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8 ENDOCRINE REGULATIONS, Vol. 51, No. 1, 8–19, 2017

doi:10.1515/enr-2017-0002

Hypoxic regulation of the expression of genes encoded estrogen related proteins in U87 glioma cells: effect of IRE1 inhibition

[1,2]Minchenko DO, [1]RIABOVOL OO, [1]RATUSHNA OO, [1]Minchenko OH

[1]Department of Molecular Biology, Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv, Ukraine; [2]Department of Pediatrics, National Bohomolets Medical University, Kyiv, Ukraine
E-mail: ominchenko@yahoo.com

Objective. The aim of the present study was to examine the effect of inhibition of endoplasmic reticulum stress signaling, mediated by IRE1 (inositol requiring enzyme 1), which is a central mediator of the unfolded protein response on the expression of genes encoded estrogen related proteins (NRIP1/RIP140, TRIM16/EBBP, ESRRA/NR3B1, FAM162A/E2IG5, PGRMC2/PMBP, and SLC39A6/LIV-1) and their hypoxic regulation in U87 glioma cells for evaluation of their possible significance in the control of glioma cells proliferation.

Methods. The expression of *NRIP1*, *EBBP*, *ESRRA*, *E2IG5*, *PGRMC2*, and *SLC39A6* genes in U87 glioma cells, transfected by empty vector pcDNA3.1 (control) and cells without IRE1 signaling enzyme function (transfected by dnIRE1) upon hypoxia, was studied by a quantitative polymerase chain reaction.

Results. Inhibition of both enzymatic activities (kinase and endoribonuclease) of IRE1 signaling enzyme function up-regulates the expression of *EBBP*, *E2IG5*, *PGRMC2*, and *SLC39A6* genes is in U87 glioma cells in comparison with the control glioma cells, with more significant changes for *E2IG5* and *PGRMC2* genes. At the same time, the expression of *NRIP1* and *ESRRA* genes is strongly down-regulated in glioma cells upon inhibition of IRE1. We also showed that hypoxia increases the expression of *E2IG5*, *PGRMC2*, and *EBBP* genes and decreases *NRIP1* and ESRRA genes expression in control glioma cells. Furthermore, the inhibition of IRE1 in U87 glioma cells decreases the effect of hypoxia on the expression of *E2IG5* and *PGRMC2* genes, eliminates hypoxic regulation of *NRIP1* gene, and enhances the sensitivity of *ESRRA* gene to hypoxic condition. Furthermore, the expression of *SLC39A6* gene is resistant to hypoxia in both the glioma cells with and without IRE1 signaling enzyme function.

Conclusions. Results of this investigation demonstrate that inhibition of IRE1 signaling enzyme function affects the expression of *NRIP1*, *EBBP*, *ESRRA*, *E2IG5*, *PGRMC2*, and *SLC39A6* genes in U87 glioma cells in gene specific manner and these changes possibly contribute to the suppression of the cell proliferation. Most of these genes are regulated by hypoxia and preferentially through IRE1 signaling pathway of endoplasmic reticulum stress.

Key words: IRE1 inhibition, hypoxia, mRNA expression, NRIP1, EBBP, ESRRA, E2IG5, PGRMC2, SLC39A6, U87 glioma cells

Corresponding author: Dmytro O. Minchenko, M.D., PhD., Department of Molecular Biology, Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Leontovycha 9, Kyiv 01601, Ukraine; e-mail: ominchenko@yahoo.com.

Estrogen receptors play an important role in the regulation of numerous metabolic and proliferative processes, including tumorigenesis, preferentially through interaction with specific response element in the target genes (Mueller et al. 2000; Cookman and Belcher 2015; Lau and To 2016; Ur Rahman and Cao 2016; Wesolowska et al. 2016; Zhu et al. 2016). Estrogen receptors are responsible for the regulation of cell growth and apoptosis as well as are involved, at least partly, in the development, proliferation, and progression of some cancers. However, the results of numerous studies are often controversial and divergent (Kim et al. 2011; Ur Rahman et al. 2016; Wesolowska et al. 2016). Moreover, the estrogen and progesterone receptors are present in astrocytomas, but higher tumor grades were associated with decreased estrogen receptor expression and increased progesterone receptor expression (Li et al. 2013; Tavares et al. 2016). At the same time, Li et al. (2013) have shown that in U87 cells estrogen receptors inhibit the cell proliferation and reduced cells in the S+G2/M phase and that hypoxia induced estrogen receptor-β5 expression in glioma as a self-protective mechanism against tumor proliferation. Recently, Cookman and Belcher (2015) have demonstrated that estrogen promotes medulloblastoma growth through estrogen receptor-β-mediated increases in IGF1R expression and activity, which induce cytoprotective mechanisms that decrease apoptosis.

Estrogen related protein LIV-1 represents zinc transporter ZIP6 also known as SLC39A6 (solute carrier family 39 member 6) and is involved in the control of gene transcription, cancer cells motility, and tumor growth (Schaner et al. 2003; Takatani-Nakase et al. 2014; Taylor et al. 2016). Furthermore, targets the SLC39A6/SNAIL pathway by miR-192 to reduce tumor metastasis in human hepatocellular carcinoma (Lian et al. 2016). Nuclear receptor interacting protein 1 (NRIP1) also known as RIP140 transcriptional coregulator is a nuclear protein that specifically interacts with the hormone-dependent activation domain AF2 of nuclear hormone receptors and modulates transcriptional activity of the estrogen receptor ESR1 and glucocorticoid receptor NR3C1 (Treuter et al. 1998). NRIP1 is a positive regulator of the circadian clock genes expression and involved in the regulation of various oncogenic signaling pathways, participates in the development and progression of solid tumors (Lapierre et al. 2015). Recently, it was shown that suppressing of NRIP1 inhibits growth of breast cancer cells *in vitro* and *in vivo* (Aziz et al. 2015). Recently, the mutations in this estrogen receptor cofactor gene were identified in 12% of patients with primary endometrial cancers (Gibson et al. 2016).

