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EXPRESSION OF HEXOKINASE AND 6-PHOSPHOFRUCTO-2-KINASE/FRUCTOSE-2,6-BISPHOSPHATASE GENES IN ERN1 KNOCKDOWN GLIOMA U87 CELLS: EFFECT OF HYPOXIA AND GLUTAMINE OR GLUCOSE DEPRIVATION

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Endoplasmic reticulum stress, as well as hypoxia and ischemia, are important factors for tumor neovascularization and growth. Cancer cells preferentially utilize glycolysis in order to satisfy their increased energetic and biosynthetic requirements. High glucose metabolism of cancer cells is caused by a combination of hypoxia-responsive transcription factors, activation of oncogenic proteins and the loss of tumor suppressor function and is realized in part by activating a family of regulatory bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) and hexokinase 2. We have studied the effect of hypoxia and ischemia on the expression of PFKFB and hexokinase genes in glioma cell line U87 under knockdown of endoplasmic reticulum–nuclei-1 (ERN1) sensing and signaling enzyme. It was shown that loss of the signaling enzyme ERN1 function leads to an increase in the expression levels of HK1, HK2, PFKFB3 and PFKFB4 mRNA. Moreover, the expression levels of all studied genes increase under hypoxia in control and ERN1-deficient glioma cells; however knockdown of ERN1 suppresses the effect of hypoxia. Besides, HK2 and PFKFB4 are more sensitive to hypoxia than HK1 and PFKFB3. Glucose or glutamine deprivation conditions have different effects on the expression levels of these genes and its effect depends mainly on ERN1 function. Expression levels of alternative splice variants of PFKFB3 and PFKFB4 mRNA change at used experimental conditions in a fashion similar to the basic PFKFB variants. Thus, the expression of hexokinase and PFKFB genes is mainly dependent on ERN1 signaling enzyme function in normal, hypoxic and ischemic conditions.

Keywords: mRNA expression, HK1, HK2, PFKFB3, PFKFB4, glioma cells, ERN1 knockdown, hypoxia, glucose and glutamine deprivation.

INTRODUCTION

High rate of glycolytic flux, even in the presence of oxygen, is a central metabolic hallmark of neoplastic tumors. The high glucose metabolism of cancer cells is caused by a combination of hypoxia-responsive transcription factors, activation of oncogenic

proteins and the loss of tumor suppressor function. Over-expression of HIF-1α or HIF-2α and MYC, activation of RAS and loss of TP53 and/or other tumor suppressor functions each have been found to stimulate glycolysis in part by activating a family of regulatory bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) and hexokinases (HK) [1, 2]. The PFKFB enzymes synthesize fructose-2,6-bisphosphate (F2,6BP) which allosterically activates 6-phosphofructo-1-kinase (PFK-1), a rate-limiting enzyme and essential control point in the glycolytic pathway [3–5]. PFKFB3 and PFKFB4 are hypoxia responsive enzymes and overexpressed in different cancer tissues [6–9]. Overexpression of these enzymes is an obligatory factor of tumor cell glycolysis [10, 11]. Recently, it was shown that nuclear targeting of 6-phosphofructo-2-kinase-3 increases proliferation via cyclin-dependent kinase [12]. Several alternative splice variants for PFKFB3 and PFKFB4 were identified in tumor cells which possibly have significance in cancer growth [13–15].

Hexokinase catalyzes the first essential step of glucose metabolism, the conversion of glucose into glucose-6-phosphate. It was shown that expression of HK2 gene is insulin-responsive, and it is involved in the increased rate of glycolysis seen in rapidly growing cancer cells. Moreover, hexokinase 2 is a key mediator of aerobic glycolysis and promotes tumor growth in human glioblastoma multiforme, the most common malignant brain tumor [2]. In contrast to normal brain tissue, which expresses predominantly HK1, glioblastoma multiforme shows increased HK2 expression. Depletion of HK2, but not of HK1 or pyruvate kinase M2, in glioblastoma cells restored oxidative glucose metabolism and increased sensitivity to cell death inducers such as radiation and temozolomide [2]. Intracranial xenografts of HK2-depleted glioblastoma multiforme cells showed decreased proliferation and angiogenesis, but increased invasion, as well as a diminished expression of hypoxia inducible factor 1α and vascular endothelial growth factor. Activation of hexokinases is regulated by Akt/PKB-mediated phosphorylation. In addition to glucose metabolism, hexokinases have been implicated in antiapoptotic and cell survival signaling. Besides that, the induction of de novo lipid synthesis from glucose in prostate cancer cells by androgen requires transcriptional up-regulation of HK2 and PFKFB2, and phosphorylation of PFKFB2 generated by the PI3K/AKT signal pathway to supply the source for lipogenesis [16].

Thus, the hexokinase and family of PFKFB proteins participate not only in the control of glucose metabolism via glycolysis, but also in the regulation of the cell cycle, proliferation, apoptosis and invasiveness [1, 2, 8, 12].

The endoplasmic reticulum is a key organelle in the cellular response to ischemia, hypoxia, and some chemicals which activate a complex set of signaling pathways named the unfolded protein response. This adaptive response is activated upon the accumulation of misfolded proteins in the endoplasmic reticulum and is mediated by three endoplasmic reticulum-resident sensors named PERK (PRK-like ER kinase), IRE1/ERN1 (Inositol Requiring Enzyme-1/Endoplasmic Reticulum to the Nuclei-1) and ATF6 (Activating Transcription Factor 6), however, endoplasmic reticulum to the nuclei-1 is the dominant sensor [17–20]. Activation of the unfolded protein response tends to limit the *de novo* entry of proteins in to the endoplasmic reticulum and facilitate both the endoplasmic reticulum protein folding and degradation to adapt cells for survival or, alternatively, to enter cell death programs through endoplasmic reticulum-associated machineries [17, 18, 21]. It participates in early cellular response to the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum, occurring under both physiological and pathological conditions.

