

HSPA5 AND DNAJB9 GENES EXPRESSION IN GLIOBLASTOMA CELLS AND NORMAL ASTROCYTES UNDER HYPOXIA AND ENDOPLASMIC RETICULUM STRESS

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Hypoxia and ER stress are obligate factors in tumor growth, however, the interaction between these factors has not been sufficiently studied. Heat shock proteins HSPA5 and DNAJB9 as a key components of the endoplasmic reticulum stress response play an important role in the growth of malignant tumors, including glioblastomas. This study aimed to investigate the interaction between hypoxia and ER stress in controlling HSPA5 and DNAJB9 expression in glioblastoma cells and normal astrocytes. Hypoxia was created with dimethyloxalylglycine, ER stress was induced with tunicamycin and thapsigargin, HSPA5 and DNAJB9 expression was studied by qPCR. It has been established that in astrocytes HSPA5 and DNAJB9 expression was resistant to hypoxia. However, in glioblastoma cells, the expression of these genes under hypoxia was increased. Tunicamycin and thapsigargin enhanced HSPA5 and DNAJB9 expression with a much stronger effect in glioblastoma cells. When these ER stress inducers were combined with hypoxia their effect was modified to a greater extent in normal astrocytes. The obtained results indicate different cell-specific sensitivity of HSPA5 and DNAJB9 expression to hypoxia and ER stress inducers.

Keywords: hypoxia, endoplasmic reticulum stress, HSPA5, DNAJB9, gene expression, glioblastoma cells, normal human astrocytes.

The HSPA5 (heat shock protein family A (Hsp70) member 5), also known as BiP (immunoglobulin heavy chain-binding protein) and GRP78 (78 kDa glucose-regulated protein), is a chaperone that plays a central role in the development and growth of malignant tumors, as a key component of the unfolded protein response, also known as endoplasmic reticulum (ER) stress [1-4]. This chaperone is actively involved in the correct folding of proteins and the degradation of misfolded proteins, as well as in the growth of glioblastomas, the most aggressive malignant primary brain tumors [5-7]. DnaJ heat shock protein family (Hsp40) member B9 (DNAJB9), also known as endoplasmic reticulum DnaJ homolog 4 ERdj4, is induced by ER stress, aberrantly expressed in cancer cells, and plays a significant role in ER stress-mediated proliferation of cancer cells, including glioblastoma cells [8, 9]. DNAJB9 is also involved in ER stress-associated degradation (ERAD) of misfolded and unfolded proteins. It also induces cell transformation under onco-

genic RAS activation, controls cancer cell metastasis, and regulates chemotherapy resistance [10-12].

Malignant tumors, including glioblastoma, to enhance their growth and survival require hypoxia and ER stress, which are key factors in tumorigenesis [13, 14]. These factors reprogram metabolic processes in malignant tumor cells by altering the expression of numerous regulatory genes [15, 16]. Cancer cells use the unfolded protein response signaling pathways to adapt to adverse environmental conditions [1]. The response to ER stress is mediated by three tightly interconnected sensory signaling pathways [16, 17]. The ERN1 (ER to nucleus signaling 1) signaling pathway mediates the response to unfolded and misfolded proteins, thereby eliminating stress and preventing apoptosis, and is a key regulator of maintaining homeostasis [1, 17]. This signaling protein has two cytoplasmic areas that reveal different enzymatic activities (protein kinase and endoribonuclease), which control the expression of numerous stress-dependent genes [16, 18-23]. ERN1

endoribonuclease activity is responsible for the alternative splicing of transcription factor XBP1 (X-box binding protein 1) that controls the expression of hundreds of stress-responsive genes, as well as the specific degradation of a subset of mRNAs through the regulated ERN1-dependent decay of messenger RNAs (RIDD) [18, 19, 21, 24]. It is known that the activity of the ERN1 protein kinase is also involved in regulating the expression of ER stress-dependent genes, and that this is mediated by JNK (c-Jun N-terminal kinase), particularly for the epiregulin gene (*EREG*) [18, 21, 22, 25].

