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Effect of hypoxia on the expression of genes encoding insulin-like growth factors and some related proteins in U87 glioma cells without IRE1 function

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Objective. The aim of the present study was to investigate the effect of hypoxia on the expression of genes encoding insulin-like growth factors (IGF1 and IGF2), their receptor (*IGF1R*), binding protein-4 (*IGFBP4*), and stanniocalcin 2 (STC2) in U87 glioma cells in relation to inhibition of endoplasmic reticulum stress signaling mediated by IRE1 (inositol requiring enzyme 1) for evaluation of their possible significance in the control of tumor growth.

Methods. The expression of *IGF1*, *IGF2*, *IGF1R*, *IGFBP4*, and *STC2* 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 qPCR.

Results. The expression of *IGF1* and *IGF2* genes is down-regulated in glioma cells without IRE1 signaling enzyme function in comparison with the control cells. At the same time, the expression of *IGF1R*, *IGFBP4*, and *STC2* genes was up-regulated in glioma cells upon inhibition of IRE1, with more significant changes for *IGFBP4* and *STC2* genes. We also showed that hypoxia does not change significantly the expression of *IGF1*, *IGF2*, and *IGF1R* genes but up-regulated *IGFBP4* and *STC2* genes expression in control glioma cells. Moreover, the inhibition of both enzymatic activities (kinase and endoribonuclease) of IRE1 in glioma cells does not change significantly the effect of hypoxia on the expression of *IGF1*, *IGF1R*, and *IGFBP4* genes but introduces sensitivity of *IGF2* gene to hypoxic condition. Thus, the expression of *IGF2* gene is resistant to hypoxia only in control glioma cells and significantly down-regulated in cells without functional activity of IRE1 signaling enzyme, which is central mediator of the unfolded protein response and an important component of the tumor growth as well as metabolic diseases.

Conclusions. Results of this study demonstrate that the expression of *IGF1* and *IGF1R* genes is resistant to hypoxic condition both in control U87 glioma cells and cells without IRE1 signaling enzyme function. However, hypoxia significantly up-regulates the expression of *IGFBP4* gene independently on the inhibition of IRE1 enzyme. These data show that proteins encoded by these genes are resistant to hypoxia except *IGFBP4* and participate in the regulation of metabolic and proliferative processes through IRE1 signaling.

Key words: hypoxia, IGF1, IGF1, IGF1R, IGFBP4, STC2, mRNA expression, IRE1 inhibition, U87 glioma cells

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Insulin-like growth factors (IGFs, IFG1 and IGF2) are secreted factors playing an important role in the regulation of numerous metabolic and proliferative processes, including tumorigenesis and metabolic diseases such are obesity and diabetes, through interaction with specific cell surface receptors (IGF receptor, and hybrid IGF1/insulin (INS) receptor (Pollak 2008; Ohlsson et al. 2009; Halje et al. 2012; Panda et al. 2013; Girnita et al. 2014). IGF1 as well as IGF2 are protein hormones that have been shown to exert several biological functions in multiple biological systems in cell proliferation, growth, migration, differentiation, and survival regulations. Thus, IGF-2 induction of aryl hydrocarbon receptor in MCF-7 breast cancer cells promotes the expression of CCND1 and the proliferation of these cells (Tomblin and Salisbury 2014). It is interesting to note that apoptosis can also be regulated via IGF1 receptor (Pan et al. 2014). There exists a specific cross talk between signaling pathways mediated by IGF and INS receptor both at the receptor and downstream signaling levels in tissue specific manner depending on the insulin receptor isoform (Limesand et al. 2013). Furthermore, formation of hybrid receptor isoforms between receptors for IGF1 and insulin as well as hybrid receptors of IGF1/INS receptor with other tyrosine kinase potentiate the transformation of cells, tumorigenesis, and tumor neovascularization (Weroha and Haluska 2012; Dai et al. 2013; Baxter 2014; Singh et al. 2014; Zhu et al. 2014). Moreover, insulin system including ligands (insulin and IGFs) and their shared receptors (INS/IGF receptors) are critical regulators of insulin signaling and glucose homeostasis (Philippou et al. 2014; Singh et al. 2014).

