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ENDOPLASMIC RETICULUM STRESS DIFFERENTIALLY ALTERS THE EFFECT OF HYPOXIA ON *PSAT1* AND *CYCLIN D1* GENE EXPRESSIONS IN NORMAL ASTROCYTES AND GLIOBLASTOMA CELLS

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Endoplasmic reticulum (ER) stress and hypoxia interaction in the progression of glioblastomas and other malignant tumors has not yet been sufficiently studied. Both PSAT1, as the ER stress-responsive phosphoserine aminotransferase, and cyclin D1 are shown to participate in tumor progression and chemoresistance. Therefore, this study aimed to elucidate the effect of endoplasmic reticulum stress on PSAT1 and CCND1 (cyclin D1) genes expression in normal human astrocytes of NHA/TS line, and U87MG glioblastoma cells. Hypoxia was created with the HIF1A prolyl hydroxylase inhibitor dimethyloxalylglycine. Tunicamycin and thapsigargin were used for ER stress induction. PSAT1 and cyclin D1 expression were examined by quantitative real-time RT-PCR. It has been established that hypoxia and tunicamycin had a similar suppressive effect on PSAT1 and CCND1 expression in normal astrocytes, but increased both genes expression in glioblastoma cells. Thapsigargin enhanced PSAT1 expression in both cell lines, but suppressed CCND1 expression in normal astrocytes without any effect on its expression in glioblastoma cells. Hypoxia modified the effect of tunicamycin and thapsigargin when these ER stress inducers were combined with hypoxia, but in different ways in normal and glioblastoma cells. These results indicate that hypoxia and ER stress relationship in the control of the studied genes expression differs in normal and tumor cells.

K e y w o r d s: hypoxia, endoplasmic reticulum stress, PSAT1, cyclin D1, gene expression, normal human astrocytes, glioblastoma cells.

Endoplasmic reticulum (ER) stress and hypoxia are key regulators of malignant tumor development, including glioblastoma, through changes in the expression of numerous genes that reprogram cellular metabolic processes for enhanced malignant tumor growth and survival [1-5]. The rapid growth of malignant tumors generates microenvironmental changes in association with nutrient deficiency and hypoxia, which initiate neoangiogenesis and increase cell proliferation [3, 6-8]. Malignant tumor cells utilize ER stress signaling pathways to adapt to stressful environmental conditions [1, 3]. The response to unfolded protein responses is mediated by three tightly interconnected sensory signaling pathways [3]. The ERN1 (endoplasmic reticulum to nucleus signaling 1) sensory signaling pathway is the most evolutionarily preserved and responds to unfolded and misfolded proteins in a program aimed at removing stress or apoptosis, making it a key regulator of cell death and life [1, 3, 9, 10]. The ERN1 signaling protein has two domains that reveal endoribonuclease and protein kinase activities, which control the expression of numerous stress-dependent genes [3, 10-12]. Endoribonuclease activity of ERN1 is involved in the creation of transcription factor XBP1s (X-box binding protein 1, splice variant) that controls the expression of numerous stress-responsive genes as well as in the degradation of a specific subset of mRNAs through the RIDD (regulated ERN1-dependent decay of messenger RNAs) [1, 10, 13-15]. Previously, we have also demonstrated that the ERN1 protein kinase activity is an important regulator of stress-dependent gene expression, possibly through JNK (c-Jun N-terminal kinase), at least the *EREG* (epiregulin) gene [11, 12, 16].

The hypoxia inducible factor 1 (HIF) plays a major role in the cellular response to hypoxia by regulating the expression of many genes involved in adaptive processes that allow cell survival under low oxygen conditions [17-19]. Hypoxia also regulates glutamine metabolism through HIF1, which is essential for cancer growth [20]. Moreover, ER stress signaling is responsible for development of hypoxia resistance in cancer cells, which is necessary for tumor growth [21, 22]. Hypoxia is known to reprogram glucose metabolism through specific changes in the expression of numerous genes that contribute to malignant tumor growth and its resistance to therapy [23-25]. Hypoxia increases the alpha subunit of the transcription factor HIF, which regulates the expression of genes with hypoxia-responsive element in promoter region [26-28]. Moreover, this transcription factor mediated metabolic reprogramming in tumor cells [29, 30]. However, more than 150 proteins have been identified that can interact with HIF1A, altering its stability and transcriptional activity through different mechanisms, including phosphorylation [31]. It was established that HIF-1 mediates the hypoxic induction of endothelin-1 gene expression in microvascular endothelial cells, and that the protein kinase inhibitor genistein abrogates the effect of hypoxia on this gene's expression [26]. We have previously demonstrated that inhibition of the signaling protein ERN1 alters the hypoxic regulation of numerous genes, indicating an interaction between ER stress and hypoxia in the regulation of gene expression, and is important for clarifying the role of HIF in cancer progression [26, 27, 32-35]. Furthermore, adaptation to the hypoxic tumor micro-environment is also critical for cancer cell proliferation [36, 37].