ESRRA (estrogen related receptor alpha) also known as NR3B1 (nuclear receptor subfamily 3 group B member 1) is a nuclear receptor that is closely related to the estrogen receptor, acts as a site-specific transcription regulator and is involved in the control of tumor growth (Hamidian et al. 2015; Ranhotra 2015; Tiwari et al. 2015). ESRRA is widely expressed in a range of tissues and accumulating evidence has supported that the high expression of ERRα correlates with poor prognosis of various human malignancies. However, knockdown of NR3B1 has antitumor effect (Casaburi et al. 2015; Zhang et al. 2015; Matsushima et al. 2016). It has been reported that mitochondrial biogenesis is required for the anchorage-independent survival and propagation of stem-like cancer cells and regulated by various transcription factors including ESRRA (De Luca et al. 2015; Wada and Nakatsuka 2016).

TRIM16 (tripartite motif containing 16) is an estrogen and anti-estrogen regulated gene and also known as EBBP (estrogen-responsive B box protein) because it contains two B box domains and a coiled-coiled region that are characteristic of the B box zinc finger protein family. Its function, however, has not yet been determined. TRIM16 inhibits hepatocellular carcinoma cell migration and invasion by suppression of ZEB2 expression, which in turn inhibited transcription of the pivotal ZEB2 target gene E-cadherin (Li et al. 2016). Moreover, recently was shown that TRIM16 inhibits cancer cell viability by a novel mechanism involving interaction and stabilization of TDP43 with consequent effects on E2F1 and pRb proteins (Kim et al. 2016). There is also data that TRIM16 inhibits neuroblastoma cells proliferation through cell cycle regulation and melanoma cells proliferation and migration through regulation of interferon beta 1 as well as induces apoptosis through activation of caspase-2 in cancer cells (Bell et al. 2013; Kim et al. 2013; Sutton et al. 2014).

Pro-apoptotic protein FAM162A (family with sequence similarity 162 member A) is localized into mitochondria and probably involved in the induction of the mitochondrial permeability transition. It is also known as HIF-1α-responsive pro-apoptotic molecule, growth and transformation-dependent protein (HGTD-P), and E2-induced gene 5 protein (E2IG5) and is responsive for activation of mitochondrial apoptotic cascades (Lee et al. 2004; Rohatgi et al. 2006; Cho et al. 2010). However, steroid receptor coactivator-interacting protein (SIP) inhibits caspase-independent apoptosis by preventing apoptosis-inducing factor to be released from mitochondria

(Wang et al. 2012). FAM162A can be considered as a valuable tumor marker of cervical neoplasia. However, the molecular mechanism of this protein involvement in tumor development and progression remains to be elucidated (Cho et al. 2009, 2010). It is interesting to note that mitochondrial dysfunction has been linked to a wide range of degenerative and metabolic diseases, cancer, and aging (Wallace et al. 2010). Pedram et al. (2006) have shown that in the mitochondria of MCF-7 breast cancer cells and endothelial cells there are high-affinity estrogen receptors, which impact mitochondrial functions and control the survival of the tumor cells. PGRMC2 (progesterone receptor membrane component 2) also known as steroid receptor protein DG6 and progesterone membrane binding protein (PMBP), is a member of non-classical progesterone signaling molecules (membrane progestin receptors) and a potential downstream effector of FOXM1 transcription factor, which plays important roles in tumorigenesis and tumor metastasis in multiple human carcinomas as tumor suppressor, migration inhibitor, and regulator of cytochrome P450 proteins (Petersen et al. 2013; Wendler and Wehling 2013; Ye et al. 2015).

It has been shown that estrogen signaling regulates major components of the unfolded protein response (UPR) and estrogen receptor expression is associated with the sensitivity of tumor cells to UPR-regulated apoptosis (Rajapaksa et al. 2016). However, involving of endoplasmic reticulum (ER) stress signaling into the regulation of different metabolic processes by estrogens has not been explored yet. The ER stress is mediated by three sensor and signaling pathways, but inositol requiring enzyme 1 (IRE1/ERN1) is a central mediator of the unfolded protein response and an important component of tumor growth, because its inhibition leads to a suppression of glioma growth through down-regulation of the angiogenesis and proliferation processes (Drogat et al. 2007; Auf et al. 2010, 2013). This stress is recognized as an important determinant of cancer and contributes to the expression profile of many regulatory genes resulting in proliferation, apoptosis, angiogenesis, and circadian clock (Clarke et al. 2014; Manie et al. 2014; Pluquet et al. 2014; Dejeans et al. 2015; Minchenko et al. 2015). The IRE1 enzyme is responsible for degradation of a specific subset of mRNA and alternative splicing of the XBP1 (X-box binding protein 1) transcription factor mRNA for control of the expression of unfolded protein response-specific genes (Acosta-Alvear et al. 2007; Aragon et al. 2009; Dejeans et al. 2012; Pluquet et al. 2013; Maurel et al. 2014, 2015).

Moreover, the ER has an essential position as a signal integrator because the signaling pathways elicited by ER stress sensors have connections with metabolic pathways and with other plasma membrane receptor signaling networks (Clarke et al. 2014; Manie et al. 2014; Chevet et al. 2015). The ER stress response-signaling pathway mediated by IRE1 is tightly associated with hypoxia and linked to the neovascularization, tumor growth, and cell death processes as well as to suppression of insulin receptor signaling through activation of c-Jun N-terminal kinase (Lenihan and Taylor 2013; Clarke et al. 2014; Minchenko et al. 2015). Recent studies have confirmed the existence of a crosstalk between the estrogen receptors and UPR (Rajapaksa et al. 2016). Therefore, estrogen signaling in breast cancer is among the regulators of the UPR and estrogen receptor expression is associated with the sensitivity of tumor cells to UPR-regulated apoptosis. Furthermore, activation of the unfolded protein response confers resistance to anti-estrogens and chemotherapeutics in estrogen receptor α-positive and breast cancers.