The insulin-like growth factor binding proteins (IGFBPs) bind and regulate the availability of both IGFs and inhibit or stimulate the growth promoting effects of the IGFs through IGF/INS receptors. Through other signaling pathways, they also regulate cell proliferation and survival as well as tumor angiogenesis and cancer cell migration (Duan et al. 2010; Azar et al. 2011; Baxter 2014; Zhu et al. 2014). IGFBPs have been understood to have many actions beyond their endocrine role in the transport of IGF (Baxter 2014; Zhu et al. 2014). IGFBP4 preferentially binds2 to IGF2 and has IGF-independent effects, including inhibition of angiogenesis and promotion of cancer cell migration (Praveen Kumar et al. 2014). In addition to interaction of IGFBPs with their canonical ligands (IGF1 and IGF2), they can also play an important role in the regulation of various processes including transcription, because they can interact with many proteins (Ingermann et al. 2010; Baxter 2014; Ellis et al 2014). IGFBP4 regulates growth and development of tissues and organs by negatively regulating the IGF signaling. Among most cancers, IGFBP4 also has a growth inhibitory role. It has been reported as a down-regulated gene, except in renal cell carcinoma and some gliomas, wherein it promotes tumor progression (Praveen Kumar et al. 2014). Thus, IGFBP4 modulates ligand-dependent estrogen receptor-alpha activation in breast cancer cells in an IGF-independent manner via activation of the Akt/PKB signaling pathway (Hermani et al. 2013). Recently, it has been shown that inhibition of tumor-associated αvβ3 integrin regulates the angiogenic switch by enhancing expression of IGFBP4, an important negative regulator of IGF-1 signaling, leading to reduced melanoma growth and angiogenesis *in vivo* (Contois et al. 2015).

The expression of IGFs, their receptors, and binding proteins are regulated at transcription and posttranscriptional levels (Panda et al. 2013). Moreover, the effect of IGF1 in the regulation of tumor cell growth depends on its mRNA splicing and posttranslational modifications, such as proteolytic processing and glycosylation (Philippou et al. 2014; Halje et al. 2012). Recently, it has been shown that a long non-coding RNA responsive to IGF/INS signaling (CRNDE) regulates genes involved in central metabolism processes (Ellis et al. 2014) and that platelet-released miR-223 promotes advanced glycation end product-induced vascular endothelial cell apoptosis via targeting insulin-like growth factor 1 receptor (Pan et al. 2014). It is interesting to note that protein kinase LKB1 expression in cancer cell may result in dephosphorylation of several tumor-enhancing RTKs, including ErbB2, hepatocyte growth factor receptor (c-Met), and IGF1R (Okon et al. 2014). There are also data indicating that liver mTOR controls IGF-I bioavailability by regulation of protein kinase CK2 and IGFBP phosphorylation in fetal growth restriction (Abu Shehab et al. 2014).

IGFs, their receptors and IGFBPs participate in endoplasmic reticulum (ER) stress, which is an important factor of cancer growth, insulin resistance, and obesity (Cao and Kaufman 2013; Minchenko et al. 2013b; Han and Kaufman 2014; Lee and Ozcan 2014; Wang and Kaufman 2012; Yadav et al. 2014; Chevet et al. 2015; Minchenko et al. 2015a,b). The endoplasmic reticulum is a dynamic intracellular organelle with exquisite sensitivity to alterations in homeostasis responsible for multiple cellular functions including protein folding and the maintenance of cellular homeostasis and provides stringent quality control systems to ensure that only correctly folded proteins transit to the Golgi and unfolded proteins are ulti-

mately degraded (Maurel et al. 2014a). It is interesting to note that secreted proteinase inhibitor stanniocalcin 2 (STC2) is a novel extracellular component of the IGF system, which potently inhibits the proteolytic activity of the growth-promoting metalloproteinase, pregnancy-associated plasma protein-A (PAPP-A) and prevents cleavage of IGFBP4 (Jepsen et al. 2015).

The ER stress is mediated by three sensors and signaling pathways, but IRE1 is a central mediator of the unfolded protein response and an important component of tumor growth. Its blockade leads to suppression of a tumor 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, obesity, and type 2 diabetes. It may contribute to the expression profile of many regulatory genes resulting in proliferation, apoptosis, angiogenesis, and peripheral insulin resistance (Minchenko et al. 2013b; Clarke et al. 2014; Han and Kaufman 2014; Manie et al. 2014; Minchenko et al. 2015a). Two distinct catalytic domains of the signaling enzyme IRE1 (protein kinase and endoribonuclease) are 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; Auf et al. 2010; Dejeans et al. 2012; Han et al. 2013; Pluquet et al. 2013; Maurel et al. 2014b).

