Mitochondria are key organelles in mammalian cells in responsible for a number of cellular functions including cell survival and energy metabolism and play an important role in the regulation of tumor growth and apoptosis through numerous metabolic pathways [1–3]. The functional activity of mitochondria is controlled through numerous nuclear-encoded mitochondrial proteins and most of these factors and enzymes are responsible for metabolic reprogramming of mitochondria in various diseases including cancer [4–7]. Thus, the level in of mitochondria associated apoptosis inducing factor 1 (AIFM1) is significantly higher in carcinomas than in normal tissues [1]. Moreover, the expression of some nuclear genes encoded mitochondrial factors and enzymes is responsible to glucose and glutamine deprivation as well as to hypoxia [6, 8–10]. Therefore, expression of ENDOG (endonuclease G), POLG (DNA directed polymerase gamma), TSFM (Ts mitochondrial translational elongation factor), and MTIF2 (mitochondrial translational initiation factor 2) genes encode mitochondrial proteins, which are related to the control of mitochondrial genome function as well as to cell proliferation, is affected by glucose or glutamine deprivation and hypoxia preferentially in diverse ways [6, 9, 10]. Furthermore, ENDOG regulates an integral network of apoptotic endonucleases, which appear to act simultaneously before cell death by destroying the host cell DNA [11, 12]. It is well known that endoplasmic
reticulum stress is a necessary component of malignant tumors growth including malignant gliomas, which are highly aggressive tumors, and inhibition of IRE1 (inositol requiring enzyme 1), which represents a dominant signaling pathway of the unfolded protein response, significantly decreases the glioma growth [13–16]. Previously was shown that inhibition of IRE1 (inositol requiring enzyme 1), which represents a dominant signaling pathway of the unfolded protein response, modifies regulation of ENDOG, POLG, TSFM, and MTIF2 genes expression by glucose or glutamine deprivations[10].

Mitochondrial NAD-dependent malic enzyme (ME2) that catalyzes the oxidative decarboxylation of malate to pyruvate is a target of TP53 depletion of this enzyme induces erythroid differentiation in human erythroleukemia cells [17, 18]. Furthermore, malic enzyme 2 is highly expressed in many solid tumors and its knockdown inhibits cell proliferation and induces cell death as well as suppresses lung tumor growth in vivo [19]. Recently was shown that embolic acid, a natural compound, can specifically inhibit the enzymatic activity of mitochondrial NADP⁺-dependent malic enzyme and induce cellular senescence [20]. The malate-aspartate shuttle is indispensable for the net transfer of cytosolic NADH into mitochondria to maintain a high rate of glycolysis and to support rapid tumor cell growth [21]. It is operated by two pairs of enzymes that localize to the mitochondria and cytoplasm, glutamate oxaloacetate transaminases (GOT), and malate dehydrogenases (MDH). Acetylation of mitochondrial GOT2 enhances the association between GOT2 and MDH2 and promotes the net transfer of cytosolic NADH into mitochondria and changes the mitochondrial NADH/NAD⁺ redox state to support ATP production. Moreover, GOT2 acetylation promotes pancreatic cell proliferation and tumor growth in vivo [21]. Recently was shown that LW6, inhibitor of MDH2, reduces cell proliferation and apoptosis [22].

Succinate dehydrogenase (SDH) complex is specifically involved in the oxidation of succinate, carries electrons from FADH to CoQ and is composed of four subunits. The iron-sulfur subunit (SDHB) is highly conserved and contains three cysteine-rich clusters which may comprise the iron-sulfur centers of the enzyme [23]. The oncogenic role of this enzyme complex has only recently been recognized and the complex is currently considered an important oncogenic signaling pathway with tumor suppressor properties [24, 25]. SDHB, as a central core subunit, responsible for the integrity of the SDH complex, the expression of SDHB is lost in all SDH-deficient neoplasms irrespective of the specific SDH subunit affected by a genetic mutation in addition to concurrent loss of the subunit specifically affected by genetic alteration [23, 26].