Hypoxia is known to contribute to malignant tumor growth and its resistance to therapy via glucose metabolism reprogramming by changing numerous gene expressions [13, 26–31]. Hypoxia regulates the expression of genes that have HRE (hypoxia-responsive element) in their promoter region by activation of the transcription factor HIF [32, 33]. Hypoxia increases the expression of alpha subunit HIF and many other genes, including those involved in glucose metabolism [34–38]. However, the final result of the effect of hypoxia on the expression level of a particular gene depends not only on the HIF transcription factor, but also on many other factors and enzymes that can significantly change the stability or transcriptional activity of HIF [39–41]. More than a hundred proteins have been identified that can alter the stability and activity of HIF1A by interacting with it through various mechanisms [39]. Previously, it was demonstrated that HIF1 mediates hypoxic induction of *EDN1* (endothelin-1) gene expression in endothelial cells, and that genistein, a protein kinase inhibitor, abolishes the effect of hypoxia on *EDN1* expression [42]. Furthermore, the hypoxic regulation of numerous genes was significantly modified by inhibition of the signaling protein ERN1, indicating an interaction between ER stress and hypoxia in regulating gene expression, and is important for clarifying the role of HIF in cancer progression [16, 17, 40, 41, 43]. The mechanisms of organ-specific changes in the expression of some genes expression under hypoxia in vivo also remain unclear, as do the differences in hypoxic regulation of gene expression in different adenocarcinoma cell lines from the same organ [44–47].

Thus, the molecular mechanism of the dependence of hypoxic gene expression on ER stress remains poorly understood. Therefore, elucidation of the mechanisms of interaction of these important factors in malignant tumor growth is relevant for the development of effective antitumor therapy methods.

This study aimed to examine the interaction between hypoxia and ER stress in the regulation of *HSPA5* and *DNAJB9* gene expressions in normal human astrocytes and glioblastoma cells using two different ER stress inducers.

Materials and Methods

To study the effects of hypoxia and ER stress on the expression of *HSPA5* and *DNAJB9* genes, normal human astrocytes (line NHA/TS) and U87MG glioblastoma cells were used, which were grown as described [22]. Cells were treated for 4 h with tunicamycin and thapsigargin for induction of ER stress (0.5 µg/ml and 2 µM, respectively), and 0.5 mM dimethylloxalylglycine (DMOG), a HIF1A prolyl hydroxylase inhibitor, which mimics the effects of hypoxia under normoxic conditions, as described [33, 35, 36]. Tunicamycin and thapsigargin were received from Sigma-Aldrich, St. Louis, MO, USA. Dimethylloxalylglycine was received from Selleck Chemicals, Huston, TX, USA. RNA extraction and reverse transcription were described previously [21]. The expression of the *HSPA5* and *DNAJB9* genes in the normal human astrocytes and glioblastoma cells was measured by real-time qPCR as described [21]. Primers for *DNAJB9* and *ACTB* were described previously [48]. The pair of primers specific for *HSPA5* was received from Sigma-Aldrich (St. Louis, MO, USA) and used for quantitative PCR: forward 5'-aggacaagaaggaggacgtg-3' and reverse 5'-atcagacgttccttcagg-3' (NM_005347.5). The results of real-time qPCR were analyzed using the Differential Expression Calculator. Statistical analysis of the achieved results was performed using GraphPad Prism 8.0.1. The values for *HSPA5* and *DNAJB9* gene expression were normalized to *ACTB* mRNA expression and expressed as a percentage of controls (100%). All values were expressed as mean ± SEM from triplicate measurements performed in four independent experiments. A value of $P < 0.05$ was considered significant in all cases. All real-time qPCR data were analyzed for the normality of distribution using a histogram and a graphical tool (normal probability plot) as described previously [21]. A normal distribution was detected for all analyzed datasets.

Results and Discussion

As shown in Fig. 1, the expression level of the *HSPA5* gene does not change significantly in normal human astrocytes under the influence of dimethylloxalylglycine, which mimics the effects of hypoxia

under normoxic conditions by inhibiting HIF1A prolyl hydroxylase.

The hypoxia model with dimethyloxallylglycine is quite successful. The effects of hypoxia are simulated under normoxic conditions, as in malignant tumors, where hypoxia develops due to inhibition of the use of available oxygen [30]. It is known that changes in HIF1A mRNA and protein expression are similar to those induced by oxygen deficiency (1%) or the iron chelator desferrioxamine in various cancer cell lines [33, 35, 36].

Induction of ER stress by tunicamycin leads to significant upregulation of *HSPA5* gene expression in normal astrocytes by 90% ($P < 0.001$) as compared to control cells (Fig. 1). At the same time, hypoxia combined with ER stress induced by tunicamycin reduces *HSPA5* gene expression in normal human astrocytes by 13% ($P < 0.05$) compared to tunicamycin-treated cells (Fig. 1). Thus, *HSPA5*/BiP gene expression in normal astrocytes is resistant to hypoxia; however, in the presence of ER stress, hy-

poxia counteracts the stress-dependent upregulation of this gene expression, indicating an interaction between hypoxia and ER stress in regulating *HSPA5* gene expression.