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Two distinct catalytic domains of the bifunctional signaling enzyme endoplasmic reticulum to the nuclei-1 were identified: a serine/threonine kinase and an endoribonuclease which contribute to ERN1 signalling. The ERN1-associated kinase activity autophosphorylates and dimerizes this enzyme, leading to the activation of its endoribonuclease domain, degradation of a specific subset of mRNA, and initiation of the pre-XBP1 (X-box binding protein 1) mRNA splicing [20–22]. Mature XBP1 mRNA splice variant encodes a transcription factor that has different C-terminus amino acid sequence and stimulates the expression of hundreds of unfolded protein response-specific genes [17, 21].

Moreover, the growing tumor requires the endoplasmic reticulum stress as well as hypoxia and ischemia, for own neovascularization and growth and the complete blockade of ERN1 signal transduction pathway has anti-tumor effects [22, 23]. The endoplasmic reticulum stress response-signalling pathway is linked to the neovascularization process, tumor growth and differentiation as well as cell death processes [23, 24]. Thus, the block of the main unfolded protein response sensor ERN1 is important at studying the role of ERN1 signalling pathways in tumor progression, especially in malignant gliomas; it is important in the development of a new understanding concerning molecular mechanisms of malignant tumors progression in relation to ischemia/hypoxia and it will help define the best targets for the design of specific inhibitors that could act as potent antitumor drugs. Gliomas are the most frequent primary brain neoplasms and represent a major challenge in cancer therapy as they are not easily accessible to current therapies.

The main goal of this work is to study the role of ERN1-signaling pathways in tumor progression by investigating the expression of hexokinase and PFKFB genes in glioma U87 cells with ERN1 loss of function under normal, hypoxic and ischemic (glucose or glutamine deprivation) conditions.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The glioma cell line U87 was obtained from ATCC (USA) and grown in high glucose (4,5 g/l) Dulbecco's modified Eagle's minimum essential medium (DMEM; Gibco, Invitrogen, 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 a 5% CO_2 incubator. In this work we used two sublines of this glioma cell line. One subline was obtained by selection of stable transfected clones with overexpression of vector, which was used for creation of dnERN1. This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of the effect of hypoxia and glutamine or glucose deprivations on the expression level of hexokinase and PFKFB genes. Second subline was obtained by selection of stable transfected clones with overexpression of ERN1 dominant/negative constructs (dnERN1) and has suppressed both protein kinase and endoribonuclease activities of this signaling enzyme [13]. The expression level of hexokinase and PFKFB genes in these cells was compared with cells, transfected by vector (control 1), but this subline was also used as control 2 for investigation the effect of hypoxia and glutamine or glucose deprivations on the expression level of hexokinase and PFKFB genes under blockade ERN1 function.

Hypoxic conditions were created in special incubator with 3% oxygen and 5% carbon dioxide levels; culture plates were exposed to these conditions for 16 hrs. For glucose or glutamine deprivation the growing medium in culture plates was replaced with a medium without glucose or without glutamine and thus exposed for 16 hrs.

The suppression level of ERN1 enzymatic activity in glioma cells that overexpress a dominant-negative construct of endoplasmic reticulum–nuclei-1 (dnERN1) was estimated by analysis of the expression of XBP1 alternative splice variant (XBP1**s**), a key transcription factor in ERN1 signaling, using cells treated by tunicamycin (10 mg/l during 2 hours).

RNA isolation. Total RNA was extracted from different tumor tissues and normal tissue counterparts as described [15, 25]. RNA pellets were washed with 75 % ethanol and dissolved in nuclease-free water.

Reverse transcription and quantitative PCR analysis. The expression levels of HK1, HK2, PFKFB3, PFKFB4 and alternative splice variants of PFKFB mRNA were measured in glioma cell line U87 and its subline with a deficiency of endoplasmic reticulum–nuclei-1 by quantitative polymerase chain reaction of complementary DNA (cDNA) using „Stratagene Mx 3000P cycler" (USA) and SYBRGreen Mix (AB gene, Great Britain). QuaniTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis as described previously [25]. Polymerase chain reaction was performed in triplicate.

For amplification of hexokinase 1 (HK1) cDNA we used forward (5′-CTTATTT-GAAGGGCGGATCA-3′ and reverse (5′-GGAATACTGTGGGTGCGTCT-3′) primers**.** The nucleotide sequences of these primers correspond to sequences 1055–1074 and 1373–1354 of human HK1 cDNA (GenBank accession number NM_000188). The size of amplified fragment is 319 bp.

The amplification of hexokinase 2 (HK2) cDNA was performed using forward primer (5′-TCTATGCCATCCCTGAGGAC-3′) and reverse primer (5′-TCTCTGCCTTCCACTC-CACT-3′). These oligonucleotides correspond to sequences 2258–2267 and 2477–2458 of human HK2 cDNA (GenBank accession number NM_000189). The size of amplified fragment is 220 bp.

