37 C) treated larvae of wild (*Oregon R*) and dNOS1-transgenic strains: *A* – Western-blot-analysis; *B* – densitometric scanning of the Western-blot

some recovery period. It was revealed that during recovery period transport of the *hsp70* transcripts from nucleus to cytoplasm is enabled [16, 17].

According to I. V. Guzhova, mRNA of stress proteins occurs in cell within few minutes after temperature increase, and the duration of its synthesis takes several hours depending on heat stress continuance with subsequent decrease after elevation of synthesis activity. At 5–6 hours time point after heat induction its level decreases to initial values. The increase in the concentration of heat shock proteins starts 1–3 hours after the heat induction and may remain at the high level for a long time [18].

It is well known that ectopic expression of at least one of key apoptotic regulatory genes (*rpr*, *hid* or *grim*) is enough for realization of apoptosis program. The activity of each gene is regulated by different upstream regulatory signals. While the regulation of *rpr* and *hid* expressions was extensively studied under the influence of a wide range of stimuli, the regulation of *grim* expression is still far from elucidation [13]. Therefore, we have focused our study on peculiarities of this gene expression.

To determine the effect of excessive nitric oxide concentrations and heat stress on the regulation of the expression of apoptosis regulatory gene *grim*, we conducted PCR amplification using specific primers. Our results proved (Fig. 4) the expression of *grim* in intact samples of both strains, which may testify to apoptosis induction, associated with natural larval metamorphosis.

However, significant difference between wild type and transgenic strains was observed in samples subjected to heat stress. In wild type *Oregon R* strain the expression level of *grim* does not change immediately after heat stress, a slight increase is observed in 2–3 hours, and a decrease – at 5–6 hours time point. Contrary to transgenic strain *HS dNOS1 Flag,* which generates increased concentrations of nitric oxide after heat stress induction, the increase in *grim* gene expression was observed after heat stress. The expression level of the gene decreases at 2–3 hours time point after heat stress with gradual coming to its normal level. In *HS dNOS1* transgenic strain there is a significant increase in *grim* expression, which decreases slightly at 2–3 hours time point and remains on the same level at 5–6 hours point.

Recent studies have demonstrated that the expression of all integrator genes *hid/rpr/grim* is transcriptionally regulated during organism development. However, if *hid* gene is expressed both in living and dying cells, *rpr* and *grim* appear to be specifically expressed in cells that are doomed to die and their expression pattern is quite similar. At the same time *rpr* and *grim* function independently of each other, as cell death induced by *grim* does not require the expression of *rpr*. Still, contrary to other integrator genes, little is known about the precise regulation of *grim* expression [13, 19].

Comparison of the results obtained in the analysis of *hsp70* gene expression with the results of *grim* expression revealed that in wild type *Oregon R* strain the activation of *hsp70* gene expression and further protein synthesis trigger protective mechanisms and suppress apoptosis. After the level of *hsp70* gene expression reduces, the cell re-enters the apoptotic way.

As the expression of *hsp70* in both strains was the same, the difference in the expression of apoptosis-

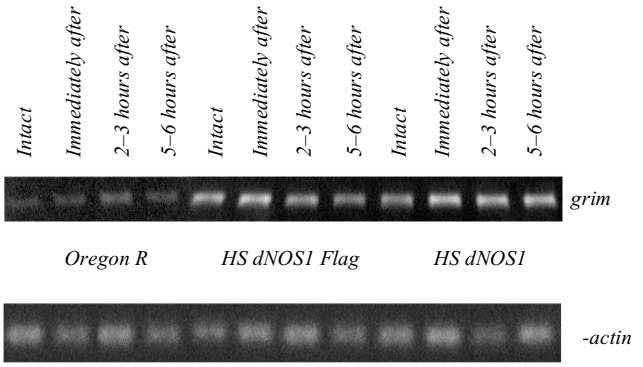

Fig. 4. *grim* mRNAs expression in intact and heat shock (37 C) treated larvae of wild (*Oregon R*) and dNOS1-transgenic strains (RT-PCR)

regulatory gene *grim* is likely to depend on increased concentrations of nitric oxide in transgenic strains. As it was mentioned earlier, the quantity of nitric oxide after heat stress induction increases drastically, protective functions of heat shock proteins become uncompetitive, and apoptosis program is enabled via apoptosis regulatory gene *grim*. However, due to short life span NO concentration decreases while the expression of *hsp70* gene still continues, which, in turn, results in apoptosis suppression. Our results demonstrated that in 5–6 hours the response to stress ceased and the process of programmed cell death re-entered its normal level.