The main goal of this work was to study the effect of the signaling enzyme IRE1 inhibition on the expression of genes encoding the estrogen receptor related proteins (NRIP1, TRIM16/EBBP, ESRRA, FAM162F/E2IG5, PGRMC2, and SLC39A6) in U87 glioma cells and regulation of these genes by hypoxia for evaluation of its possible significance in the control of glioma growth through IRE1 mediated by ER stress signaling.

Materials and Methods

Cell lines and culture conditions. The glioma cell line U87 was obtained from ATCC (U.S.A.) and grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's minimum essential medium (Gibco, Invitrogen, Carlsbad, CA, U.S.A.) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., U.S.A.), penicillin (100 U/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 cells. One subline was obtained by selection of stable transfected clones with overexpression of vector pcDNA3.1, which was used for creation of dnIRE1. This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of the effect of hypoxia on the expression level of estrogen receptor and related genes. Second subline was obtained by selection of stable transfected clone with overexpression of IRE1 dominant/negative construct (dnIRE1) and has suppressed both protein kinase and endoribonuclease activities of this signal-

ing enzyme (Auf et al. 2010). The expression level of studied 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 on gene expressions under blockade of both enzymatic activities of IRE1. The efficiency of IRE1 suppression in this glioma cell subline was estimated previously (Auf et al. 2010, 2013) by determining the expression level of the XBP1 alternative splice variant, a key transcription factor in the IRE1 signaling, and the level of the phosphorylated isoform IRE1 using cells treated by tunicamycin (0.01 mg/ml during 2 h) for induction of ER stress. Both used in this study sublines of glioma cells are grown with the addition of geneticin (G418) while these cells carrying empty vector pcDNA3.1 or dnIRE1 construct.

Hypoxic condition was created in special incubator with 3% oxygen and 5% carbon dioxide levels; culture plates with complete DMEM were exposed to these conditions for 16 h.

RNA isolation. Total RNA was extracted from glioma cells using the Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). RNA pellets was 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 ND1000 (PEQLAB, Biotechnologie GmbH).

Reverse transcription and quantitative PCR analysis. The expression levels of NRIP1 (nuclear receptor interacting protein 1), FAM162F/E2IG5 (family with sequence similarity 162 member A/E2-induced gene 5 protein), TRIM16/EBBP (tripartite motif containing 16/estrogen-responsive B box protein), ESRRA/NR3B1 (estrogen related receptor alpha/nuclear receptor subfamily 3 group B member 1), PGRMC2/PMBP (progesterone receptor membrane component 2/progesterone membrane binding protein), and SLC39A6/LIV-1 (solute carrier family 39 member 6/estrogen regulated protein LIV-1) mRNAs as well as ACTB mRNA were measured in control U87 glioma cells and cells with a deficiency of IRE1 by quantitative polymerase chain reaction in real-time using qPCR „RotorGene RG-3000" (Corbett Research, Germany) and Applied Biosystems 7500 (Applied Biosystems, U.S.A.) using SYBRGreen Mix (ABgene, Thermo Fisher Scientific, Epsom, Surrey, UK). QuaniTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) was used for cDNA synthesis as described previously (Minchenko et al. 2015). Polymerase chain reaction was performed in triplicate.

For amplification of ESRRA, also known as NR3B1, cDNA we used forward (5'–tgtctcatctgctggtggtt–3' and reverse (5'–acgctctgcagtactgacat–3') primers. The nucleotide sequences of these primers correspond to sequences 814–833 and 1012–993 of human ESRRA cDNA (GenBank accession number NM_004451). The amplification of NRIP1 cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'–ctccggatgacatcagagct–3' and reverse – 5'–cgcaaggaggaggagaagaa–3'. The nucleotide sequences of these primers correspond to sequences 180–199 and 390–371 of human NRIP1/RIP140 cDNA (GenBank accession number NM_003489). For amplification of FAM162A, also known as E2-induced gene 5 protein (E2IG5) or HIF-1 alpha-responsive proapoptotic molecule, cDNA we used forward (5'– tcctcatatggtcaggtcgc–3' and reverse (5'– gctgcttcctctttcagacg–3') primers. The nucleotide sequences of these primers correspond to sequences 299–318 and 533–514 of human FAM162A cDNA (GenBank accession number NM_014367).

The amplification of TRIM16, also known as estrogen-responsive B box protein (EBBP), cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'–ggcaacctgggagagtaact–3' and reverse – 5'–agtctttctctgccaggtcc–3'. The nucleotide sequences of these primers correspond to sequences 3717–3736 and 3934–3915 of human TRIM16cDNA (GenBank accession number NM_004491). For amplification of SLC39A6, also known as estrogen regulated protein LIV-1 or zinc transporter ZIP6, cDNA we used two oligonucleotides primers: forward – 5'–cagccaacccaatgaaacca–3' and reverse – 5'–agggcaaaggtcaggatcaa–3'. The nucleotide sequences of these primers correspond to sequences 4327–4346 and 4517–4498 of human SLC39A6 cDNA (GenBank accession number NM_012319). The amplification of PGRMC2, also known as steroid receptor protein DG6, cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'–ttggagtgagagcagggaag–3' and reverse – 5'–gcctctcagccaataacgtg–3'. The nucleotide sequences of these primers correspond to sequences 3868–3887 and 4031–4012 of human PGRMC2 cDNA (GenBank accession number NM_006320). The amplification of ACTB (beta-actin) cDNA for real time RCR analysis was performed using forward – 5'–ggacttcgagcaagagatgg–3' and reverse - 5'–agcactgtgttggcgtacag–3' primers. These primers nucleotide sequences correspond to 747–766 and 980–961 of human ACTB cDNA (GenBank accession number NM_001101). The expression of beta-actin mRNA was used as con-