At the same time, the mechanisms of organ-specific changes in *VEGF* and different *PFKFB* gene expressions under hypoxia in vivo remain enigmatic [38, 39]. Moreover, differences in the efficiency of hypoxic regulation of gene expression were also observed in various adenocarcinoma cell lines from both the same and different organs [40]. However, despite significant advances in the study of mechanisms regulating hypoxic gene expression, much remains to be elucidated, particularly in understanding the molecular mechanisms of action of numerous factors that modify the effects of hypoxia, especially ER stress [21, 32, 41-43]. Thus, hypoxia stimulates a variety of adaptive responses, many mediated via the hypoxia inducible factors family of transcriptional complexes [44]. Furthermore, hypoxia-inducible factor 1 is an oxygen-sensing transcriptional regulator orchestrating a complex of adaptive cellular responses to hypoxia [45]. For this investigation we select two genes, which have relation to tumor growth: *PSAT1* (phosphoserine aminotransferase 1) and *CCND1* (cyclin D1). PSAT1 is an ER stress-responsive enzyme responsible for serine synthesis and necessary for tumor cell proliferation and therapeutic resistance [12, 46, 47]. Furthermore, overexpressed PSAT1 stimulates cancer cell growth and increases their chemoresistance via the modulation of the cell cycle [48]. Cyclin D1, which alters cell cycle progression, is overexpressed in a variety of human cancers and participates in glioma and other tumor progression and chemoresistance [49-51].

This study aimed to investigate the dependence of hypoxic regulation of the expression of *PSAT1* and *CCND1* genes from ER stress, induced by tunicamycin and thapsigargin, in normal human astrocytes and U87MG glioblastoma cells. This is necessary to identify differences in the response of these gene expressions to different stress factors in normal and tumor cells.

Materials and Methods

In this investigation, normal human astrocytes, line NHA/TS, and U87MG glioblastoma cells were used. Cells were grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's minimum essential medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., USA), penicillin (100 units/ml, Gibco), and streptomycin (0.1 mg/ml, Gibco) at 37°C in incubator with 5% CO_2 as described [16]. 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 dimethyloxalylglycine, a HIF1A prolyl hydroxylase inhibitor, which mimics the effects of hypoxia under normoxic conditions, as described [23]. In these works, it was shown that the effect of dimethyloxalylglycine and real hypoxic conditions (1% oxygen) on the expression level of the alpha subunit of HIF and HIF-dependent genes was similar. Tunicamycin and thapsigargin were received from Sigma-Aldrich and used for the induction of ER stress. Hypoxia was introduced by the HIF1A prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG), which mimics the effects of hypoxia under normoxic conditions, which was received from Selleck Chemicals, Huston, TX, USA. RNA was obtained from NHA/TS and glioblastoma cells using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad,

CA, USA). The RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water. The expression of the *PSAT1* and cyclin D1 genes in normal astrocytes and glioblastoma cells was examined by quantitative real-time RT-PCR using "QuantStudio 5 Real-Time PCR System" (Applied Biosystems, USA) as described [11]. Primers for *PSAT1* and ACTB were described previously [32]. The pair of primers specific for *CCND1* was received from Sigma-Aldrich (St. Louis, MO, USA) and used for quantitative PCR: forward 5′-gcatgttcgtggcctctaag-3′ and reverse 5′-gtgtttgcggatgatctgt-3′ (NM_053056.3). The results of quantitative PCR were analyzed using the "Differential Expression Calculator". For analysis, statistics, and graph presentation of obtained scientific results, the GraphPad Prism 8.0.1 package was used. The values of studied gene expression were normalized to the expression of ACTB mRNA and expressed as a percentage of controls (100%). All values were expressed as mean±SEM from triplicate measurements performed in 4 independent experiments. A value of $P < 0.05$ was considered significant in all cases. All experimental qPCR data were analyzed for the normality of distribution using a graphical tool (normal probability plot) and a histogram as described previously [11]. A normal distribution was observed for all analyzed datasets.