The mitochondrial NADP⁺-dependent isocitrate dehydrogenase (IDH2) catalyzes the conversion of isocitrate to α-ketoglutarate and concomitantly produce reduced NADPH from NADP⁺. Mutations in the genes encoding IDH1 and IDH2 have recently been found in a variety of human cancers, most commonly glioma, and acute myeloid leukemia [27]. Expression of mutant IDH impairs cellular differentiation in various cell lineages and promotes tumor development in cooperation with other cancer genes, including the TET family of 5’-methylcytosine hydroxylases, which catalyze a key step in the removal of DNA methylation, cohesin and CCCTC-binding factor [27, 28]. Moreover, IDH2 mutations promote glioma genesis by disrupting chromosomal topology and allowing aberrant regulatory interactions that induce oncogene expression [28]. Recently was shown that IDH2 plays an essential role protecting cells against oxidative stress-induced damages and that a deficiency in this enzyme leads to mitochondrial dysfunction and the production of reactive oxygen species in cancer cells [29].

Malignant gliomas are highly aggressive tumors with very poor prognosis and to date there is no efficient treatment available. The moderate efficacy of conventional clinical approaches therefore underlines the need for new therapeutic strategies. Glutamine and glucose are important substrates for glutaminolysis and glycolysis, which are important to glioma development and a more aggressive behaviour [30–32]. Recently was shown that glioblastoma proliferation under glutamine deprivation depends on glutamate-derived glutamine synthesis through glutamine synthetase activity, which fuels nucleotide biosynthesis and supports growth of glutamine-restricted glioblastoma [33, 34]. A better knowledge of tumor responses to glucose or glutamine deprivation conditions is required to elaborate therapeutical strategies of cell.
sensibilization, based on the blockade of survival mechanisms [10, 35, 36].

The aim of this study was investigating the effect of glucose or glutamine deprivation conditions on the expression of nuclear genes encoding mitochondrial enzymes ME2, MDH2, GOT2, SDHB, SDHD, and IDH2, which related to cell proliferation and apoptosis, in glioma cells in relation to inhibition of signaling enzyme IRE1.

Materials and Methods

Cell Lines and Culture Conditions. In this study we used two sublines of U87 glioma cells, which are growing 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), streptomycin (0.1 mg/ml; Gibco) and penicillin (100 units/ml; Gibco) at 37°C in a 5% CO2 incubator. One subline was obtained by selection of stable transfected clones with overexpression of vector (pcDNA3.1), which was used for creation of dominant-negative constructs (dnIRE1). This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of effects of glucose or glutamine deprivations on the expression level of MDH2, ME2, GOT2, SDHB, SDHD, and IDH2 genes. Second subline was obtained by selection of stable transfected clones with overexpression of dnIRE1 and has suppressed both protein kinase and endoribonuclease activities of this bifunctional sensing and signaling enzyme of endoplasmic reticulum stress. The expression level of studied nuclear genes encoded mitochondrial proteins in these cells was compared with cells, transfected by vector (control 1). The subline, which overexpress dnIRE1, was also used as control 2 for investigation the effect of glucose or glutamine deprivation conditions on the expression level of studied in cells with inhibited signaling enzyme IRE1 function. Clones were received by selection at 0.8 mg/ml geneticin (G418) and grown in the presence of this antibiotic at lower concentration (0.4 mg/ml). Glucose and glutamine deprivation conditions were created by changing the complete DMEM medium into culture plates on medium without glucose or glutamine (from Gibco) and plates were exposed to these conditions for 16 h.

The suppression level of IRE1 both enzymatic activity in glioma cells that overexpress a dominant-negative construct of inositol requiring enzyme-1 was estimated previously [37] by determining the expression level of XBP1 alternative splice variant (XBP1s), a key transcription factor in IRE1 signaling, using cells treated by tunicamycin (0.01 mg/ml during 2 hrs). Efficiency of XBP1s inhibition was 95%. Moreover, the proliferation rate of glioma cells with mutated IRE1 is decreased in 2 fold [38]. Thus, the blockade of both kinase and endoribonuclease activity of signaling enzyme IRE1 has significant effect on proliferation rate of glioma cells.