Therefore, taking into consideration the previously obtained data, it was assumed that nitric oxide can play a crucial role in cellular signaling and organogenesis. It is believed that NO regulates cell proliferation in the imaginal discs of developing larvae and participates in the development of visual system. One of the mechanisms of regulating visual system development may be associated with the ability of NO to induce or suppress apoptosis. Thus, it was demonstrated that exogenous donors of nitric oxide in $Lobe^{RSV}$-mutants may stimulate the apoptosis in imaginal discs and therefore contribute the reduction of ommatidia number in the eyes of flies. However, many aspects of NO signaling in apoptosis machinery and its precise role in apoptosis induction and progression are yet to be revealed [20].

Thus, it is suggested that anti-apoptotic functions of Hsp70 in *D. melanogaster* larvae are realized via decreased expression of such apoptosis regulatory genes as, for instance, *grim*, whereas the increase in nitric oxide level is present in transgenic strains after heat stress induction in addition to increased expression of Hsp70. This increase in NO is likely to suppress anti-apoptotic function of Hsp70 and to stimulate the expression of one out of three key apoptosis-inducing genes – *grim*.

Despite the fact that a large number of key apoptosis regulatory proteins are well known, the precise molecular mechanisms of their activities are still far from elucidation. Detailed study of apoptosis mechanisms on the molecular level will provide better understanding of the pathology development and therefore will facilitate the elaboration of novel therapeutical approaches.

This experimental article is not a complete study, additional experimental analysis and investigations in the given direction are to be performed.

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Зв'язок між синтазою оксиду азоту DNOS1, Hsp70 і апоптоз-регулюючим геном *grim* у *Drosophila melanogaster* після індукції теплового стресу

Резюме

Мета. *Дослідити взаємозв'язок між синтазою оксиду азоту, білком теплового шоку (БТШ70) та апоптоз-регулюючим геном grim у D. melanogaster.* ***Методи***. *Індукція теплового шоку (37 C, 1 год) у личинок 3-го віку лінії Oregon R і трансгенних ліній з додатковими копіями dNOS1-гена. Для аналізу експресії генів dNOS, hsp70 и grim використано методи ЗТ-ПЛР і Вестерн-блот.* ***Результати***. *Показано, що в трансгенних лініях додаткові копії гена dNOS1 активно експресуються відразу після теплового стресу. Виявлено, що в усіх використаних лініях рівень експресії hsp70 і його білкового продукту підвищується після індукції стресу і знижується через 2–3 год, у той час як рівень експресії гена grim у трансгенних лініях за таких же умов підвищується, а в контролі зменшується при збереженні високого рівня експресії hsp70.* ***Висновки***. *Значний рівень БТШ70 негативно впливає на експресію гена grim, додатковий синтез NO нейтралізує антиапоптичну дію БТШ70 і підвищує рівень експресії grim. Таким чином, ми передбачаємо наявність конкурентного взаємозв'зку між антиапоптичною функцією БТШ70 і про-апоптичною дією оксиду азоту.*

Ключові слова: апоптоз, Drosophila, білки теплового шоку, оксид азоту, grim.

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Связь между синтазой оксида азота DNOS1, Hsp70 и апоптоз-регулирующим геном *grim* у *Drosophila melanogaster* после индукции теплового стресса

Резюме

Цель. *Исследовать взаимосвязь между синтазой оксида азота, белком теплового шока (БТШ70) и апоптоз-регулирующим геном grim у D. melanogaster.* ***Методы***. *Индукция теплового шока (37 С, 1 ч) у личинок 3-го возраста линии Oregon R и трансгенных линий с дополнительными копиями dNOS1 гена. Для анализа экспрессии генов dNOS, hsp70 и grim использовали методы ОТ-ПЦР и Вестерн-блот.* ***Результаты***. *Показано, что в трансгенных линиях дополнительные копии гена dNOS1 активно экспрессируются сразу после теплового стресса. Выявлено, что во всех используемых линиях уровень экспрессии hsp70 и его белкового продукта повышается после индукции стресса и снижается через 2–3 ч, в то время как уровень экспрессии grim в трансгенных линиях в этих же условиях возрастает, а в контроле уменьшается при сохранении высокого уровня экспрессии hsp70.* ***Выводы***. *Значительный уровень БТШ70 негативно влияет на экспрессию гена grim, дополнительный синтез NO нейтрализует антиапоптическое действие БТШ70 и увеличивает уровень экспрессии grim. Таким образом, мы предполагаем наличие конкурентной взаимосвязи между антиапоптической функцией БТШ70 и про-апоптическим действием оксида азота.*

Ключевые слова: апоптоз, Drosophila, белки теплового шока, оксид азота, grim.

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