The ER stress response-signaling pathway 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 (JNK) and subsequent serine phosphorylation of IRS1 (Langlais et al. 2011; Lenihan and Taylor 2013; Clarke et al. 2014; Minchenko et al. 2014). 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 (Bravo et al. 2013; Cao and Kaufman, 2013; Han et al. 2013; Clarke et al. 2014; Manie et al. 2014). There are data indicating that hypoxia may affect the expression of most INS and IGF related genes in IRE1 dependent manner (Minchenko et al. 2013b, 2015a). It has been also shown that hypoxia in children decreases the level of IGF1 and IGF2 proteins in plasma (Custodio et al. 2012).

The main goal of this work was to study the role of expression of genes encoding the IGF1, IGF2, and their receptor (IGF1R) as well as IFG binding protein-4 and stanniocalcin 2 (STC2) in U87 glioma cell line and its subline with IRE1 loss of function upon hypoxia for evaluation of its possible significance in the control of tumor growth through IRE1 mediated 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 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 cells. One subline was obtained by selection of stable transfected clones with overexpression of vector pcDNA3.1, which was used for creation of dn-IRE1. 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 different *IGF* 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 signaling 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 has been estimated previously (Minchenko et al. 2013b; 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). Both sublines of glioma cells used in this study are grown with the addition of geneticin (G418) while these cells carrying empty vector pcDNA3.1 or dn-ERN1 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 insulin-like growth factors (IGF1 and IGF2), IGF1 receptor (IGF1R), IGF binding protein-4 (IGFBP4), and stanniocalcin 2 (STC2) 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 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. 2012). Polymerase chain reaction was performed in triplicate.

For amplification of IGF1 cDNA, we used forward (5'–catgtcctcctcgcatctct–3' and reverse (5'–ggtgcgcaatacatctccag–3') primers. The nucleotide sequences of these primers correspond to sequences 297–316 and 551–532 of human IGF1 cDNA (GenBank accession number NM_000618). The amplification of IGF2 cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'–caatggggaagtcgatgctg–3' and reverse – 5'–ggaaacagcactcctcaacg–3'. The nucleotide sequences of these primers correspond to sequences 763–782 and 969–950 of human IGF2 cDNA (GenBank accession number NM_000612). For amplification of IGF1R cDNA we used forward (5'–gccactactactatgccggt–3' and reverse (5'–gtgcatccttggagcatctg–3') primers. The nucleotide sequences of these primers correspond to sequences 875–856 and 1111–1092 of human IGF1R cDNA (GenBank accession number NM_000875). The amplification of IGFBP4 cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'–caccccaacaacagcttcag–3' and reverse – 5'–agttggggatggggatgatg–3'. The nucleotide sequences of these primers correspond to sequences 679–698 and 922–903 of human IGFBP4 cDNA (GenBank accession number NM_001552). For amplification of STC2 cDNA we used two oligonucleotides primers: forward – 5'–gttcatgaccctggctttgg–3' and reverse – 5'–gcgtgggccttacatttcaa–3'. The nucleotide sequences of these primers correspond to sequences 1334–1353 and 1648–1629 of human STC2 cDNA (GenBank accession number NM_003714). 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 control 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" and statistical analysis – as described previously (Bochkov et al 2006). The values of *IGF1, IGF2, IGF1R,* and *IGFBP4* gene expressions were normalized to the expression of beta-actin mRNA and represent as percent of control (100%). All values are expressed as mean ± SEM from triplicate measurements performed in four independent experiments. 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 *IGF1, IGF2, IGF1R, IGFBP4* and *STC2* genes in U87 glioma cells. To investigate a possible role of ER stress signaling mediated by IRE1 bifunctional enzyme in the expression level of IGFs, IGF1 receptor and IGF binding proteins 4 and 5 mRNA, we studied the effect of IRE1 inhibition by dnIRE1 on these genes expression in U87 glioma cells. As shown in Figure 1a, the expression level of IGF1 mRNA was significantly down-regulated (2.3 times) in U87 glioma cells by inhibition of IRE1 signaling enzyme in comparison with the control cells. The expression level of IGF2 mRNA in glioma cells without IRE1 signaling enzyme function was also down-regulated (1.6 times) in comparison with the control glioma cells. The level of IGF2 protein was also significantly decreased (Figure 1b). At the same time, inhibition of IRE1 by dnIRE1 slightly but statistically significantly up-regulated the expression level of IGF receptor (IGF1R) mRNA (+26%) in glioma cells in comparison with the control cells (Figure 1a). We next investigated the expression of *IGFBP4* gene in relation to the inhibition of IRE1 signaling enzyme function. As shown in Figure 1a, the expression level of this IGF binding protein-4 was strongly increased (3.8 times) in glioma cells with suppressed function of IRE1 signaling enzyme. More strong induction was shown for *STC2* gene (6.1 times). Thus, the inhibition of ER stress signaling mediated by IRE1 enzyme affects the