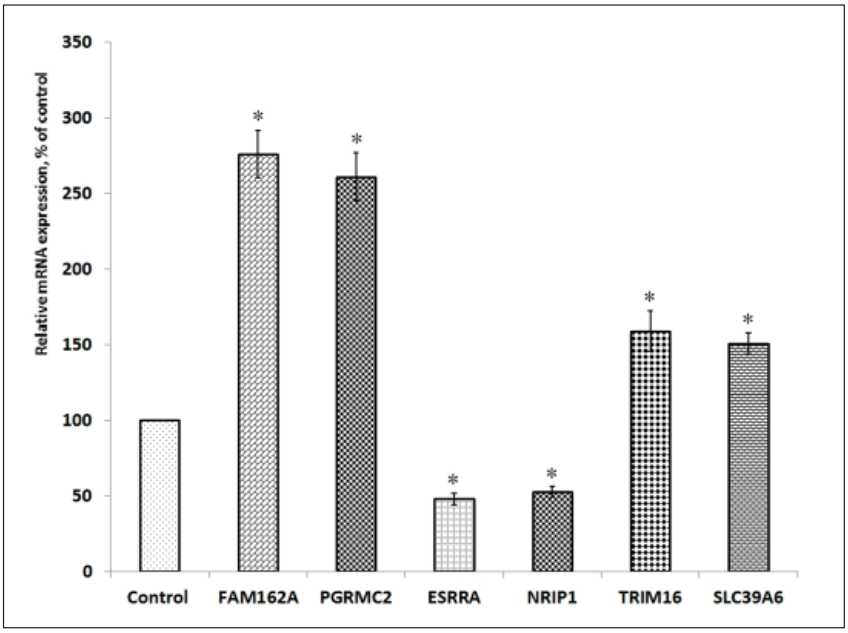

Figure 1. Effect of IRE1 inhibition by dnIRE1 on the expression level of genes encoded estrogen responsible prtoteins: FAM162F/E2IG5 (family with sequence similarity 162 member A/E2-induced gene 5 protein), PGRMC2/PMBP (progesterone receptor membrane component 2/progesterone membrane binding protein), ESRRA/NR3B1 (estrogen related receptor alpha/nuclear receptor subfamily 3 group B member 1), NRIP1 (nuclear receptor interacting protein 1), TRIM16/EBBP (tripartite motif containing 16/estrogen-responsive B box protein), and SLC39A6/LIV-1 (solute carrier family 39 member 6/estrogen regulated protein LIV-1) in U87 glioma cells measured by qPCR. Values of these mRNA expressions were normalized to beta-actin mRNA level and represented as percent for control (cells transfected be empty vector, 100%); n=4; *p<0.01 vs. control.

trol of analyzed RNA quantity. The primers were received from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Quantitative PCR analysis was performed using a special computer program "Differential expression calculator". The values of *NRIP1,* TRIM16, *ESRRA, FAM162A, PGRMC2,* and *SLC39A6* gene expressions were normalized to the expression of beta-actin (ACTB) mRNA and represent as percent of control (100%). All values are expressed as mean ± SEM from triplicate measurements performed in four independent experiments. Statistical analysis was performed as described previously (Bochkov et al. 2006). The amplified DNA fragments were also analyzed on a 2% agarose gel and that visualized by SYBR* Safe DNA Gel Stain (Life Technologies, Carlsbad, CA, U.S.A.).

Results

Inhibition of IRE1 affects the expression of *FAM162A/E2IG5, PGRMC2/PMBP, ESRRA/NR3B1, NRIP1/RIP140, TRIM16/EBBP, and SLC39A6/LIV1* genes in U87 glioma cells. To investigate a possible role of ER stress signaling mediated by IRE1 bifunctional enzyme in the expression level of genes for estrogen receptor related proteins, we studied the effect of IRE1 inhibition by dnIRE1 on these genes expression in U87 glioma cells. As shown in Figure 1, the expression level of FAM162A mRNA is significantly up-regulated (+176%) in U87 glioma cells by inhibition of IRE1 signaling enzyme in comparison with the control cells. The expression level of PGRMC2 mRNA in glioma cells without IRE1 signaling enzyme function is also up-regulated (+161%) in comparison with the control glioma cells. At the same time, inhibition of IRE1 by dnIRE1 is significantly down-regulated the expression level of ESRRA (–52%) and NRIP1 mRNA (–47%) in glioma cells in comparison with the control cells (Figure 1). We next investigated the expression of *TRIM16*, and *SLC39A6* genes in relation to the inhibition of IRE1 signaling enzyme function. As also showed in Figure 1, the expression level of TRIM16 and SLC39A6 mRNAs is significantly increased (+59% and +51%, respectively) in glioma cells with suppressed function of IRE1 signaling enzyme. Therefore, the inhibition of ER stress signaling mediated by IRE1 enzyme affects the expression level of the estrogen receptor related genes in a gene-specific manner.