It has also been shown that induction of ER stress by another stress-inducer, thapsigargin, leads to stronger upregulation *HSPA5* gene expression in normal human astrocytes compared to cells treated with tunicamycin. From the data presented in Fig. 1, it is clear that *HSPA5* gene expression in normal astrocytes increases by 996% ($P < 0.001$) under the impact of thapsigargin compared to control cells. However, when thapsigargin was used with hypoxia, this effect was also reduced: by 21% ($P < 0.05$) compared to cells treated with thapsigargin alone (Fig. 1). Thus, *HSPA5* gene expression in normal human astrocytes is altered upon induction of ER stress; however, the magnitude of changes in the *HSPA5* gene expression significantly depends on the nature of ER stress and the mechanisms of its initiation. It is known that tunicamycin and thapsigargin induce ER

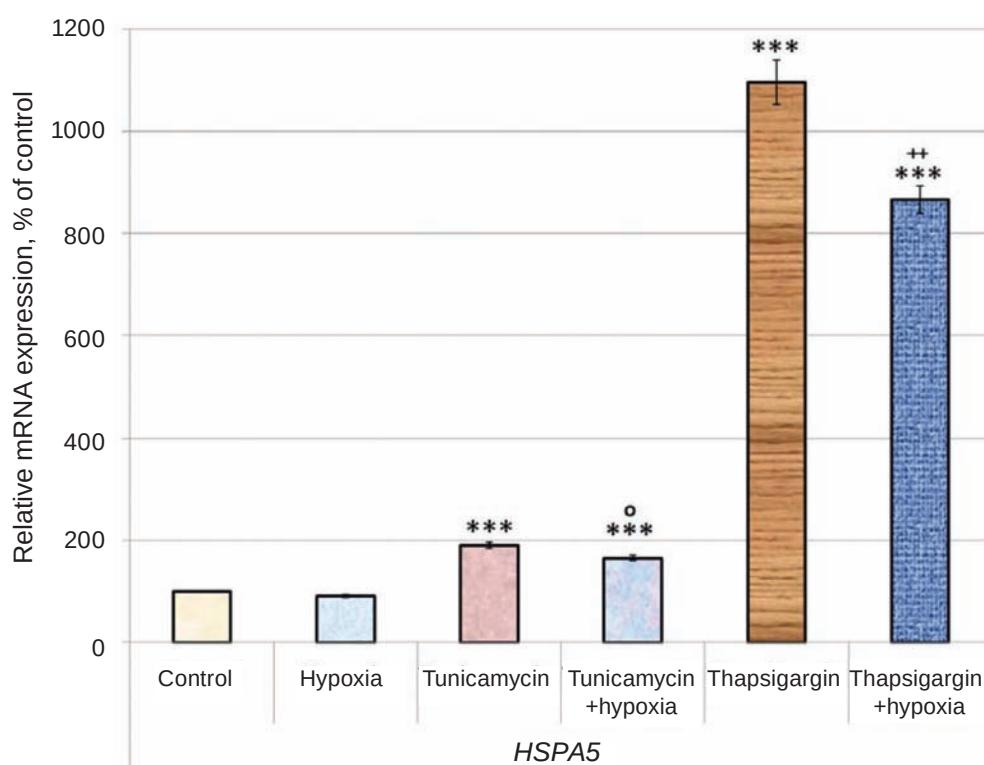


Fig. 1. The effect of hypoxia and endoplasmic reticulum stress introduced by tunicamycin or thapsigargin on the expression of the *HSPA5* (heat shock protein family A (Hsp70) member 5), also known as BiP (immunoglobulin heavy chain-binding protein) gene in the normal human astrocytes line NHA/TS, measured by quantitative PCR. The values of this gene expression were normalized to beta-actin mRNA and represented as a percent of the control; mean $\pm$ SEM; *** $P < 0.001$ vs control; ° $P < 0.05$ vs tunicamycin; ++ $P < 0.01$ vs thapsigargin

stress through different mechanisms. The large difference in the magnitude of changes in *HSPA5* gene expression under the action of these two ER stress inducers is determined by the different mechanisms of their action and the different types of ER stress. At the same time, despite the different magnitudes of changes in *HSPA5* gene expression introduced by tunicamycin and thapsigargin, hypoxia reduced the effect of both tunicamycin and thapsigargin on the expression level of this gene in normal human astrocytes.

As shown in Fig. 2, the expression of *HSPA5* gene is sensitive to hypoxia in glioblastoma cells: the level of this gene expression is increased by 30% ($P < 0.05$).