The amplification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 cDNA for real time RCR analysis was performed using two oligonucleotide primers: forward – 5′-CTTGTCGCTGATCAAGGTGA-3′ and reverse – 5′-TTCTGCTCCTCCACGAACTT-3′. The nucleotide sequences of these primers correspond to sequences 1011–1030 and 1253–1234 of human PFKFB3 cDNA (GenBank accession number NM_004566). The size of amplified fragment is 243 bp.

Two other primers were used for real time RCR analysis of the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 cDNA: forward – 5′-GGAGTTCAAT-GTTGGCCAGT-3′ and reverse – 5′-TCAGGATCCACACAGATGGA-3′. The nucleotide sequences of these primers correspond to sequences 230–249 and 505–486 of human PFKFB4 cDNA (GenBank accession number NM_004567). The size of amplified fragment is 276 bp.

The amplification of alternative splice variant of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 cDNA with deletion of 174 bp fragment for real time RCR analysis was performed using two oligonucleotide primers: forward – 5′-CGGGAGAG-GTCAGAGAACAT-3′ and reverse – 5′-CTCTCCGGAAGAAGTCCTCA-3′. The nucleotide sequences of these primers correspond to sequences 34–53 and 197–178 of this human PFKFB3 alternative splice variant (GenBank accession number AJ272440). The size of amplified fragment is 164 bp.

Two other primers were used for real time RCR analysis of the expression of alternative splice variant of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 cDNA with deletion of 21 bp fragment: forward – 5′-AGAGCGAGCTCAACCTCAAG-3′ and reverse – 5′-ACGCCCTTCCACTGTTCATA-3′. The nucleotide sequences of these

primers correspond to sequences 889–908 and 1090–1071 of this human PFKFB4 alternative splice variant (GenBank accession number AY707863). The size of amplified fragment is 212 bp.

The amplification of beta-actin cDNA was performed using forward – 5′-CGTAC-CACTGGCATCGTGAT-3′ and reverse – 5′-GTGTTGGCGTACAGGTCTTT-3′ primers. The expression of beta-actin mRNA was used as control of analyzed RNA quantity. The primers were received from „Sigma" (USA).

The amplification of XBP1 cDNA was performed using HotStarTaq Master Mix Kit („QIAGEN", Germany), „MasterCycler Personal" („Eppendorf", Germany) and primers: forward – 5′-GGAGTTAAGACAGCGCTTGG-3′ and reverse – 5′-TCACCCCTCCAG-AACATCTC-3′. The nucleotide sequences of these primers correspond to sequences 441–460 and 608–589 of XBP1 mRNA (GenBank accession number NM_005080). The size of amplified fragment is 168 bp for non spliced variant and 142 bp for alternative splice variant (XBP1s).

An analysis of quantitative PCR was performed using special computer program „Differential expression calculator" and statistic analysis – in Excel program. The amplified DNA fragments were analyzed on a 2% agarose gel and that visualized by 5x Sight DNA Stain (EUROMEDEA).

RESULTS

In this study, we have used human glioma cell line U87 and genetically modified variant of these cells (deficient in the endoplasmic reticulum stress signaling enzyme ERN1) to investigate the expression of different genes that encode hexokinase and PFKFB proteins as well as the involvement of the endoplasmic reticulum stress signaling system in the effect of hypoxia and glutamine or glucose deprivation on the expression of these genes. The level of suppression of the enzymatic activity of ERN1 was estimated by analysis of the expression of XBP1 and its splice variant, shorter isoform (XBP1s), a key transcription factor in ERN1 signaling, in U87 glioma cells that overexpress a dominant-negative construct of endoplasmic reticulum–nuclei-1 (dnERN1) compared with the control glioma cells transfected with a vector. As shown in Fig. 1, inductor of endoplasmic reticulum stress, tunicamycin (10 mg/l), strongly induces alternative splicing of XBP1 only in control glioma cells, while having no effect on this process in cells transfected with dnERN1.

We have found that HK1, HK2, PFKFB3 and PFKFB4 genes are expressed in the human glioma cell line U87 and the levels of their expression mainly depend upon the
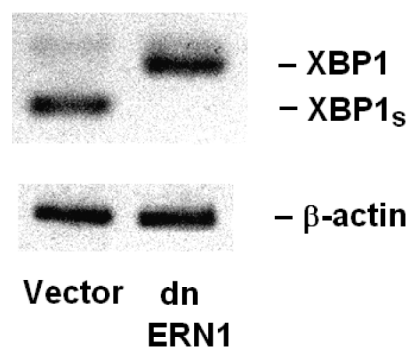

Fig. 1. Effect of tunicamicin (10 mg/l) on the expression of transcription factor XBP1 and its alternative splice variant (XBP1s) mRNA in glioma cell line U87 stable transfected with vector, which was used as control, and its subline with blockade of signaling enzyme endoplasmic reticulum–nuclei-1 (ERN1) stable transfected with dnERN1