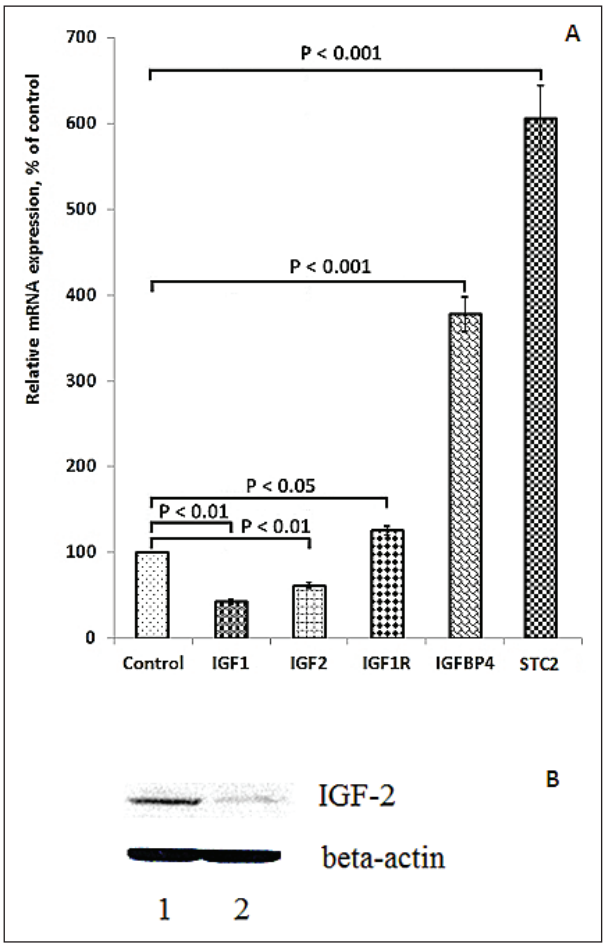

Figure 1. Inhibition of IRE1 by dnIRE1 affects the expression level of insulin-like growth factors IGF1 and IGF2, IGF1 receptor (IGF1R), IGF binding protein-4 (IGFBP4), and stanniocalcin 2 (STC2) mRNA (a) and IGF2 protein (b) in U87 glioma cells assessed by qPCR (a) and Western blot (b). Values of these mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control (cells transfected be empty vector, 100 %); n=4. 1 – control; 2 – dnIRE1.

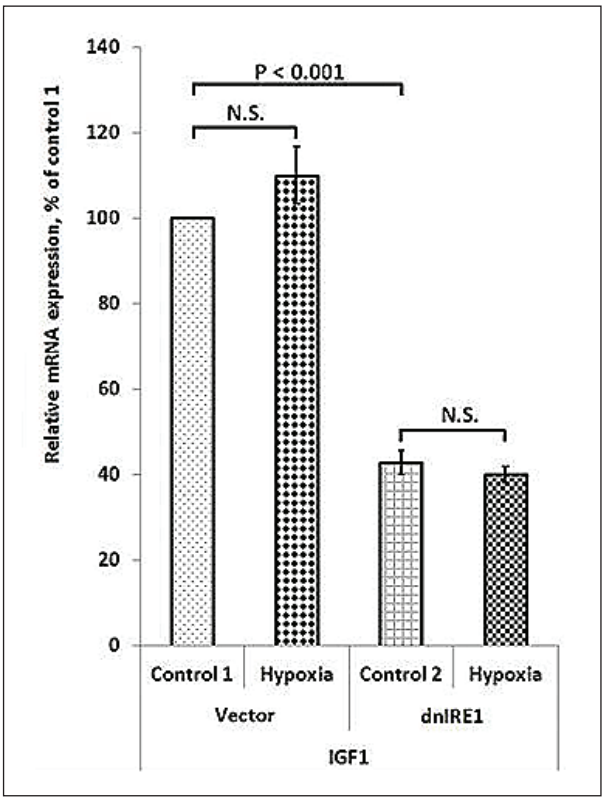

Figure 2. Effect of hypoxia on the expression level of insulin-like growth factor 1 (IGF1) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of IGF1 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100 %); n=4.

expression level of insulin-like growth factors, IGF receptor-1, IGF binding protein-4 and STC2 in gene-specific manner.