RNA isolation. Total RNA was extracted from glioma cells as previously described [39]. The RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water. For additional purification RNA samples were re-precipitated with 95% ethanol and re-dissolved again in nuclease-free water. RNA concentration and spectral characteristics were measured using NanoDrop Spectrophotometer.

Reverse transcription and quantitative PCR analysis. QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) was used for cDNA synthesis as described previously [37]. The expression level of MDH2, ME2, GOT2, SDHB, SDHD, and IDH2 mRNA were measured in glioma cell line U87 and its subline (clone 1C5) by real-time quantitative polymerase chain reaction using “7900 HT Fast Real-Time PCR System” (Applied Biosystems) or “Mx 3000P QPCR” (Stratagene, USA) and Absolute qPCR SYBRGreen Mix (Thermo Fisher Scientific, ABgene House, Epsom, Surrey, UK). Polymerase chain reaction was performed in triplicate.

The amplification of cDNA of the mitochondrial malate dehydrogenase 2 (MDH2; EC_number=“1.1.1.37”) was performed using forward primer (5’-CCTGTCTCAACACCATGCA-3’) and reverse primer (5’-GCCGAAGATTTGTGGGTT-3’). These oligonucleotides correspond to sequences 478 — 497 and 646 — 627 of human MDH2 cDNA (GenBank accession number NM_005918). The size of amplified fragment is 169 bp.

For amplification of the mitochondrial NAD+-dependent malic enzyme 2 (ME2; EC_number=“1.1.1.38), also known as pyruvic-malic carboxylase, cDNA we used next primers: forward 5’-TTCTCTGTAACACCATGCA-3’
and reverse 5′-TGGGCTTGCTTCAGGTTCT-3′. The nucleotide sequences of these primers correspond to sequences 1695–1714 and 1910–1891 of human ME2 cDNA (GenBank accession number NM_001161). The size of amplified fragment is 216 bp.

The amplification of the succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial (SDHB; EC_number=“1.3.5.1”), also known as succinate dehydrogenase complex iron sulfur subunit B, cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward — 5′- AGAAC- TGGACGGGCTCTAC-3′ and reverse — 5′- TGTTGCCAGCGGTATAGAGAG-3′. The nucleotide sequences of these primers correspond to sequences 684–703 and 884–865 of human SDHB cDNA (GenBank accession number NM_002396). The size of amplified fragment is 201 bp.

For amplification of the succinate dehydrogenase [ubiquinone] cytochrome b small subunit, mitochondrial SDHD, also known as succinate-ubiquinone reductase membrane anchor subunit, cDNA we used forward 5′- ATACACTGTCACCGAGCCA-3′ and reverse 5′-CCCCAGTGACCATGAAGAGT-3′ primers. The nucleotide sequences of these primers correspond to sequences 229–248 and 401–382 of human SDHD cDNA (GenBank accession number NM_001101). The size of amplified fragment is 173 bp.

The amplification of the glutamic-oxaloacetic transaminase 2, mitochondrial (GOT2; EC_number=“2.6.1.1”), also known as kynurenine aminotransferase IV (KAT4) and aspartate aminotransferase, mitochondrial (mitAAT), cDNA into real time RCR analysis was performed using two oligonucleotides primers: forward — 5′- ATACACTTTGTCACCGAGCCA-3′ and reverse — 5′- CCCCAGTGACCATGAAGAGT-3′ primers. The nucleotide sequences of these primers correspond to sequences 461–480 and 707–688 of human GOT2 cDNA (GenBank accession number NM_002168). The size of amplified fragment is 233 bp.

The amplification of the beta-actin (ACTB) cDNA was performed using forward — 5′- GGACTTCGAGCAAGAGATGG-3′ and reverse — 5′- AGCAGTGTGTCGCGTACAG -3′ primers. These primers nucleotide sequences correspond to 747–766 and 980–961 of human ACTB cDNA (GenBank accession number NM_001101). The size of amplified fragment is 234 bp. The expression of beta-actin mRNA was used as control of analyzed RNA quantity.