Results and Discussion

Studies have shown that the expression level of the PSAT1 gene does not change significantly in normal human astrocytes under the influence of dimethyloxalylglycine, which mimics the effects of hypoxia under normoxic conditions by inhibiting HIF1A prolyl hydroxylase (Fig. 1).

It has previously been shown that changes in the expression of HIF1A mRNA and protein are similar to those induced by oxygen deficiency (1%) or the iron chelator desferrioxamine in different cancer cell lines [19, 40, 41]. At the same time, induction of ER stress by tunicamycin leads to downregulation of phosphoserine aminotransferase 1 gene expression in normal human astrocytes by 32% ($P < 0.01$) as compared to control (Fig. 1). By investigating the

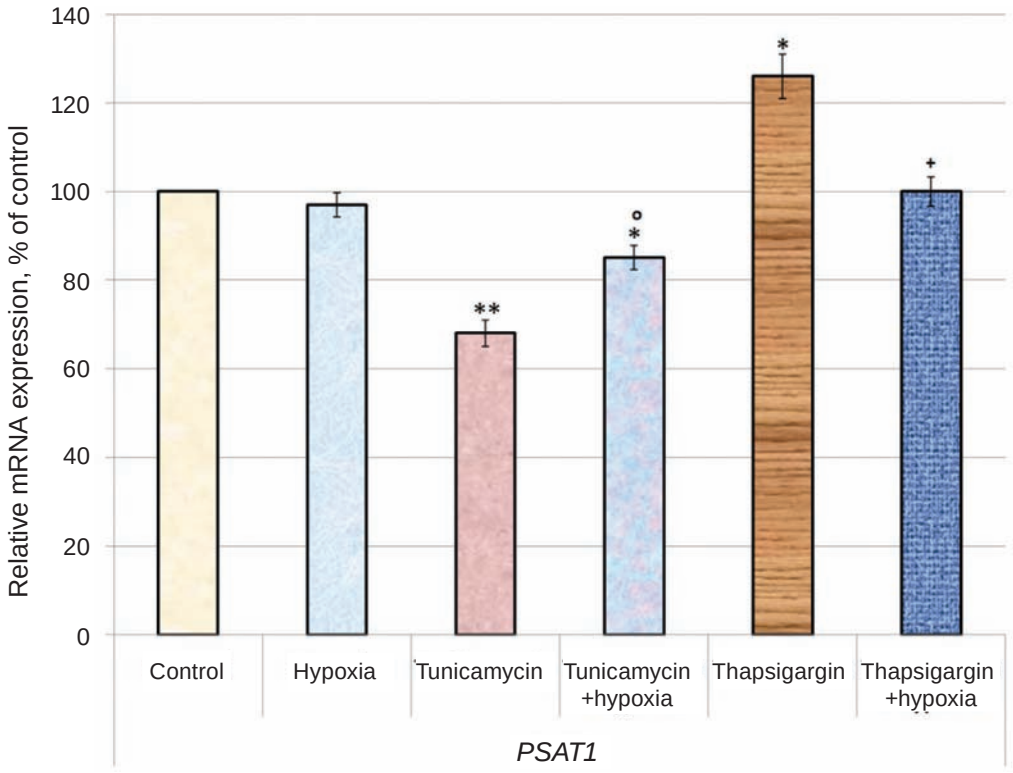

*Fig. 1. The effect of hypoxia and endoplasmic reticulum stress induced by tunicamycin or thapsigargin on the expression of the PSAT1 (phosphoserine aminotransferase 1) gene in the normal human astrocyte line NHA/TS, measured by quantitative PCR. The values of this gene expression were normalized to beta-actin mRNA and represented as a percent of the control; mean ± SEM; *$P < 0.05$ and **$P < 0.01$ vs control; $^{O}P < 0.05$ vs tunicamycin; $^{+}P < 0.05$ vs thapsigargin*

interaction of hypoxia and endoplasmic reticulum stress induced by tunicamycin in the regulation of *PSAT1* gene expression in normal human astrocytes, we demonstrated that hypoxia reduces the effect of tunicamycin on the expression of this gene by 25% ($P < 0.05$) compared to tunicamycin-treated cells (Fig. 1). Thus, *PSAT1* gene expression in normal human astrocytes is resistant to hypoxia; however, in the presence of tunicamycin, hypoxia counteracts the stress-dependent suppression of this gene expression, affecting the interaction between hypoxia and stress in the regulation of gene expression, particularly phosphoserine aminotransferase 1.