Tunicamycin also increased *HSPA5* gene expression (by 770%, $P < 0.001$) in the glioblastoma cells in comparison to the control; however, when tunicamycin is combined with hypoxia, the level of this gene expression is reduced by 22% ($P < 0.05$) when compared with the effect of tunicamycin alone

(Fig. 2). At the same time, it is upregulated by 581% ($P < 0.001$) in comparison to control and by 424% ($P < 0.001$) when compared with the effects of hypoxia alone (Fig. 2). Thus, the effect of tunicamycin on the expression of *HSPA5* gene in glioblastoma cells is stronger than in normal astrocytes, indicating higher sensitivity of this gene expression in glioblastoma cells to ER stress, induced by tunicamycin.

A strong increase in the expression level of the *HSPA5* gene was also detected in glioblastoma cells upon induction of ER stress by thapsigargin, as in normal astrocytes, but more pronounced (by 1222%, $P < 0.001$) when compared with control cells (Fig. 2). However, hypoxia did not modify the effect of thapsigargin on the expression level of the *HSPA5* gene in glioblastoma cells at their combined action (by 1203 % ($P < 0.001$) compared to control cells and by 902 % ($P < 0.001$) compared to hypoxia alone (Fig. 2).

Thus, upon induction of ER stress by thapsigargin, no significant difference was found in the

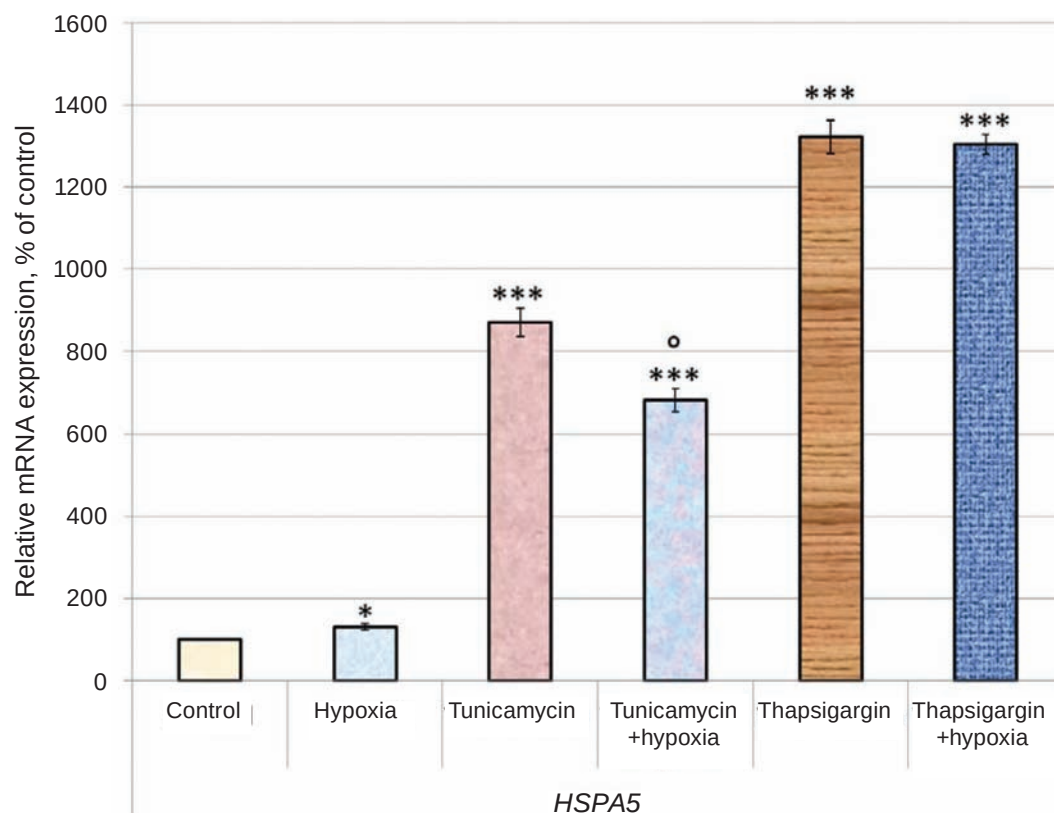


Fig. 2. The effect of hypoxia and endoplasmic reticulum stress introduced by tunicamycin or thapsigargin on the expression of the *HSPA5* (heat shock protein family A (Hsp70) member 5) gene in the glioblastoma cells line U87MG, measured by quantitative PCR. The values of *HSPA5* gene expression were normalized to beta-actin mRNA and represented as a percent of the control; mean $\pm$ SEM; *** $P < 0.001$ vs control; ° $P < 0.05$ vs tunicamycin

magnitude of changes in *HSPA5* gene expression between normal astrocytes and glioblastoma cells, which is probably due to the increased sensitivity of normal astrocytes to the action of this ER stress inducer. However, upon induction of ER stress by thapsigargin, no modifying effect of hypoxia on the effect of thapsigargin on the level of *HSPA5* gene expression was detected upon their combined action in glioblastoma cells, which was manifested upon the action of tunicamycin, as well as in normal human astrocytes upon the action of both ER stress inducers.