Effect of hypoxia on the expression of *FAM162A, PGRMC2, ESRRA, NRIP1, TRIM16, and SLC39A6* genes in control glioma cells and cells without IRE1 enzyme function. Next, we investigated the effect of the hypoxia on the expression of *FAM162A*, *PGRMC2*, *ESRRA*, *NRIP1*, *TRIM16*, and *SLC39A6* genes in glioma cells in relation to inhibition of IRE1 signaling enzyme function. We found that expression level of *FAM162A* gene is strongly up-regulated by hypoxia in control glioma cells (+303% in comparison with control 1) and that inhibition of IRE1 signaling enzyme function decreases effect of hypoxia on this gene expression (+108% in comparison with control 2) (Figure 2). As shown in Figure 3, hypoxia

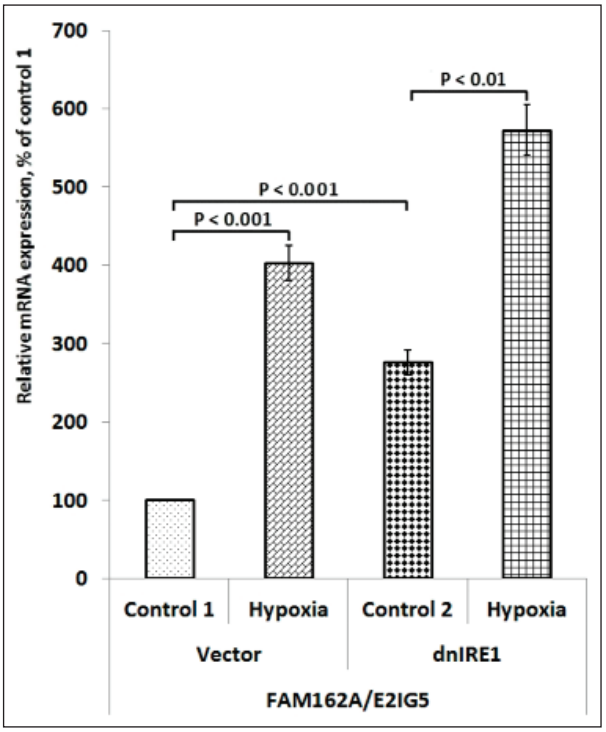

Figure 2. Effect of hypoxia on the expression level of FAM162F/E2IG5 (family with sequence similarity 162 member A/E2-induced gene 5 protein), also known as HIF-1 alpha-responsive pro-apoptotic molecule and growth and transformation-dependent protein, mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of this mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100%); n=4.

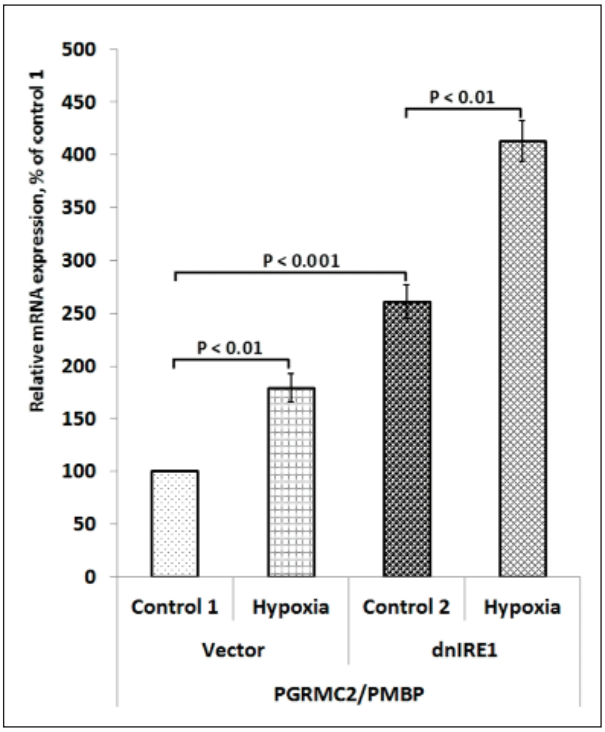

Figure 3. Effect of hypoxia on the expression level of PGRMC2/PMBP (progesterone receptor membrane component 2/progesterone membrane binding protein) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of this mRNA expressions were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); n=4.

also significantly up-regulates the expression level of *PGRMC2* gene in control glioma cells in comparison with the control 1 (+79%). At the same time, inhibition of IRE1 signaling enzyme slightly decreases the sensitivity of this gene expression to hypoxia. Therefore, in glioma cells without IRE1 signaling enzyme function, the expression level of *PGRMC2* gene is up-regulated on 58% in comparison with corresponding control (control 2; Figure 3).

Investigation of *ESRRA* gene expression showed that hypoxia down-regulated its expression both in the control glioma cells and cells without IRE1 signaling enzyme function. Thus, as shown in Figure 4, the expression level of *ESRRA* gene is slightly but statistically significantly decreased by hypoxia (–16%) in control glioma cells in comparison with control 1, but inhibition of IRE1 significantly enhances effect of hypoxia on this gene expression (–42%) in comparison with control 2. We also studied the hypoxic regulation of *NRIP1* gene expression in glioma cells in relation to inhibition of IRE1 signaling enzyme function and found that in control glioma cells hypoxia is down-regulated (–32%) the expression level of *NRIP1* gene in comparison with control 1 (Figure 5). At the same time, the inhibition of IRE1 signaling enzyme function completely eliminated the hypoxic regulation of this gene expression. Therefore, IRE1 signaling pathway is necessary for hypoxic regulation of this gene expression.

As shown in Figure 6, the expression level of *TRIM16* gene is slightly but statistically significantly up-regulated (+20%) in control glioma cells exposure upon hypoxic condition in comparison with control 1. Furthermore, inhibition of IRE1 did not change the effect of hypoxia on the expression of *TRIM16* gene (+20%) in comparison with control 2. Investigation of SLC39A6, also known as estrogen regulated protein LIV-1, showed that hypoxia does not change significantly its expression in both the control glioma cells as well as cells without IRE1 enzyme function (Figure 7). As shown in Figure 8, inhibition of IRE1 signaling enzyme modifies hypoxic regulation of the