It has also been shown that induction of ER stress by thapsigargin leads to oppositely directed changes in *PSAT1* gene expression in normal human astrocytes compared to tunicamycin-treated cells. From the data presented in Fig. 1, it is clear that the level of *PSAT1* gene expression in normal human astrocytes increases by 26% ($P < 0.05$) under the influence of thapsigargin compared to control cells. However, when thapsigargin was used with hypoxia, this effect was eliminated (Fig. 1). Thus, the expression of the *PSAT1* gene in normal human astrocytes changes upon induction of ER stress; however, the nature of changes in *PSAT1* gene expression significantly depends on the nature of ER stress and the mechanisms of its initiation. It is known that tunicamycin and thapsigargin initiate ER stress through different mechanisms, and it is possible that this is what determines both the magnitude and direction of changes in gene expression, and different genes in different ways. At the same time, despite the differently directed changes in *PSAT1* gene expression induced by tunicamycin and thapsigargin, hypoxia reduced the effects of both tunicamycin and thapsigargin on the expression level of this gene in normal human astrocytes.

Completely different changes in *PSAT1* gene expression were found in glioblastoma cells under hypoxia and ER stress inducers compared to normal human astrocytes (Fig. 2).

Hypoxia significantly increased the expression of the *PSAT1* gene (by 68%, $P < 0.001$) in glioblas-

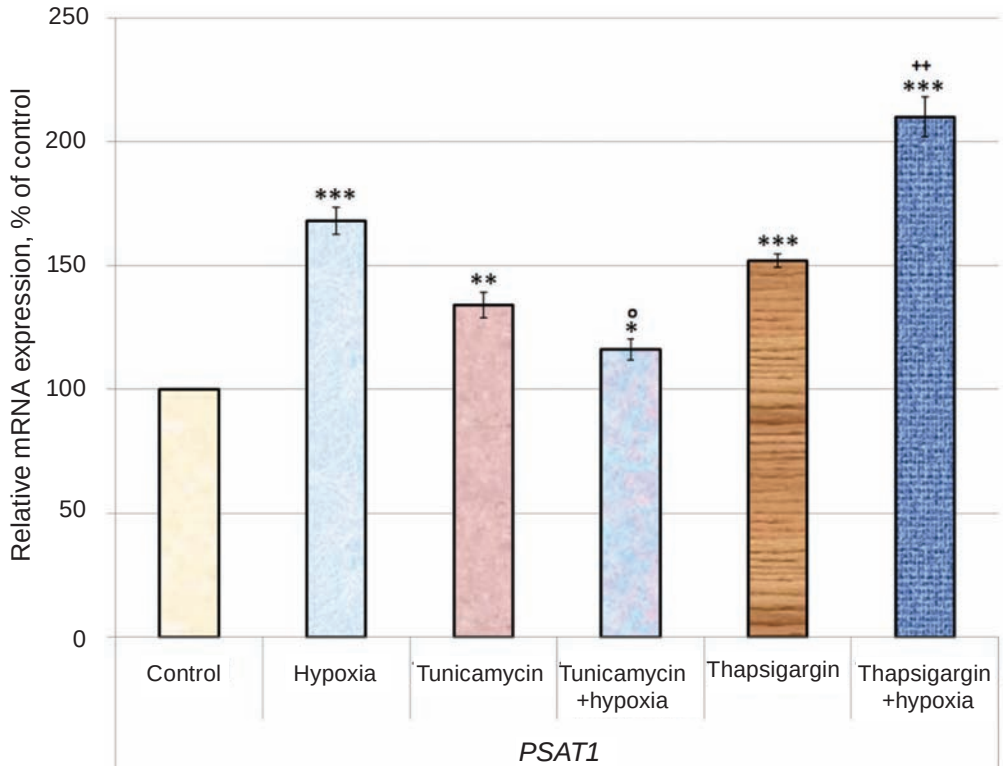

*Fig. 2. The effect of hypoxia and endoplasmic reticulum stress induced by tunicamycin or thapsigargin on the expression of the PSAT1 gene in the glioblastoma cell line U87MG, measured by quantitative PCR. The values of PSAT1 gene expression were normalized to beta-actin mRNA and represented as a percent of the control; mean ± SEM; *P < 0.05, ** P < 0.01, and ***P < 0.001 vs control; °P < 0.05 vs tunicamycin; ++P < 0.01 vs thapsigargin*