The primers were received from Sigma-Aldrich (St. Louis, MO, USA). The quality of amplification products was analyzed by melting curves and by electrophoresis using 2% agarose gel. An analysis of quantitative PCR was performed using special computer program “Differential Expression Calculator”. The values of ME2, MDH2, IDH2, SDHB, SDHD, and GOT2 mRNA expressions were normalized to the expression of beta-actin mRNA and represented as percent of control 1 (100%).

Statistical analysis. All values are expressed as mean ± SEM from triplicate measurements performed in 4 independent experiments. Statistical analysis was performed according to Student’s t-test using Excel program as described previously [40].

Results and Discussion

We have studied the effect of glucose or glutamine deprivation conditions on the expression of nuclear genes encoded mitochondrial proteins ME2, MDH2, GOT2, SDHB, SDHD, and IDH2 in two sublines of U87 glioma cells in relation to inhibition of IRE1 signaling enzyme, which is a major component of the unfolded protein response. It was shown that in control glioma cells (transfected by empty vector) glutamine deprivation condition significantly down-regulates (−24%) the expression of nuclear gene for mitochondrial malic enzyme 2, that catalyzes the oxidative decarboxylation of malate to pyruvate (Fig. 1). Moreover, in cells without functional activity of IRE1 signaling enzyme the expression of this gene is down-regulated (−60%) upon complete DMEM and is resistant to glutamine deprivation. At the same time, glucose deprivation condition does not change significantly the expression of ME2 gene in control glioma cells, but inhibition of IRE1 signaling enzyme function leads to
strong up-regulation of ME2 gene expression upon glucose deprivation (+60%). Thus, inhibition of IRE1 signaling enzyme function in U87 glioma cells by dnIRE1 removes the sensitivity of the expression of ME2 gene to glucose deprivation condition and introduces sensitivity of this gene expression to glucose deprivation condition (Fig. 1).

As shown in Fig. 2, the expression level of malate dehydrogenase 2 (MDH2) gene is resistant to in control glioma cells, but inhibition of IRE1 signaling enzyme introduces up-regulation of this gene expression (+27%) upon glutamine deprivation. Moreover, glucose deprivation condition does not change significantly MDH2 gene expression in both control and IRE1 knockout glioma cells (Fig. 1). Thus, the IRE1 signaling enzyme suppresses the sensitivity of MDH2 gene expression to glutamine deprivation condition only.

We have also studied the expression of GOT2 gene, which encoded mitochondrial glutamic-oxaloacetic transaminase 2. As shown in Fig. 3, exposure the control glioma cells upon glutamine deprivation condition is strongly down-regulated the expression of GOT2 gene in control glioma cells (−45%) as compared to control cells, but glucose deprivation does not significantly change this gene expression in these glioma cells. At the same time, inhibition of IRE1 signaling enzyme in glioma cells removes the sensitivity of this gene expression to glutamine deprivation condition and introduces up-regulation of this gene expression (+26%) upon glucose deprivation. The expression level of this gene in cells with inhibited IRE1 signaling enzyme function is significantly down-regulated in the medium with glucose and glutamine (−38%; Fig. 3). Thus, inhibition of IRE1 signaling enzyme modifies the effect of glucose and glutamine deprivation on the expression of GOT2 gene in U87 glioma cells.

We next investigated the effect of glucose and glutamine deprivation conditions on the expression of succinate dehydrogenase [ubiquinone] iron-sulfur subunit (SDHB) gene in control U87 glioma cells and cells with inhibited function of signaling enzyme IRE1. As shown in Fig. 4, the expression level of SDHB mRNA is down-regulated (−27%) in control glioma cells treated by glutamine deprivation condition; however, effect of glucose deprivation condition on this gene expression was slightly lesser (−18%). In cells with suppressed function of IRE1 signaling enzyme the expression level of SDHB gene

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**Fig. 1.** Effect of glutamine and glucose deprivations on the expression level of mitochondrial malic enzyme 2 (ME2) mRNA in control U87 glioma cells stable transfected with vector (Vector) and cells with inhibited function of signaling enzyme IRE1 (dnIRE1) measured by qPCR. Hereinafter values of ME2 mRNA expressions were normalized to beta-actin mRNA and represented as percent of control 1 (100%) mean ± SEM; NS – no significant changes.