Next, we studied the impact of hypoxia and endoplasmic reticulum stress inducers tunicamycin and thapsigargin on the expression of the DnaJ heat shock protein family (Hsp40) member B9 (*DNAJB9*) gene in normal human astrocytes. (Fig. 3).

As shown in Fig. 3, the expression of the *DNAJB9* gene in normal human astrocytes is resistant to hypoxia compared to control cells. However, the level of this gene expression is significantly increased in normal astrocytes exposed to tunicamycin by 134% ($P < 0.001$) compared to control cells.

However, under the combined action of hypoxia with tunicamycin, the effect of tunicamycin on the expression level of the *DNAJB9* gene in normal astrocytes is increased by 12% ($P < 0.05$) compared to the action of tunicamycin alone (Fig. 3). At the same time, the expression of the *DNAJB9* gene in normal human astrocytes was very sensitive to thapsigargin action. It was found that thapsigargin increased the expression level of this gene by 1659% ($P < 0.001$) compared to control cells, but when combined with hypoxia, the effect was reduced by 15% ($P < 0.05$) compared to the action of thapsigargin alone, but remained higher by 1302% as compared to control cells (Fig. 3).

At the same time, the sensitivity of *DNAJB9* gene expression to hypoxia and to both ER stress inducers in glioblastoma cells differs significantly from that in normal astrocytes. As shown in Fig. 4, the expression level of the *DNAJB9* gene is increased by 74% ($P < 0.001$) in hypoxia-treated glioblastoma cells compared to control cells.

It was found that tunicamycin increases the expression of the *DNAJB9* gene by 879% ($P < 0.001$)

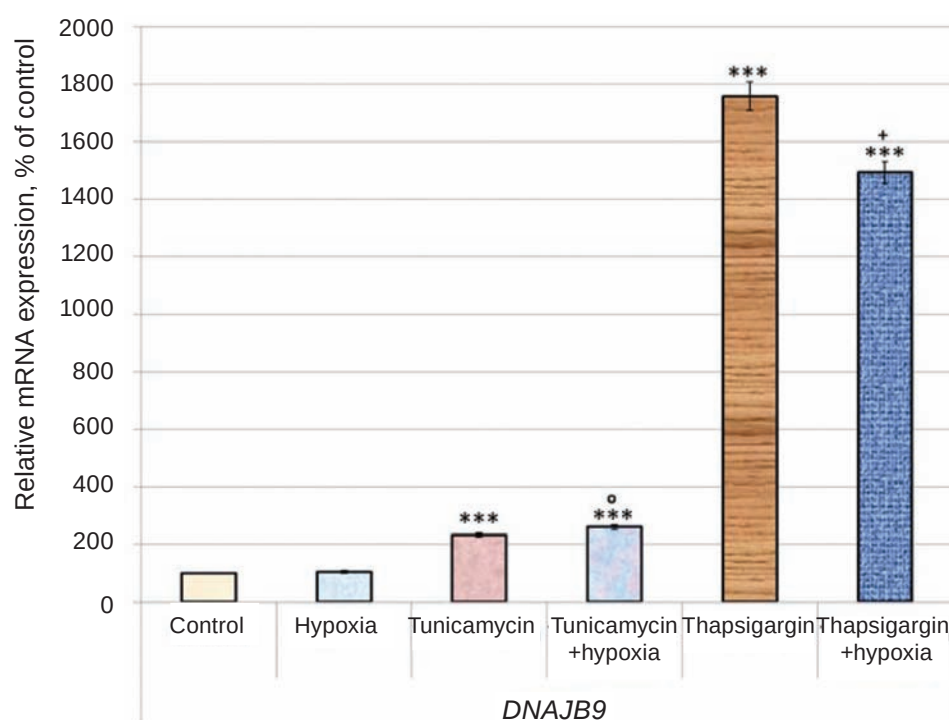


Fig. 3. The impact of hypoxia and endoplasmic reticulum stress introduced by tunicamycin or thapsigargin on the expression of the *DNAJB9* (DnaJ heat shock protein family (Hsp40) member B9) gene in the normal human astrocytes line NHA/TS, measured by quantitative PCR. The values of this gene expression were normalized to beta-actin mRNA and represented as a percent of the control; mean $\pm$ SEM; *** $P < 0.001$ vs control; ° $P < 0.05$ vs tunicamycin; + $P < 0.05$ vs thapsigargin