Рис. 1. Вплив тунікаміцину (10 мг/л) на рівень експресії мРНК транскрипційного фактора XBP1 та його альтернативного сплайс-варіанта (XBP1s) у клітинах гліоми лінії U87, стабільно трансфекованих вектором, які слугували контролем, та в сублінії цих клітин із пригніченою активністю сигнального ензиму ендоплазматичний ретикулум–ядро-1 (ERN1), стабільно трансфекованих dnERN1

function of the ERN1 signaling enzyme. As shown in Fig. 2, the expression level of HK1 mRNA in glioma cells, deficient in the signaling enzyme ERN1, does not change significantly compared with the control 1 value. However, HK2, PFKFB3 and PFKFB4 mRNA expression levels are increased in glioma cells with none functional ERN1 signalling enzyme (Fig. 2).
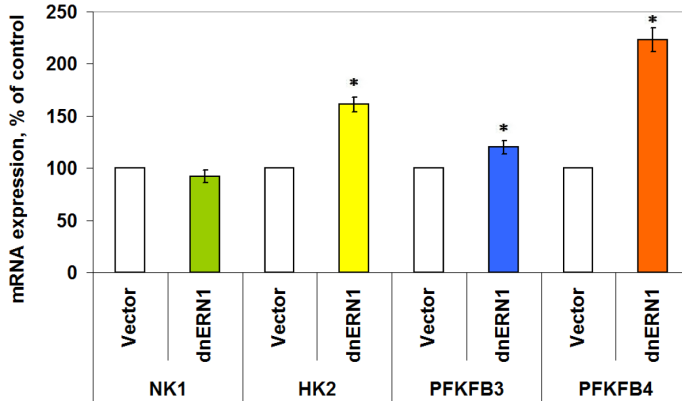

Fig. 2. Effect of blockade of signaling enzyme ERN1 on the expression of hexokinase 1 (HK1), hexokinase 2 (HK2), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) and PFKFB4 mRNA in glioma cell line U87 (Vector) and its subline with ERN1 deficiency (dnERN1) measured by quantitative polymerase chain reaction. Values of HK1, HK2, PFKFB3 and PFKFB4 mRNA expressions were normalized to the expression of beta-actin mRNA and represent as percent of control (100%); *n* = 4; * – P < 0,05 as compared to control

Рис. 2. Вплив блокади сигнального ензиму ERN1 на рівень експресії мРНК гексокінази 1 (НК1), гексокінази 2 (НК2), 6-фосфофрукто-2-кінази/фруктозо-2,6-бісфосфатази-3 (PFKFB3) та PFKFB4 у клітинах гліоми лінії U87 (Vector = контроль) та сублінії цих клітин з дефіцитом ERN1 (dnERN1), визначений методом кількісної полімеразної ланцюгової реакції. Значення рівнів експресії мРНК НК1, НК2, PFKFB3 та PFKFB4 нормалізували по експресії мРНК бета-актину і виражали у відсотках щодо контролю, прийнятого за 100%; *n* = 4; * – P < 0,05 порівняно з контролем

Exposure of cells to hypoxia for 16 hrs leads to an increase of HK1 mRNA expression level (+35%) in control glioma cells, as compared to control 1, but in glioma cells with suppressed function of the ERN1 signaling enzyme hypoxia had no effect on the HK1 mRNA expression level, as compared to control 2 (Fig. 3). Exposure of cells to glucose deprivation condition also leads to an increase of HK1 mRNA expression level, but in glioma cells with suppressed function of the ERN1 signaling enzyme effect of glucose deprivation was slightly less: +31% in control glioma cells, as compared to control 1, and +24% in cells with a none functional ERN1, as compared to control 2. However, no significant changes were found in both glioma cell types under glutamine deprivation condition (Fig. 3).

At the same time, effect of hypoxia on the level of hexokinase 2 mRNA expression was significantly greater in both tested cell types, compared with the HK1 values. Thus, HK2 mRNA expression level was increased by hypoxia more than in 11 fold in control glioma cells, as compared to control 1, and only in 5 fold in ERN1 loss of function cells, as compared to control 2 (Fig. 4). Exposure of glioma cells to glutamine or glucose deprivation conditions leads to an increase of HK2 mRNA expression level in control cells: +51% and +120%, respectively, as compared to control 1. In the glioma cells with a suppressed function of the ERN1 signaling enzyme, however, the expression level of this

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mRNA decreased (-40%) in glutamine deprivation condition and did not changed significantly in glucose deprivation condition, as compared to control 2 (Fig. 4).

It was also shown that the exposure of glioma cells to hypoxia leads to an increase of PFKFB3 and PFKFB4 mRNA expression levels, but in glioma cells with suppressed function of the signaling enzyme ERN1 effect of hypoxia was significantly less: +43% and 246%, respectively, in control glioma cells, as compared to control 1, and +21% and 43%, respectively, in cells, as compared to control 2 (Fig. 5 and 6). Thus, hypoxia responsibility of PFKFB4 mRNA expression was much higher than PFKFB3. At the same time, no significant changes were found in the expression level of PFKFB3 mRNA in none-functional ERN1 glioma cells both in glutamine and glucose deprivation conditions, but in control glioma cells the expression level of this mRNA is decreased (-21%) in glucose deprivation condition, only (Fig. 5). Moreover, the expression level of PFBFB4 mRNA increases in glucose deprivation conditions in control glioma cells (+24%), but decreases in glioma cells with the suppressed function of ERN1 enzyme (- 42%) (Fig. 6). As shown in Fig. 6, in glutamine deprivation conditions no significant changes were found in the expression levels of PFKFB4 mRNA in control glioma cells, however, in cells with none-functional ERN1 expression levels of this mRNA decreased (-29%).