toma cells compared to control cells (Fig. 2). Tunicamycin also increased *PSAT1* gene expression (by 34%, $P < 0.01$) in the glioblastoma cells in comparison to control; however, when tunicamycin is combined with hypoxia, the level of *PSAT1* gene expression is reduced to a value that is significantly lower than the effects of either tunicamycin or hypoxia (Fig. 2). Thus, the effect of the combined action of tunicamycin with hypoxia in glioblastoma cells is reduced by 13% ($P < 0.05$) when compared with the effect of tunicamycin alone and by 31% ($P < 0.05$) when compared with the effects of hypoxia alone. At the same time, upon induction of ER stress by thapsigargin, an increase in the expression level of the *PSAT1* gene was detected in glioblastoma cells, as in normal astrocytes, but more pronounced (by 52%, $P < 0.001$) (Fig. 2). However, the combined action of thapsigargin with hypoxia in glioblastoma cells did not result in inhibition, but rather an increase in the expression level of the *PSAT1* gene: by 110% ($P < 0.001$) compared to control, by 38% ($P < 0.01$) compared to thapsigargin, and by 25% ($P < 0.05$) compared to hypoxia (Fig. 2).

Next, we studied the impact of hypoxia and endoplasmic reticulum stress inducers tunicamycin and thapsigargin on the expression of the *CCND1* (cyclin D1) gene in the normal human astrocytes (Fig. 3).

Hypoxia was shown to reduce the expression level of the *CCND1* gene by 28 % ($P < 0.05$) in the normal human astrocytes as compared to control cells (Fig. 3). Moreover, the level of this gene expression is also decreased in normal astrocytes treated with tunicamycin and thapsigargin by 55 % in both cases ($P < 0.001$) compared to control cells (Fig. 3). However, the combined action of tunicamycin with hypoxia on the expression level of the *CCND1* gene in glioblastoma cells decrease the effect of tunicamycin by 16% ($P < 0.05$) compared to tunicamycin alone (Fig. 3). At the same time, the combined impact of thapsigargin with hypoxia on this gene expression leads to additional suppression of its expression by 16% ($P < 0.05$) compared to thapsigargin alone, by 47% ($P < 0.01$) compared to hypoxia, and by 62% ($P < 0.001$) compared to control (Fig. 3).