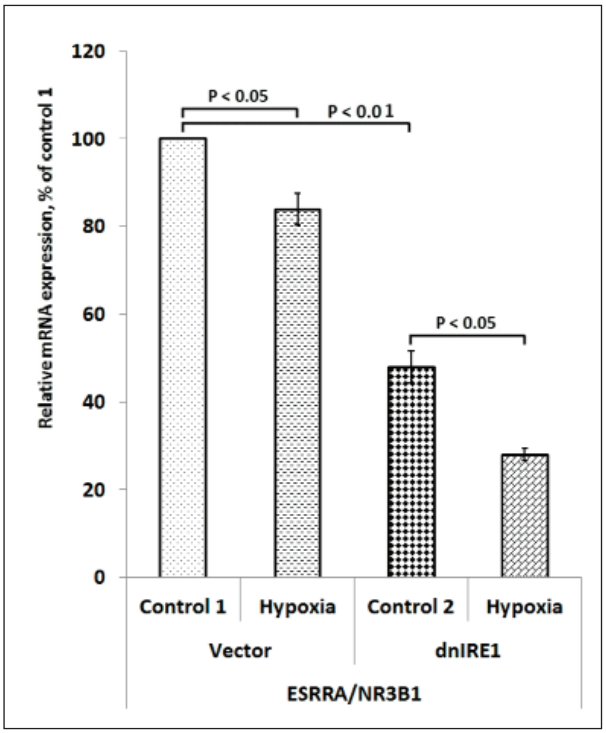

Figure 4. Effect of hypoxia on the expression level of ESRRA/NR3B1 (estrogen related receptor alpha/nuclear receptor subfamily 3 group B member 1) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of ESRRA mRNA expressions were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); n=4.

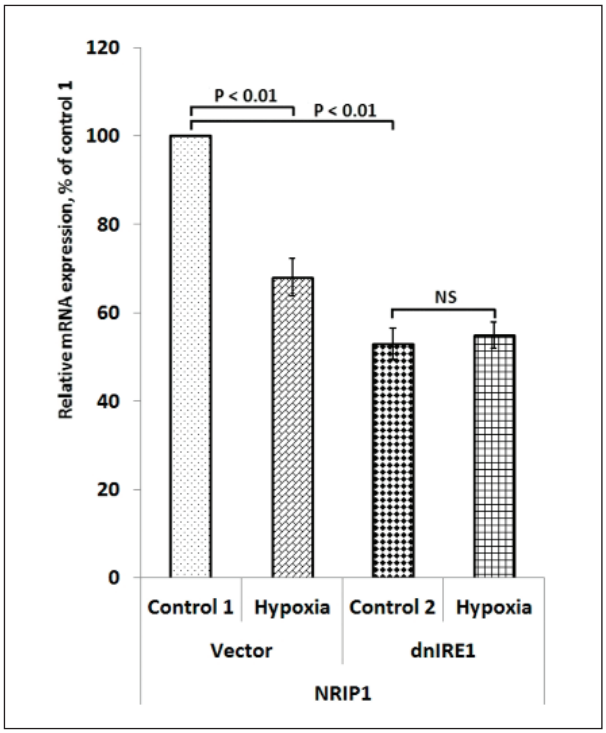

Figure 5. Effect of hypoxia on the expression level of nuclear receptor interacting protein 1 (NRIP1) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of NRIP1 mRNA expressions were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); n=4.

expression level of *FAM162A*, *PGRMC2*, *ESRRA*, and *NRIP1* genes in glioma cells, but had no effect on *TRIM16* and *SLC39A6* gene expressions. Figure 9 contains schematic representation of the hypoxic regulation of studied genes expression in glioma cells in relation to inhibition of IRE1 signaling enzyme function.

Discussion

In this work, we studied the expression of a subset of genes encoding the estrogen receptor related proteins in U87 glioma cells upon hypoxia and after inhibition of IRE1, the major signaling pathway of the unfolded protein response. It is important for evaluation of possible significance of these genes in the control of glioma growth through ER stress signaling mediated by IRE1 and hypoxia. It is known that stress signaling pathways are involved in numerous metabolic pathways and inhibition of the activity of IRE1 signaling enzyme in glioma cells had antitumor effects (Auf et al. 2010, 2013; Manie et al. 2014; Minchenko et al. 2015).

The data of this study demonstrate that inhibition of IRE1 signaling enzyme function significantly affects the expression level of all studied estrogen receptor related genes, indicating their participation in IRE1 mediated network of the unfolded protein response. The changes in these genes expression in cells without IRE1 signaling enzyme function possible contribute in the suppression of glioma cell proliferation and tumor growth, because there are data indicating that the estrogen receptor related factors play an important role in the control of cell proliferation and apoptosis (Cho et al. 2010; Lapierre et al. 2015; Tiwari et al. 2015). It is possible that increased expression of *FAM162A*, *PGRMC2*, *TRIM16* and *SLC39A6* genes as well as decreased expression of *ESRRA*, and *NRIP1* genes (Figure 1) may contribute to the suppression of the proliferation and glioma growth from glioma cells with IRE1 knockdown (Auf et al. 2010, 2013; Aziz et al. 2015; Casaburi et al. 2015; Minchenko et al. 2015; Zhang et al. 2015; Matsushima et al.

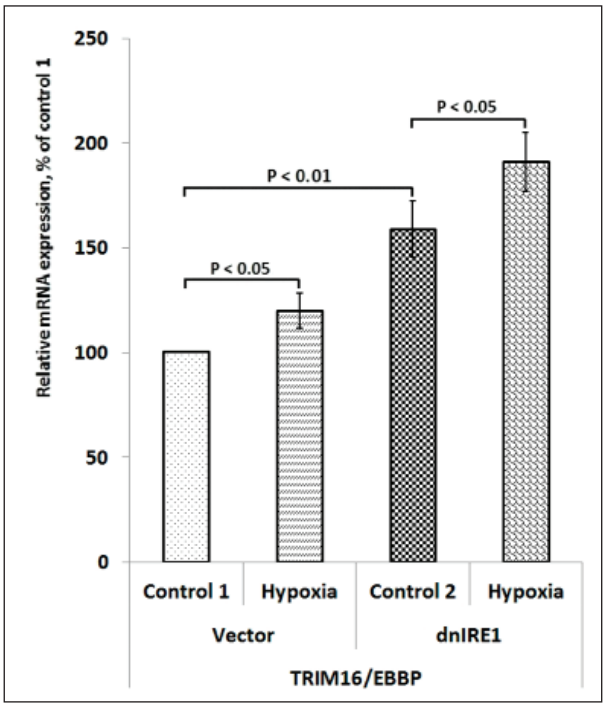

Figure 6. Effect of hypoxia on the expression level of TRIM16/EBBP (tripartite motif containing 16/estrogen-responsive B box protein) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of this mRNA expressions were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); n=4.