in glioblastoma cells as compared to control cells (Fig. 4). These results indicate a significantly greater sensitivity of *DNAJB9* gene expression to tunicamycin stress induction in glioblastoma cells compared with normal astrocytes. Furthermore, hypoxia did not change the impact of tunicamycin on the expression level of the *DNAJB9* gene in glioblastoma cells (Fig. 4). As shown in Fig. 4, thapsigargin also significantly increased the expression of the *DNAJB9* gene in glioblastoma cells by 1461% ($P < 0.001$), although this increase is smaller compared to that in normal astrocytes (Fig. 3). However, hypoxia did not significantly modify the impact of thapsigargin on this gene expression at the combined their action (Fig. 4). Moreover, the impact of thapsigargin on *DNAJB9* gene expression in normal astrocytes was 7.5 times greater than that of tunicamycin, whereas in glioblastoma cells, it was only 1.6 times greater.

The summarized results of this investigation are presented in Fig. 5.

They demonstrate the relationships between hypoxia and ER stress in the regulation of *HSPA5* and *DNAJB9* gene expression in normal human astrocytes and glioblastoma cells, as well as a signifi-

cant difference in the actions of the two ER stress inducers, tunicamycin and thapsigargin, with different mechanisms of stress induction. Results of this study demonstrate that the effect of hypoxia on the expression of the *HSPA5* and *DNAJB9* genes is cell-specific, depends on the cell type, and is manifested only in tumor cells, particularly glioblastoma cells (Fig. 5). The obtained data are consistent with those previously reported, which demonstrated the dependence of hypoxic regulation of numerous gene expressions on ER stress, in particular its signaling protein ERN1 [40-43]. Moreover, our results showed that the expression of the *HSPA5* and *DNAJB9* genes has different sensitivity to ER stress induced by tunicamycin and thapsigargin, which significantly depends on the cell type, is also cell-specific, and is more pronounced mainly in glioblastoma cells (Fig. 5). Thus, this investigation demonstrates the presence of non-canonical mechanisms of gene expression control by hypoxia, namely the dependence of hypoxic regulation on endoplasmic reticulum stress. Furthermore, a dependence of the expression of the *HSPA5* and *DNAJB9* genes on the type of ER stress was revealed, which is determined by the mo-