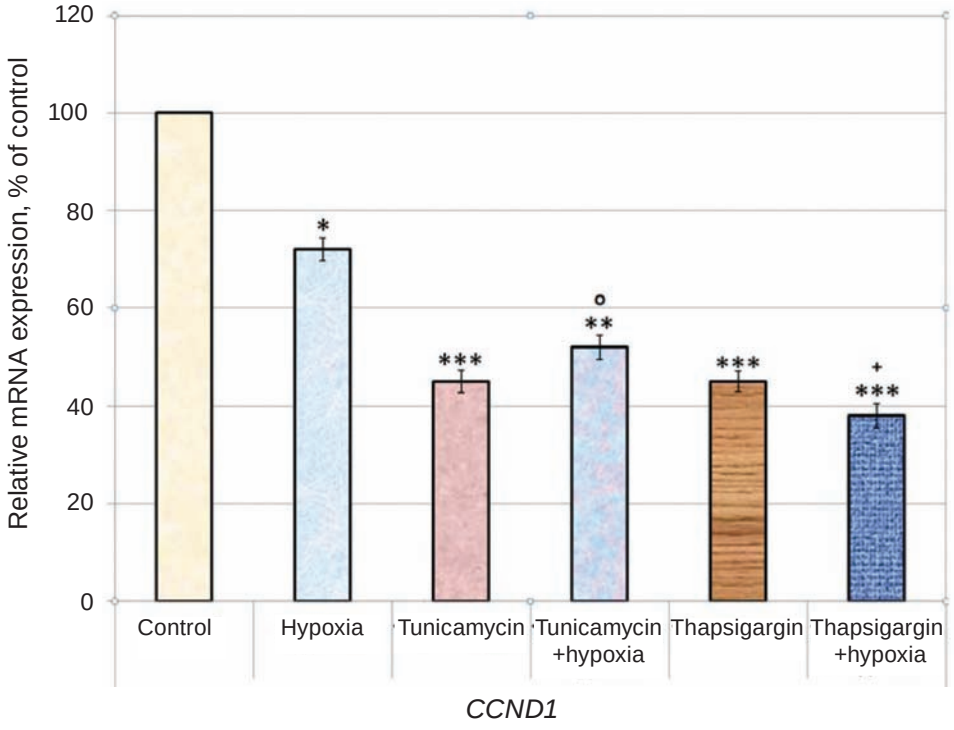

*Fig. 3. The impact of hypoxia and endoplasmic reticulum stress induced by tunicamycin or thapsigargin on the expression of the CCND1 (cyclin D1) gene in the normal human astrocyte line NHA/TS, measured by quantitative PCR. The values of CCND1 gene expression were normalized to beta-actin mRNA and represented as a percent of the control; mean ± SEM; *P < 0.05, **P < 0.01, and ***P < 0.001 vs control; °P < 0.05 vs tunicamycin; +P < 0.05 vs thapsigargin*

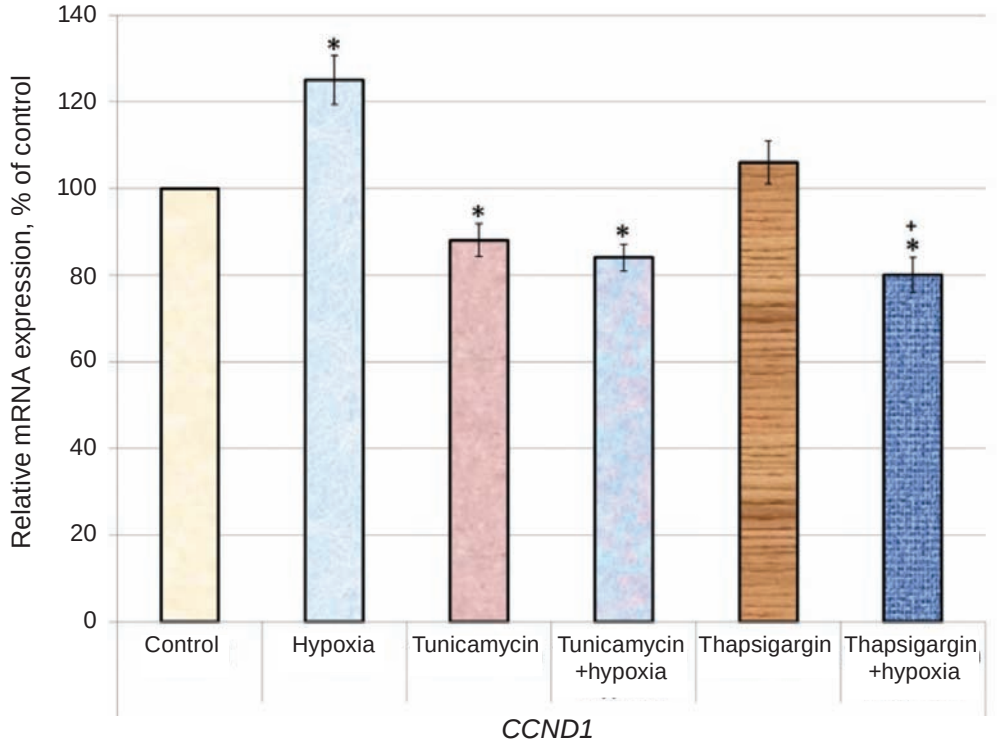

*Fig. 4. The impact of hypoxia and endoplasmic reticulum stress induced by tunicamycin or thapsigargin on the expression of the CCND1 (cyclin D1) gene in the glioblastoma cell line U87MG, measured by quantitative PCR. The values of CCND1 gene expression were normalized to beta-actin mRNA and represented as a percent of the control; mean ± SEM; *P < 0.05 vs control; $^{+}$P < 0.05 vs thapsigargin*

It was also shown that the expression level of the *CCND1* gene is increased by 25% ($P < 0.05$) in hypoxia-treated glioblastoma cells compared to control cells (Fig. 4).