Results of this investigation of the expression of alternative splice variants of PFKFB3 and PFKFB4 mRNA are shown in Fig. 7 and 8. It was also shown that expression levels of alternative splice variants of PFKFB3 and PFKFB4 mRNA change at all used experimental conditions in a similar to the basic variants manner. Thus, the expression level of both of the alternative splice variant of PFKFB3 and PFKFB4 mRNA increase in glioma cells with a none-functional ERN1 signalling enzyme, but more strongly the increase was shown for the PFKFB4 splice variant. Exposure of glioma cells to hypoxia leads to an increase of the
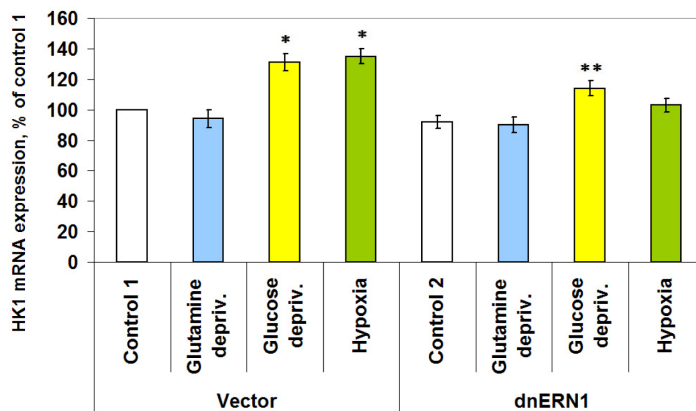

Fig. 3. Effect of hypoxia and glucose or glutamine deprivation on the expression of hexokinase 1 (HK1) mRNA in glioma cell line U87 (Vector = control) and its subline with a deficiency of the signaling enzyme ERN1 (dnERN1) measured by quantitative polymerase chain reaction. Values of HK1 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent for control (100%); n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2

Рис. 3. Вплив гіпоксії та дефіциту глюкози або глютаміну на рівень експресії мРНК гексокінази 1 (НК1) у клітинах гліоми лінії U87 (Vector = контроль) та сублінії цих клітин з дефіцитом ERN1 (dnERN1), визначений методом кількісної полімеразної ланцюгової реакції. Значення рівня експресії мРНК НК1 нормалізували по експресії мРНК бета-актину і виражали у відсотках щодо контролю, прийнятого за 100%; n = 4; * – P < 0,05 порівняно з контролем1; ** – P < 0,05 порівняно з контролем 2
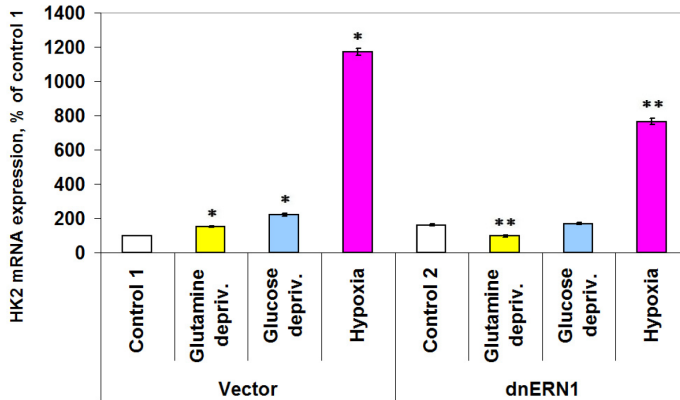
Fig. 4. Effect of hypoxia and glucose or glutamine deprivation on the expression of hexokinase 2 (HK2) mRNA in glioma cell line U87 (Vector = control) and its subline with loss of the signaling enzyme ERN1 function (dnERN1) measured by quantitative polymerase chain reaction. Values of HK2 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent for control (100%); n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2

Рис. 4. Вплив гіпоксії та дефіциту глюкози або глютаміну на рівень експресії мРНК гексокінази 2 (HK2) в клітинах гліоми лінії U87 (Vector = контроль) та субліній цих клітин без функції сигнального ензиму ERN1 (dnERN1), визначений методом кількісної полімеразної ланцюгової реакції. Значення рівня експресії мРНК HK2 нормалізували по експресії мРНК бета-актину і виражали у відсотках щодо контролю, прийнятого за 100%; n = 4; * – P < 0,05 порівняно з контролем 1; ** – P < 0,05 порівняно з контролем 2
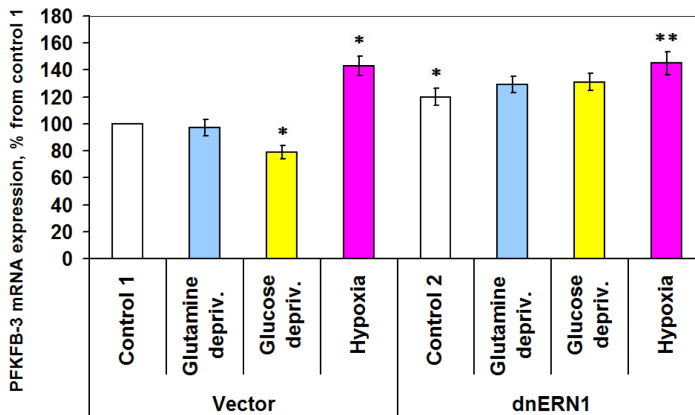

Fig. 5. Effect of hypoxia and glucose or glutamine deprivation on the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) mRNA in glioma cell line U87 (Vector = control) and its subline with loss of the signaling enzyme ERN1 function (dnERN1) measured by quantitative polymerase chain reaction. Values of PFKFB3 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent for control (100 %); n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2