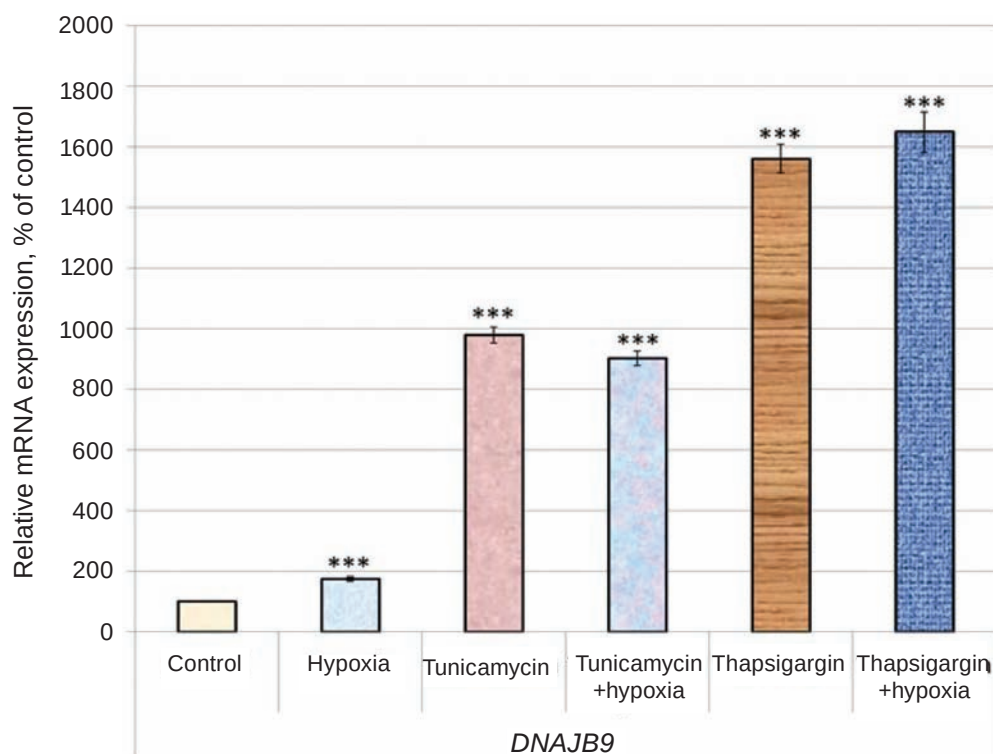


Fig. 4. The impact of hypoxia and endoplasmic reticulum stress introduced by tunicamycin or thapsigargin on the expression of the *DNAJB9* gene in the glioblastoma cells line U87MG, measured by quantitative PCR. The values of *DNAJB9* gene expression were normalized to beta-actin mRNA and represented as a percent of the control; mean $\pm$ SEM; *** $P < 0.001$ vs control

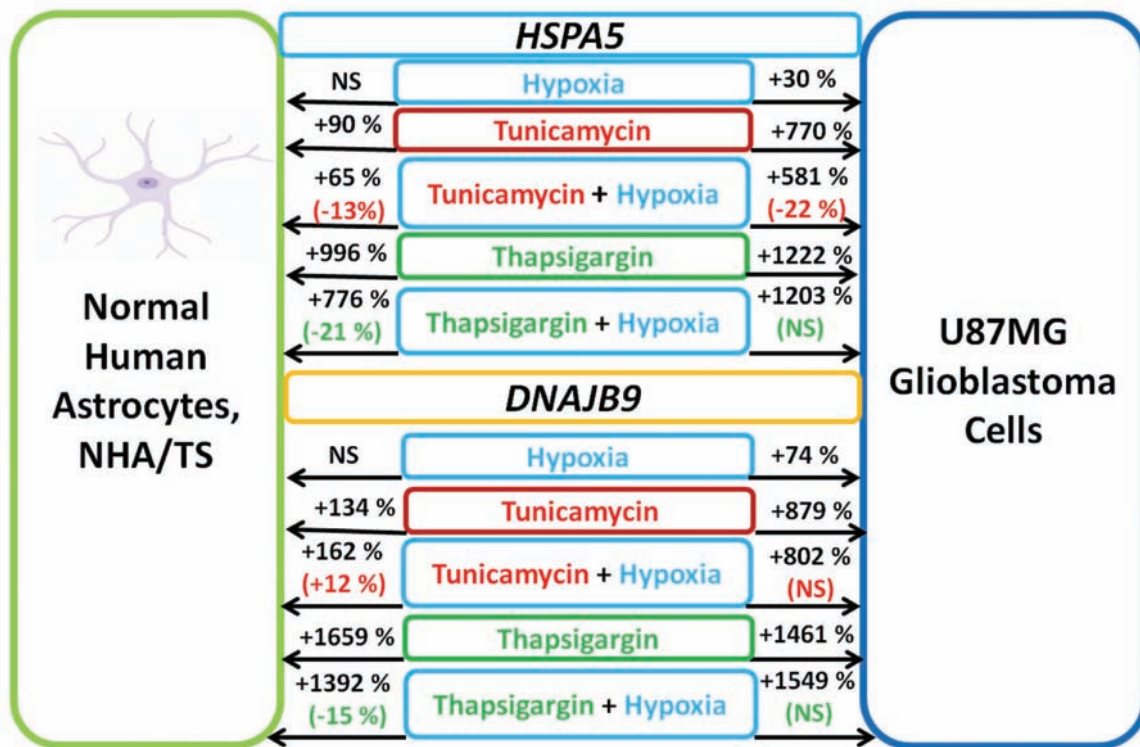


Fig. 5. Schematic representation of the interaction between hypoxia and endoplasmic reticulum stress introduced by tunicamycin or thapsigargin in the regulation of *HSPA5* and *DNAJB9* gene expressions in normal human astrocytes, line NHA/TS, and U87MG glioblastoma cells. Black numbers indicate changes in *HSPA5* and *DNAJB9* gene expressions compared to the control, which is set at 100%. Red-colored numbers represent the combined effect of tunicamycin with hypoxia on these gene expressions compared to tunicamycin alone. Green-colored numbers represent the combined effect of thapsigargin with hypoxia on the expression of *HSPA5* and *DNAJB9* genes compared to tunicamycin alone. NS – no significant differences

lecular mechanisms of the development of this stress. Moreover, the relationship between hypoxia and ER stress in the regulation of *HSPA5* and *DNAJB9* gene expression differs between normal astrocytes and glioblastoma cells. To a large extent, this difference may be due to stress-dependent reprogramming of hypoxic regulation in malignant tumor cells under the influence of ER stress.