Tunicamycin decreases the expression of the *CCND1* gene by 12% ($P < 0.05$) in glioblastoma cells as compared to control cells (Fig. 4). Furthermore, the effect of hypoxia on the expression level of the *CCND1* gene was eliminated at the combined action of tunicamycin and hypoxia in glioblastoma cells. At the same time, the expression of the *CCND*1 gene was resistant to thapsigargin in glioblastoma cells; however, it was decreased by 25% ($P < 0.05$) at the combined action of thapsigargin and hypoxia compared to thapsigargin alone and by 36% ($P < 0.01$) compared to hypoxia (Fig. 4).

Thus, hypoxia affects the expression of the *CCND1* gene in normal astrocytes and glioblastoma cells in opposite directions. Moreover, hypoxia modifies the effect of tunicamycin and thapsigargin on this gene expression in normal astrocytes, but in different ways.

Results of this investigation are summarized in Fig. 5, which demonstrates the interaction between hypoxia and ER stress induced by two different compounds, tunicamycin and thapsigargin, in the control of *PSAT1* and *CCND1* gene expressions in normal astrocytes and glioblastoma cells.

This study focused on investigating non-canonical mechanisms of hypoxia-induced gene expression regulation and its dependence on endoplasmic reticulum stress, induced by two different compounds. As a result of the conducted studies, the dependence of hypoxic regulation of gene expression on ER stress, as well as the dependence of the effects of ER stress on hypoxia, was revealed. Moreover, a dependence on the type of ER stress was revealed, which is determined by the mechanisms of its development. Thus, a relationship between hypoxia and ER stress in the control of gene expression has been revealed, which differs in normal and tumor cells. To a large extent, this difference is due to stress-dependent reprogramming of hypoxic regulation in malignant tumor cells. The revealed patterns should be taken into account when developing methods to combat malignant tumors.

Conclusions. Thus, hypoxia decreased the expression of the *CCND1* gene and did not significantly