**Fig. 2.** Effect of glutamine and glucose deprivations on the expression level of mitochondrial malate dehydrogenase 2 (MDH2) mRNA in control U87 glioma cells stable transfected with vector (Vector) and cells with inhibited function of signaling enzyme IRE1 (dnIRE1) measured by qPCR.
was resistant to both glucose and glutamine deprivation conditions. Furthermore, the inhibition of IRE1 signaling enzyme function down-regulated the expression of this gene in glioma cells growing with complete (with glucose and glutamine) medium.

At the same time, the expression of gene encoding other subunit of succinate dehydrogenase, subunit D (succinate dehydrogenase [ubiquinone] cytochrome b small subunit) is resistant to both glucose and glutamine deprivation conditions in control U87 glioma cells as compared to control 1 (Fig. 5). However, inhibition of IRE1 signaling enzyme function by dnIRE1 introduces sensitivity of SDHD gene expression to both glucose and glutamine deprivation conditions in comparison with the control 2 glioma cells: –31% upon glucose deprivation condition and –15% upon glutamine deprivation condition. As shown in Fig. 5, the expression of this gene in glioma cells growing in complete medium IRE1 knockdown down-regulates the expression of SDHD gene (~38%).

Investigation of the expression of IDH2 gene, which encodes mitochondrial isocitrate dehydrogenase 2 (NADP⁺) enzyme, shown that glutamine deprivation condition significantly down-regulates
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this gene expression in control and IRE1 knockdown glioma cells: +44% and +56%, correspondingly (Fig. 6). The expression of IDH2 gene is also down-regulated by glucose deprivation condition in both types of used glioma cells, but effect was significantly lesser: +16% and +15%, correspondingly (Fig. 6). Thus, inhibition of IRE1 signaling enzyme function in U87 glioma cells by dnIRE1 enhances effect of glutamine deprivation condition and does not affected the sensitivity of this gene expression to glucose deprivation (Fig. 7). Next we studied the effect of inhibition of IRE1 signaling enzyme on the expression of IDH2 gene in complete DMEM. As shown in Fig. 6, the level of IDH2 mRNA expression is strongly up-regulated (+262%) in glioma cells without IRE1 signaling enzyme function in comparison with the control glioma cells (control 1).

Therefore, glutamine deprivation suppresses the expression level of most studied nuclear genes encoding important mitochondrial enzymes (ME2, SDHB, IDH2, and GOT2) in control glioma cells. This data agrees to the hypothesis that glutamine as an important substrate for glutaminolysis is necessary to glioma development and a more aggressive behaviour [31, 33, 34]. At the same time, we have shown that glucose deprivation condition does not affect the expression of ME2, MDH2, SDHD, and GOT2 genes in control glioma cells. These results support idea for the differential use of glucose and glutamine in cell proliferation [32]. Furthermore, the effect of glucose or glutamine deprivation conditions on the expression level of nuclear genes encoded all studied mitochondrial proteins is depended of IRE1 signaling enzyme function, except MDH2 and IDH2 genes upon glucose deprivation.

Moreover, inhibition of IRE1 signaling enzyme function modifies the effect of glucose and glutamine deprivations on the expression level of these genes in gene specific manner like many other previously studied genes [41–43].