Рис. 5. Вплив гіпоксії та дефіциту глюкози або глютаміну на рівень експресії мРНК 6-фосфофрукто-2-кінази/фруктозо-2,6-бісфосфатази-3 (PFKFB3) у клітинах гліоми лінії U87 (Vector = контроль) та субліній цих клітин без функції сигнального ензиму ERN1 (dnERN1), визначений методом кількісної полімеразної ланцюгової реакції. Значення рівня експресії мРНК PFKFB3 нормалізували по експресії мРНК бета-актину і виражали у відсотках щодо контролю, прийнятого за 100 %; n = 4; * – P < 0,05 порівняно з контролем 1; ** – P < 0,05 порівняно з контролем 2
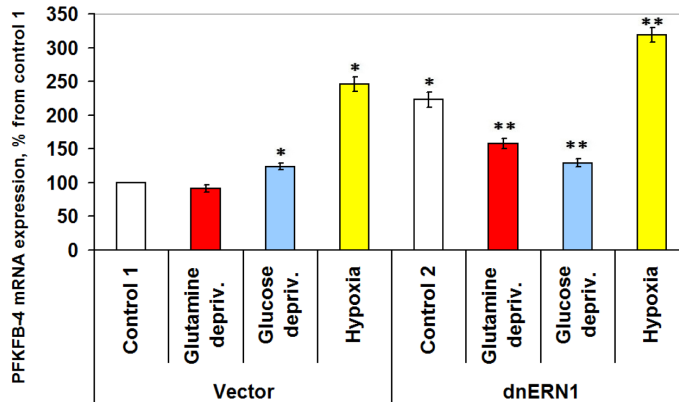
Fig. 6. Effect of hypoxia and glucose or glutamine deprivation on the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 (PFKFB4) mRNA in glioma cell line U87 (Vector = control) and its subline with loss of the signaling enzyme ERN1 function (dnERN1) measured by quantitative polymerase chain reaction. Values of PFKFB4 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent for control (100 %); n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2

Рис. 6. Вплив гіпоксії та дефіциту глюкози або глютаміну на рівень експресії мРНК 6-фосфофрукто-2-кінази/фруктозо-2,6-бісфосфатази-4 (PFKFB4) у клітинах гліоми лінії U87 (Vector = контроль) та субліній цих клітин без функції сигнального ензиму ERN1 (dnERN1), визначений методом кількісної полімеразної ланцюгової реакції. Значення рівня експресії мРНК PFKFB4 нормалізували по експресії мРНК бета-актину і виражали у відсотках щодо контролю, прийнятого за 100%; n = 4; * – P < 0,05 порівняно з контролем 1; ** – P < 0,05 порівняно з контролем 2
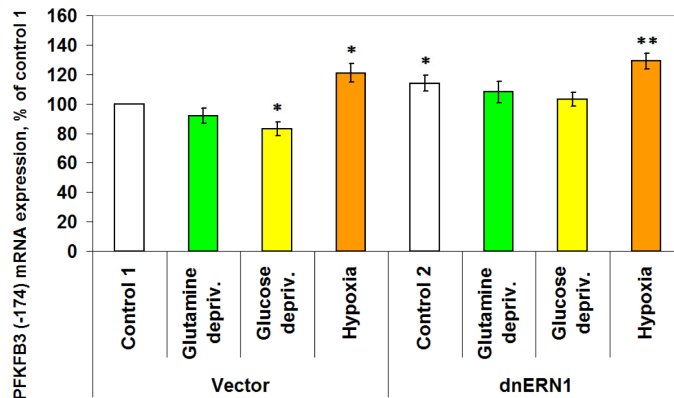

Fig. 7. Effect of hypoxia and glucose or glutamine deprivation on the expression of alternative splice variant of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) mRNA with deletion of 174 bases (-174) in glioma cell line U87 (Vector = control) and its subline with loss of the signaling enzyme ERN1 function (dnERN1) measured by quantitative polymerase chain reaction. Values of splice variant of PFKFB3 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent for control (100%); n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2

Рис. 7. Вплив гіпоксії та дефіциту глюкози або глютаміну на рівень експресії мРНК альтернативного сплайс-варіанта 6-фосфофрукто-2-кінази/фруктозо-2,6-бісфосфатази-3 (PFKFB3) з делецією 174 основ (-174) у клітинах гліоми лінії U87 (Vector = контроль) та субліній цих клітин без функції сигнального ензиму ERN1 (dnERN1), визначений методом кількісної полімеразної ланцюгової реакції. Значення рівня експресії мРНК сплайс-варіанта PFKFB3 нормалізували по експресії мРНК бета-актину і виражали у відсотках щодо контролю, прийнятого за 100%; n = 4; * – P < 0,05 порівняно з контролем 1; ** – P < 0,05 порівняно з контролем 2
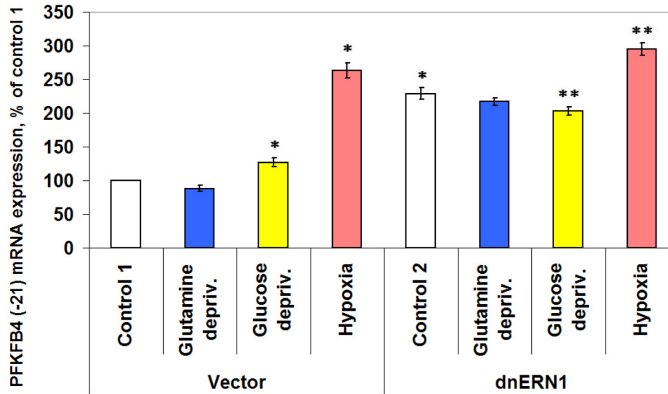
Fig. 8. Effect of hypoxia and glucose or glutamine deprivation on the expression of alternative splice variant of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 (PFKFB4) mRNA with deletion of 21 bases (-21) in glioma cell line U87 (Vector = control) and its subline with loss of the signaling enzyme ERN1 function (dnERN1) measured by quantitative polymerase chain reaction. Values of splice variant of PFKFB4 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent for control (100%); n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2