Hypoxia is known to contribute to malignant tumor growth as well as to its resistance to therapy through metabolism reprogramming by changing numerous gene expressions [28, 29, 31]. It is known that hypoxic regulation of gene expression is mediated by the transcription factor HIF, which is a heterodimer that binds to the HRE (hypoxia-responsive element) in the promoter region of target genes [31-33]. At the same time, the final result of hypoxic regulation of gene expression depends not only on the transcription factor HIF, but also on many other factors that can significantly modify its stability

or transcriptional activity [39, 40]. It was demonstrated that more than a hundred proteins can alter the stability and activity of HIF1A by interacting with it through various molecular mechanisms [39]. Previously, we demonstrated that HIF mediates hypoxic induction of *EDN1* gene expression in microvascular endothelial cells, and that a protein kinase inhibitor, genistein, reduces the hypoxic regulation of this gene expression [42]. In this work, we demonstrated the presence of a close interaction between hypoxia and ER stress in the regulation of *HSPA5* and *DNAJB9* gene expression, which is in good agreement with the results of previous studies showing the effect of ERN1 inhibition on hypoxic regulation of the expression of many genes [40, 42, 43]. Thus, the dependence of hypoxic regulation of gene expression on the signaling protein ERN1 indicates an interaction between ER stress and hypoxia in regulating gene expression, and is important for clarifying the role of HIF in cancer progression [17,

40, 41, 43]. The revealed dependence of hypoxic regulation of gene expression on the type of ER stress in both normal and tumor cells must be taken into account when developing antitumor therapies.

Conclusions. Thus, the expression of the *HSPA5* and *DNAJB9* genes is resistant to hypoxia in normal human astrocytes; however, in glioblastoma cells, the expression of both genes is increased by hypoxia, indicating cell-specificity of hypoxic regulation. It was demonstrated that tunicamycin increased the expression of both *HSPA5* and *DNAJB9* genes in normal and tumor cells; however, the effect was much stronger in glioblastoma cells. Moreover, hypoxia modifies the effect of tunicamycin and thapsigargin when these ER stress inducers are combined with hypoxia preferentially in normal astrocytes. However, the detailed molecular mechanism underlying the interaction between hypoxia and different ER stress pathways remains complex and requires further investigation. Elucidating the features of these molecular mechanisms of interaction between ER stress and hypoxia signaling pathways in the control of gene expression, not only in malignant tumor cells but also in normal cells, is important both for understanding the pathways of malignant neoplasm development and for combating them.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ЕКСПРЕСІЯ ГЕНІВ *HSPA5* ТА *DNAJB9* У КЛІТИНАХ ГЛІОБЛАСТОМИ ТА НОРМАЛЬНИХ АСТРОЦИТАХ В УМОВАХ ГІПОКСІЇ ТА СТРЕСУ ЕНДОПЛАЗМАТИЧНОГО РЕТИКУЛУМУ

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Гіпоксія та стрес ендоплазматичного ретикулуму (ЕР-стрес) є ключовими факторами росту пухлини; однак взаємодія цих факторів вивчена ще недостатньо. Протеїни теплового шоку *HSPA5* та *DNAJB9* як ключові компоненти реакції на стрес ендоплазматичного ретикулума відіграють важливу роль у рості злоякісних пухлин, зокрема гліобластом. Метою цього дослідження було вивчення взаємодії гіпоксії та ЕР-стресу у регуляції експресії *HSPA5* і *DNAJB9* у клітинах гліобластоми і нормальних астроцитах. Гіпоксію моделювали диметилксалілгліцином, ЕР-стрес індукували тунікаміцином та тапсигаргіном, експресію *HSPA5* і *DNAJB9* досліджували методом кількісної ПЛР. Встановлено, що експресія *HSPA5* і *DNAJB9* в астроцитах була стійкою до гіпоксії. Однак, у клітинах гліобластоми експресія цих генів збільшувалася за гіпоксії. Тунікаміцин і тапсигаргін посилювали експресію генів *HSPA5* і *DNAJB9* більш виражено у клітинах гліобластоми. За поєднаної дії цих індукторів стресу ЕР з гіпоксією їх ефект був більш вираженим у нормальних астроцитах. Отримані результати свідчать про різну клітинно-специфічну чутливість експресії *HSPA5* та *DNAJB9* до гіпоксії та індукторів стресу ендоплазматичного ретикулуму.

Ключові слова: гіпоксія, стрес ендоплазматичного ретикулума, *HSPA5*, *DNAJB9*, експресія генів, клітини гліобластоми, нормальні астроцити людини.

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