We have also shown that inhibition of IRE1 signaling enzyme function down-regulates the expression level of all studied nuclear genes for mitochondrial proteins except IDH2 gene and this data correlated well with suppression of these glioma cell proliferation [15, 16], because almost all of these genes are highly expressed or mutated in many solid tumors and their knockdown

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**Fig. 6.** Effect of glutamine and glucose deprivations on the expression level of mitochondrial (NADP+) dependent isocitrate dehydrogenase 2 (IDH2) mRNA in control U87 glioma cells stable transfected with vector (Vector) and cells with inhibited function of signaling enzyme IRE1 (dnIRE1) measured by qPCR

**Fig. 7.** Comparative effect of glutamine and glucose deprivations on the expression level of IDH2 gene in two types of glioma cells: control cells transfected by vector (Vector) and cells with a deficiency of the signaling enzyme IRE1 (dnIRE1) measured by quantitative PCR
inhibits cell proliferation and induces cell death [18–25]. Induction of IDH2 gene expression in glioma cells with inhibited IRE1 signaling enzyme argues with data that IDH2 plays an essential role protecting cells against oxidative stress-induced damage and that a deficiency in this enzyme leads to mitochondrial dysfunction and the production of reactive oxygen species in cancer cells [29]. It is possible that inhibition of IRE1 signaling enzyme suppresses oxidative phosphorylation in mitochondria through down-regulation of ME2, MDH2, SDHB, SDHD, and GOT2 genes expression in glioma cells with native IRE1 signaling enzyme. Thus, our results agree with data [16–22, 24] that the suppression of IRE1 signaling enzyme function significantly decreases the glioma cell proliferation and that reduced level of ME2, MDH2, GOT2, SDHB, and SDHD genes expression can contribute to regulation of cell death processes and to suppression of glioma cell proliferation upon inhibition of IRE1-mediated endoplasmic reticulum stress signaling.

Thus, a better knowledge of tumor responses to glucose or glutamine deprivation conditions is required to elaborate therapeutical strategies of cell sensibilization, based on the blockade of survival mechanisms [10, 35, 36]. However, it is not clear yet the functional significance of changes in ME2, MDH2, GOT2, SDHD, SDHB, and IDH2 gene expressions and prospective studies are still needed to confirm.

Results of this study clearly demonstrated that the expression levels of almost all studied nuclear genes (ME2, MDH2, GOT2, SDHD, SDHB, and IDH2), which encoding key mitochondrial enzymes, responsible for control of cell proliferation and apoptosis and participate in malignant tumor growth, are affected by glutamine and glucose deprivations in glioma cells in gene-specific manner preferentially through IRE1 signaling branch of endoplasmic reticulum stress.

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10. Tsybald D. O., Minchenko D. O., Riabovol O. O., Ratushna O. O., Minchenko O. H. IRE1 knockdown modifies glucose and glutamine deprivation effects on the expression of proliferation related genes in U87 glioma.


The aim of the work was to study the influence of glutamine and glucose deficiency on the expression of nuclear genes that code mitochondrial proteins in glioma line U87 cells under IRE1 inhibition. It was shown that glutamine deficiency decreases the expression of genes NADP+-dependent mitochondrial isocitrate dehydrogenase 2 (IDH2), malate enzyme 2 (ME2), mitochondrial aspartateaminotransferase (GOT2) and subunit B of succinate dehydrogenase (SDHB) in control transduced with vector without insertion glioma gene specifically. At the same time, the level of expression of genes malate dehydrogenase 2 (MDH2) and subunit D of succinate dehydrogenase in these cells under glutamine deficiency does not change significantly. It was also established that inhibition of the function of the signaling enzyme IRE1 in glioma line U87 cells modifies the effect of glutamine deficiency on the expression of all studied genes. The expression of most studied genes is resistant to glucose deficiency, except genes IDH2 and SDHB, whose level slightly decreases. Inhibition of IRE1 in glioma line U87 cells modifies the effect of glucose deficiency on the expression of genes ME2, SDHB, SDHD and GOT2. Thus, the deficiency of glucose and glutamine changes the level of expression of most nuclear genes that code mitochondrial proteins, depending on the functional activity of enzyme IRE1, which is a central mediator of the stress response in the endoplasmic reticulum and controls processes of proliferation and cell death.

**Key words:** deficiency of glutamine and glucose, mitochondrial proteins, inhibition of IRE1, glioma line U87.

Ключові слова: дефіцит глутаміну та глікози, мітохондріальні протеїни, пригнічення IRE1, клітини глиоми лінії U87.