Рис. 8. Вплив гіпоксії та дефіциту глюкози або глютаміну на рівень експресії мРНК альтернативного сплайс-варіанта 6-фосфофрукто-2-кінази/фруктозо-2,6-бісфосфатази-4 (PFKFB4) з делецією 21 основи (-21) у клітинах гліоми лінії U87 (Vector = контроль) та субліній цих клітин без функції сигнального ензиму ERN1 (dnERN1), визначений методом кількісної полімеразної ланцюгової реакції. Значення рівня експресії мРНК сплайс-варіанта PFKFB4 нормалізували по експресії мРНК бета-актину і виражали у відсотках щодо контролю, прийнятого за 100%; n = 4; * – P < 0,05 порівняно з контролем 1; ** – P < 0,05 порівняно з контролем 2

expression of alternative splice variants of PFKFB3 and PFKFB4 mRNA in both cell types, but in glioma cells with a suppressed function of the ERN1 signaling enzyme the effect under hypoxia was significantly less: +21% and 263%, respectively, in control glioma cells, as compared to control 1, and +13% and 29%, respectively, in ERN1 loss of function cells, as compared to control 2 (fig. 7 and 8). Thus, hypoxia responsibility of the expression of the splice variant of PFKFB4 mRNA was much higher than PFKFB3. At the same time, no significant changes were found in the expression level of splice variants of PFKFB3 and PFKFB4 mRNA in both glioma cell types in glutamine deprivation conditions. In glucose deprivation condition, however, we observed an increase of the expression level of PFKFB4 mRNA splice variant in control glioma cells, only (+27%).

DISCUSSION

Bifunctional transmembrane signaling enzyme endoplasmic reticulum–nuclei-1 is a major proximal sensor of the unfolded protein response and participates in the early cellular response to the accumulation of misfolded proteins in the endoplasmic reticulum [23, 24]. It is known that the endoplasmic reticulum stress sensing and signal transduction pathways are linked to the neovascularization process, tumor growth, and cellular death processes [23]. Moreover, growing tumor requires endoplasmic reticulum stress, as well as ischemia and hypoxia both of which initiate endoplasmic reticulum stress. It is important for tumor neovascularization and growth as well as for inhibition of apoptotic processes [22, 24]. In this work, we have studied the expression of several genes that encode hexokinase and PFKFB proteins in glioma cells with ERN1 knock-

down for the purpose of evaluating the dependence of these genes upon the ERN1 signaling enzyme function. Results of this investigation clearly demonstrate that the expression levels of hexokinase-2, PFKFB3 and PFKFB4, which enhance glucose metabolism via glycolysis as well as increase tumor growth and invasiveness, increase in glioma cells with a none-functional ERN1 signaling enzyme. This data does not completely correlate with the anti-tumour effects of this ERN1 blockade, but does correlate with the enhanced invasiveness [22, 24, 25]. It is well known that hexokinase-2 and PFKFB participate in the control of glycolysis, proliferation, and invasiveness and are components of the endoplasmic reticulum stress system, its activity, however, is controlled by different mechanisms, not only by the ERN1 signaling system [1, 2, 10, 23]. Moreover, similar results were received with alternative splice variants of PFKFB3 and PFKFB4 which have enhanced kinase activity. It is possible that this type of alternative splicing does not have specialized function in IRN1 signaling system in glioma cells.

In this study we have examined the effect of hypoxia on the expression of two hexokinase genes and two PFKFB genes and shown that different hexokinase and PFKFB genes have significant difference in hypoxia responsibility. More strong induction of the expression level was shown for HK2 and PFKFB4 mRNA compared with the HK1 and PFKFB3 values. These results correlate with the data concerning biological significance of different hexokinases and PFKFB in tumor growth [2, 8, 9, 13]. We have also shown that blockade of the activity of the signaling enzyme endoplasmic reticulum–nuclei-1 leads to a significant reduction of hypoxic effect on the expression of these genes in glioma cells. This data demonstrated that effect of hypoxia on the expression of hexokinase and PFKFB genes is mediated by the ERN1 signaling system, at least in part. Investigation of XBP1 mRNA expression clearly demonstrated that blockade of the activity of ERN1 signaling enzyme by dnERN1 construct completely suppresses the formation of alternative splice variant of XBP1 and the main biological function of ERN1.

In this study we have also shown that there is a difference in sensitivity of hexokinase and PFKFB genes to glutamine or glucose deprivation conditions both in control glioma cells and in cells with a none-functional ERN1. Thus, the expression levels of HK1 and PFKFB3 genes are not responsible to glutamine deprivation condition in both used cell types, but expression levels of HK2 and PFKFB4 are significantly suppressed at this experimental condition only in glioma cells with a none-functional ERN. It is possible that the increase of HK2 and PFKFB4 expression in cells without activity of ERN1 enzyme is sensitive to the glutamine deprivation.

Thus, results of this investigation demonstrate that the expression of genes encoding hexokinase and PFKFB proteins in glioma cells is mainly regulated by hypoxia, glutamine or glucose deprivation and depends upon the activity of signaling enzyme endoplasmic reticulum–nuclei-1. However, the detailed molecular mechanisms the regulation of hexokinase and PFKFB genes as well as its alternative splicing by ERN1 signaling system under hypoxic and nutrient deprivation conditions are complex and warrants further study.