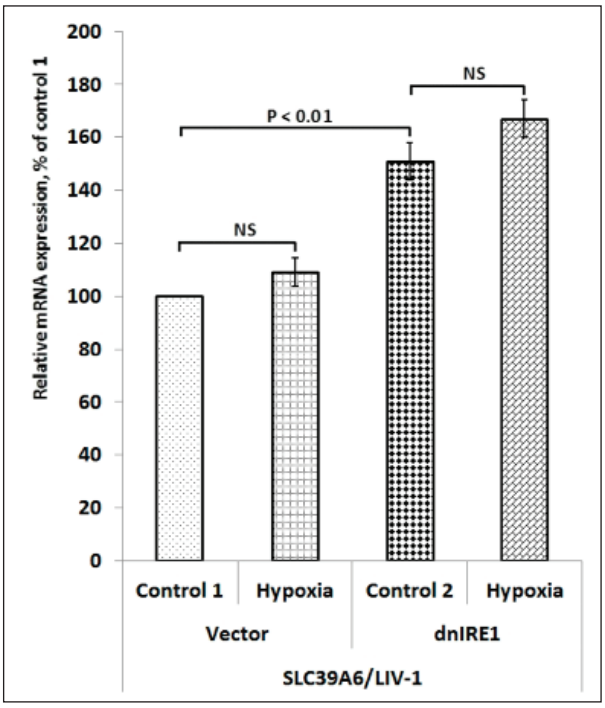

Figure 7. Effect of hypoxia on the expression level of SLC39A6/LIV-1 (solute carrier family 39 member 6/estrogen regulated protein LIV-1), also known as zinc transporter ZIP6, mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of SLC39A6 mRNA expressions were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); n=4.

2016). Therefore, suppressing of NRIP1, which modulates transcriptional activity of the estrogen receptor, as well as knockdown of a site-specific transcription regulator ESRRA/NR3B1 inhibits growth of the cancer cells *in vitro* and *in vivo* (Aziz et al. 2015; Zhang et al. 2015; Matsushima et al. 2016).

Furthermore, IRE1 knockdown-mediated increased expression of a pro-apoptotic protein FAM162A, which is E2-induced gene 5 protein, has antitumor effect through activation of the mitochondrial apoptotic cascades (Lee et al. 2004; Rohatgi et al. 2006; Cho et al. 2010). IRE1 inhibition also upregulates the expression level of PGRMC2, a potential downstream effector of FOXM1 transcription factor, which plays important roles in the tumorigenesis and tumor metastasis in multiple human carcinomas as tumor suppressor and migration inhibitor (Wendler and Wehling 2013; Petersen et al. 2013; Ye et al. 2015). Another estrogen-responsive protein, TRIM16, which inhibits hepatocellular carcinoma cell migration and invasion by suppression of ZEB2 expression (Li et al. 2016) as well as suppresses cancer cell viability by involving interaction and stabilization of TDP43 with consequent effects on E2F1 and pRb proteins (Kim et al. 2016), is also up-regulated in glioma cells without IRE1 signaling enzyme function. Moreover, TRIM16 inhibits neuroblastoma cells proliferation through cell cycle regulation and induces apoptosis via activation of caspase-2 (Bell et al. 2013; Kim et al. 2013). Therefore, our results conform to data that FAM162A, PGRMC2, TRIM16, and SLC39A6 have mainly anti-proliferative functions through interaction with different transcription co-regulators and signaling pathways of ER stress in cell specific manner.

Consequently, our results demonstrate that all the genes studied are ER stress responsive and consequently have an essential role in the control of cell proliferation, but functional significance and molecular mechanisms of induction or suppression of these genes expression upon inhibition of IRE1 are not clear yet and many details remain to be elucidated. Thus, the ER has an important position as a signal integrator in both normal and malignant cells because