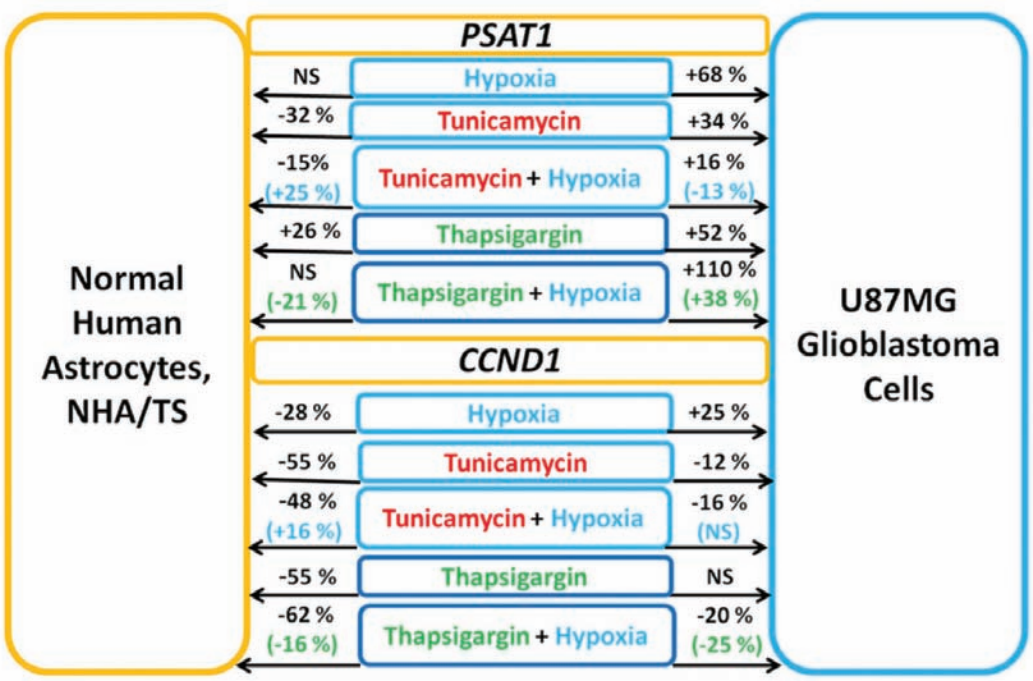

Fig. 5. Schematic representation of the interaction between hypoxia and endoplasmic reticulum stress induced by tunicamycin or thapsigargin in the regulation of PSAT1 and CCND1 gene expressions in normal human astrocytes, line NHA/TS, and U87MG glioblastoma cells. Black numbers indicate changes in PSAT1 and CCND1 gene expressions compared to the control, which is set at 100%. Blue numbers represent the combined effect of tunicamycin with hypoxia on these gene expressions compared to tunicamycin alone. Green numbers represent the combined effect of thapsigargin with hypoxia on the expression of PSAT1 and CCND1 genes compared to thapsigargin alone

change *PSAT1* gene expression in normal human astrocytes. However, in glioblastoma cells, the expression of both these genes increased under hypoxia. It was demonstrated that tunicamycin has a similar suppressive effect on *PSAT1* and *CCND1* gene expression in normal astrocytes, but increased *PSAT1* expression in glioblastoma cells. At the same time, thapsigargin enhanced the expression of the *PSAT1* gene in both cell lines, but more significantly in glioblastoma cells. The expression of the *CCND1* gene is suppressed under thapsigargin in normal astrocytes, but its expression was resistant to this compound in glioblastoma cells. Moreover, hypoxia modifies the effect of tunicamycin and thapsigargin when these endoplasmic reticulum stress inducers are combined with hypoxia, but in different ways in normal and tumor cells. However, the molecular mechanism of the interaction between hypoxia and endoplasmic reticulum stress is complex and requires further investigation. This is necessary to identify differences in the response of these gene expressions to different stress factors in normal and tumor cells and to understand the pathways of malignancy. The revealed patterns should be taken into account when developing methods to combat malignant tumors.

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

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СТРЕС ЕНДОПЛАЗМАТИЧНОГО РЕТИКУЛУМА ПО-РІЗНОМУ ЗМІНЮЄ ВПЛИВ ГІПОКСІЇ НА ЕКСПРЕСІЮ ГЕНІВ *PSAT1* ТА ЦИКЛІНУ D1 У НОРМАЛЬНИХ АСТРОЦИТАХ І КЛІТИНАХ ГЛІОБЛАСТОМИ

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Взаємодія стресу ендоплазматичного ретикулума (ЕР) та гіпоксії у прогресуванні гліобластом та інших злоякісних пухлин вивчена ще недостатньо. Показано, що PSAT1, як фосфосерин амінотрансфераза, що реагує на стрес ЕР, так і циклін D1 беруть участь у прогресії пухлини та хіміорезистентності. Отже, метою цього дослідження було з'ясувати вплив стресу ендоплазматичного ретикулума на експресію генів *PSAT1* та *CCND1* (циклін D1) у нормальних астроцитах людини лінії NHA/TS та клітинах гліобластоми U87MG. Гіпоксію створювали за допомогою інгібітора пролілгідроксилази HIF1A диметилоксалілгліцину. Тунікаміцин та тапсигаргін використовували для індукції стресу ендоплазматичного ретикулума. Експресію *PSAT1* та циклін D1 досліджували за допомогою кількісної зворотної ПЛР у реальному часі. Було встановлено, що гіпоксія та тунікаміцин мали подібний супресивний вплив на експресію *PSAT1* та *CCND1* у нормальних астроцитах, але збільшували експресію обох генів у клітинах гліобластоми. Тапсигаргін посилював експресію *PSAT1* в обох клітинних лініях, але пригнічував експресію *CCND1* у нормальних астроцитах без будь-якого впливу на його експресію в клітинах гліобластоми. Гіпоксія змінювала ефект тунікаміцину та тапсигаргіну, коли ці індуктори стресу ЕР поєднувалися з гіпоксією, але по-різному в нормальних клітинах та клітинах гліобластоми. Ці результати вказують на те, що взаємозв'язок між гіпоксією та стресом ЕР у контролі експресії досліджуваних генів відрізняється в нормальних та пухлинних клітинах.

Ключові слова: гіпоксія, стрес ендоплазматичного ретикулума, *PSAT1*, циклін D1, експресія генів, нормальні астроцити людини, клітини гліобластоми.

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