CONCLUSIONS

The major finding reported here is that the expression of HK2, PFKFB3 and PFKFB4 genes, which encode the key regulatory enzymes, are dependent on the function of ERN1 signaling enzyme both in normal and hypoxic or nutrient deprivation conditions. This data clearly demonstrated that hexokinase and PFKFB proteins are members of ERN1 signaling system associated with endoplasmic reticulum stress.

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ЕКСПРЕСІЯ ГЕНІВ ГЕКСОКІНАЗИ ТА 6-ФОСФОФРУКТО-2-КІНАЗИ/ФРУКТОЗО-2,6-БІСФОСФАТАЗИ В U87 КЛІТИНАХ ГЛІОМИ З ПРИГНІЧЕНОЮ ФУНКЦІЄЮ ERN1: ЕФЕКТ ГІПОКСІЇ ТА ДЕФІЦИТУ ГЛЮТАМІНУ АБО ГЛЮКОЗИ

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Стрес ендоплазматичного ретикулуму, як і гіпоксія та ішемія, є важливим фактором неоваскуляризації та росту злоякісних пухлин. Пухлинні клітини використовують переважно гліколіз для забезпечення своїх підвищених енергетичних та біосинтетичних потреб. Високий рівень метаболізму глюкози у клітинах пухлин забезпечується комбінацією залежних від гіпоксії транскрипційних факторів, активацією онкогенних протеїнів і втратою функції пухлинних супресорів; частково він реалізується шляхом активації родини регуляторних 6-фосфофрукто-2-кіназ/фруктозо-2,6-бісфосфатаз (PFKFB) та гексокінази 2. Ми дослідили ефект гіпоксії та ішемії на експресію генів PFKFB та гексокіназ у клітинах гліоми лінії U87 з пригніченою функцією сенсорно-сигнального ензиму ERN1 (від ендоплазматичного ретикулуму до ядра-1). Показано, що втрата функції сигнального ензиму ERN1 приводить до збільшення рівня експресії мРНК HK1, HK2, PFKFB3 та PFKFB4. Більше того, рівень експресії всіх досліджених генів збільшується за умов гіпоксії в контрольних та в дефіцитних за геном ERN1 клітинах гліоми; хоча пригнічення функції ERN1 знижувало ефект гіпоксії. Крім того, було показано, що HK2 та PFKFB4 є більш чутливими до гіпоксії порівняно з HK1 та PFKFB3. За умов дефіциту глютаміну чи глюкози рівень експресії цих генів змінювався по-різному і здебільшого залежав від функції ERN1. Рівень експресії альтернативних сплайс-варіантів мРНК PFKFB3 та PFKFB4 змінювався за цих умов експерименту подібно до основного варіанта. Таким чином, експресія генів гексокіназ та PFKFB переважно залежить від функції сигнального ензиму ERN1 за нормальних умов, гіпоксії та ішемії.

ЭКСПРЕССИЯ ГЕНОВ ГЕКСОКИНАЗЫ И 6-ФОСФОФРУКТО-2-КИНАЗЫ/ФРУКТОЗО-2,6-БИСФОСФАТАЗЫ В U87 КЛЕТКАХ ГЛИОМЫ С УГНЕТЕННОЙ ФУНКЦИЕЙ ERN1: ЭФФЕКТ ГИПОКСИИ И ДЕФИЦИТА ГЛЮТАМИНА ИЛИ ГЛЮКОЗЫ

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Стресс эндоплазматического ретикулума, как и гипоксия и ишемия, является важным фактором неоваскуляризации и роста злокачественных опухолей. Опухолевые клетки используют преимущественно гликолиз для обеспечения своих повышенных энергетических и биосинтетических потребностей. Высокий уровень метаболизма глюкозы в клетках опухолей обеспечивается комбинацией зависимых от гипоксии транскрипционных факторов, активацией онкогенных протеинов и потерей функции супрессоров опухолей и частично реализуются путем активации семейства регуляторных 6-фосфофрукто-2-киназ/фруктозо-2,6-бисфосфатаз (PFKFB) и гексокиназы-2. Мы изучили эффект гипоксии и ишемии на экспрессию генов PFKFB и гексокиназ в клетках глиомы линии U87 с угнетенной функцией сенсорно-сигнального энзима ERN1 (от эндоплазматического ретикулума до ядра-1). Показано, что потеря функции сигнального энзима ERN1 приводит к увеличению уровня экспрессии мРНК НК1, НК2, PFKFB3 и PFKFB4. Более того, уровень экспрессии всех изученных генов увеличивается при гипоксии в контрольных и дефицитных по гену ERN1 клетках глиомы; хотя угнетение функции ERN1 снижало эффект гипоксии. Кроме того, было показано, что НК2 и PFKFB4 являются более чувствительными к гипоксии по сравнению с НК1 и PFKFB3. При дефиците глютамина или глюкозы уровень экспрессии этих генов изменялся по-разному и преимущественно зависел от функции ERN1. Уровень экспрессии альтернативных сплайс-вариантов мРНК PFKFB3 и PFKFB4 изменялся в этих условиях эксперимента подобно основному варианту. Таким образом, экспрессия генов гексокиназ и PFKFB преимущественно зависит от функции сигнального энзима ERN1 как в нормальных условиях, так и в условиях гипоксии и ишемии.

Ключевые слова*:* экспрессия мРНК, НК1, НК2, PFKFB3, PFKFB4, клетки глиомы, угнетение ERN1, гипоксия, дефицит глюкозы или глютамина.