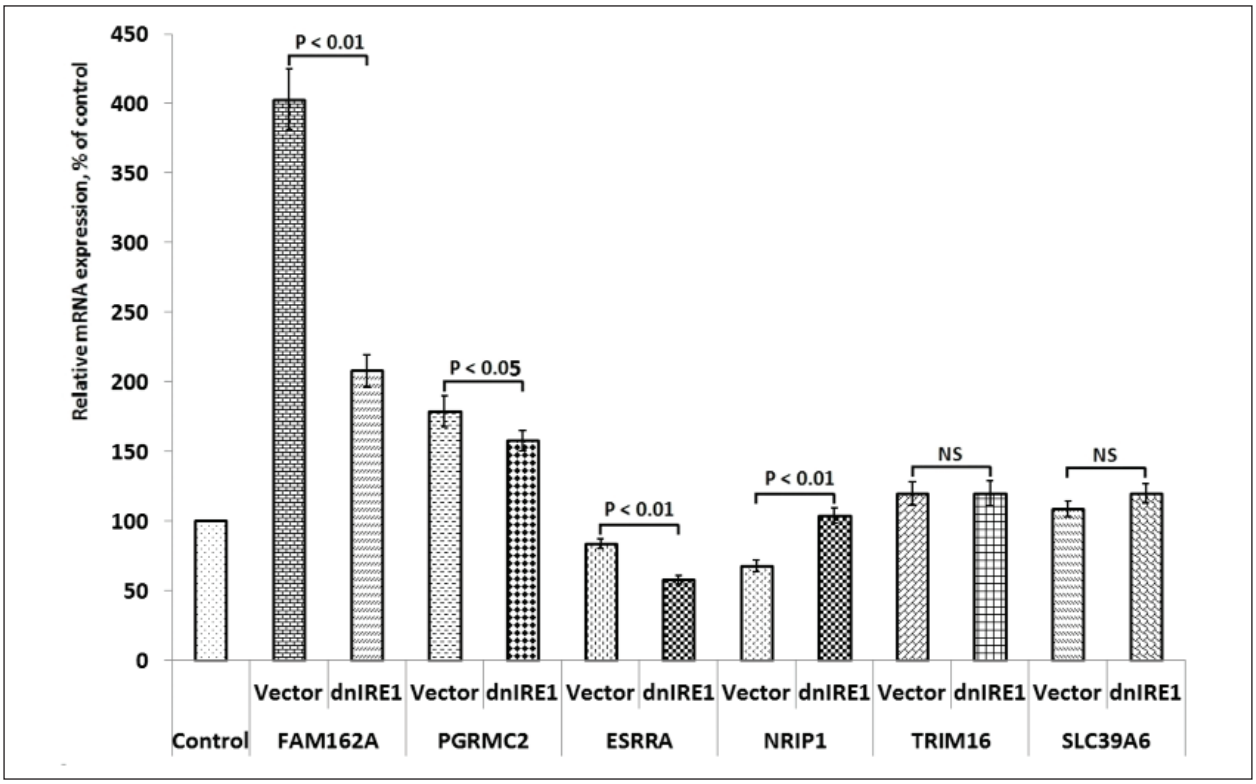

Figure 8. Effect of hypoxia on the expression levels of FAM162A, PGRMC2, ESRRA, NRIP1, TRIM16, and SLC39A6 mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 (dnIRE1) measured by qPCR. Values of these mRNA expressions were normalized to β-actin mRNA level and presented as percent of control (100%) both in cells transfected by vector and dnIRE1; n=4.

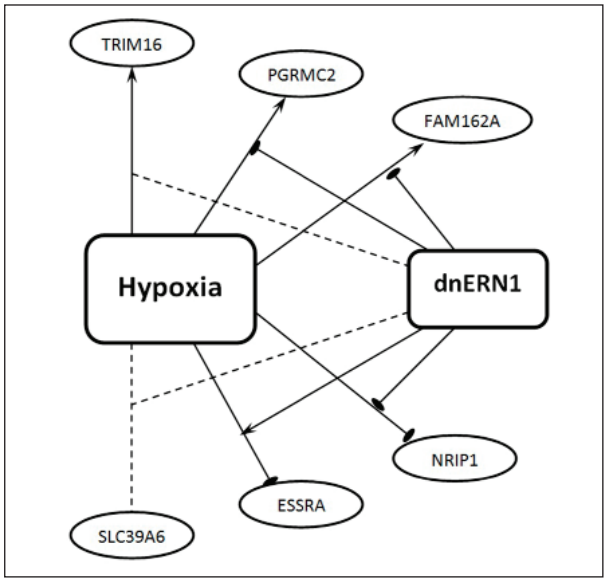

Figure 9. Schematic representations of key results on the hypoxic regulation of studied genes expression in glioma cells in relation to inhibition of IRE1 signaling enzyme function.
← – up-regulation of gene expression; ⊦ – down-regulation of gene expression; - - - – no significant changes.

it signaling pathways is involved in numerous metabolic pathways (Manie et al. 2014; Pluquet et al. 2014; Chevet et al. 2015).

The investigation of hypoxic regulation of the expression of genes encoded estrogen receptor related proteins, in respect to inhibition of IRE1 signaling, is very important for the understanding of malignant tumor growth mechanisms, because low oxygen has essential role in the control of proliferation and tumor progression (Lenihan and Taylor 2013; Minchenko et al. 2015). We are showing that hypoxia leads to up-regulation of the expression of *FAM162A*, *PGRMC2*, and *TRIM16* genes in control glioma cells and that inhibition of IRE1 signaling enzyme function modifies hypoxic regulation of *FAM162A* and *PGRMC2* genes. Therefore, inhibition of IRE1 signaling enzyme declines the effect of hypoxia on the expression level of *FAM162A* and *PGRMC2* genes in glioma cells and possibly reflects multifactorial mechanism of these genes regulation by hypoxia.

At the same time, IRE1 knockdown eliminates hypoxia effect on the expression of *NRIP1* gene level and

does not change significantly the hypoxic regulation of *TRIM16* gene. Thus, inhibition of IRE1 modifies the sensitivity of *NRIP1* gene expression to hypoxia in glioma cells, but hypoxic regulation of *TRIM16* gene expression is not mediated by IRE1 signaling. Therefore, these results conform the data that hypoxic regulation has preferentially a multifactorial mechanism mediated by IRE1 signaling (Minchenko et al. 2015). However, it is not clear yet why hypoxia up-regulates the expression of most studied genes encoded estrogen receptor related proteins, which expression is increased by IRE1 inhibition and is correlated with anti-proliferative effect.

This study provides an unique insight into the molecular mechanisms regulating the expression of genes encoded estrogen receptor related factors and their correlation with slower cell proliferation in cells harboring dnIRE1, attesting to the fact that expression of these genes is controlled by ER stress signaling in gene specific manner and that ER stress is a necessary component of the malignant tumor growth and cell survival. Moreover, the expression of some studied genes upon hypoxia is significantly depended from IRE1 signaling enzyme function. However, the detailed molecular mechanisms of this regulation are complex and warrants further studies.

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