Effect of hypoxia on the expression of *IGF1, IGF2, IGF1R, IGFBP4,* and *STC2* genes in control glioma cells and cells without IRE1 enzyme function. We next investigated the effect of hypoxia on the expression of *IGF1, IGF2, IGF1R,* and *IGFBP4* genes in glioma cells in relation to inhibition of IRE1 signaling enzyme function. We found that expression levels of *IGF1* and *IGF1R* genes were resistant to hypoxia both in the control glioma cells and cells without IRE1 signaling enzyme function in comparison with corresponding controls (control 1 and 2) (Figure 2, Figure 4). As shown in Figure 3, hypoxia also did not change significantly the expression level of *IGF2* gene in control glioma cells in comparison with the control 1. At the same time, inhibition of IRE1 signaling enzyme introduced the sensitivity of *IGF2* gene expression to hypoxia. Thus, in glioma cells without IRE1 signaling enzyme function the expression level of *IGF2* gene was significantly down-regulated (2.5 times) in comparison with corresponding control (control 2; Figure 3).

As shown in Figure 5, the expression level of *IGFBP4* gene was strongly affected by inhibition of IRE1 signaling enzyme function in both control and IRE1 deficient glioma cells. Thus, inhibition of IRE1 enzyme function by dnIRE1 strongly up-regulated the expression level of IGFBP4 mRNA (1.9 times) in

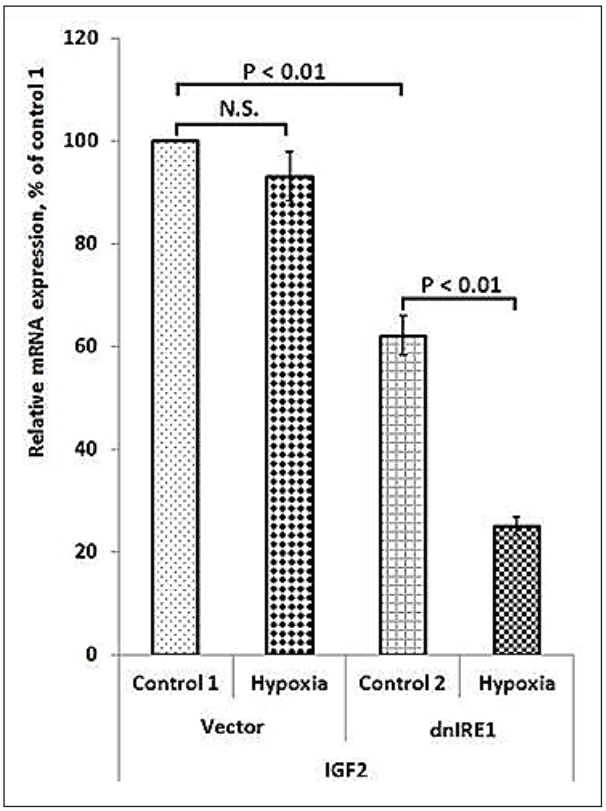

Figure 3. Effect of hypoxia on the expression level of insulin-like growth factor 2 (IGF2) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of IGF2 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100 %); n=4.

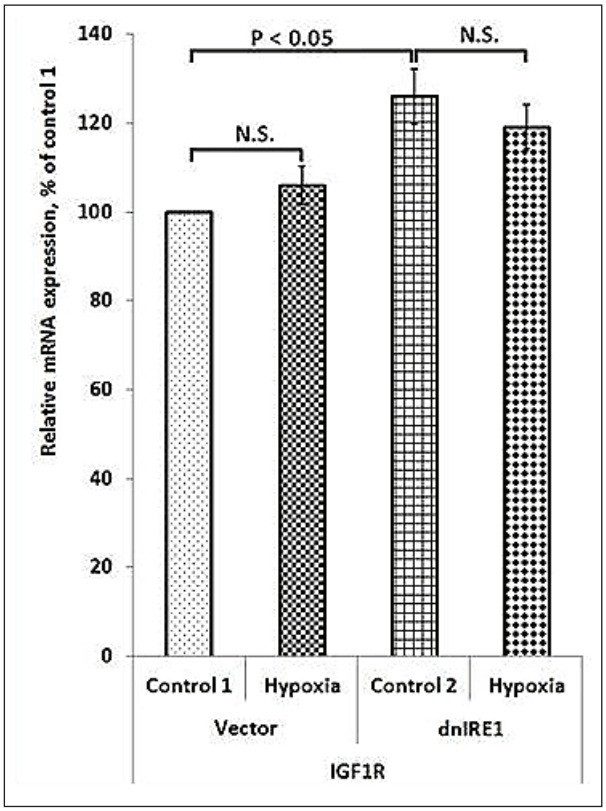

Figure 4. Effect of hypoxia on the expression level of insulin-like growth factor 1 receptor (IGF1R) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of IGF1R mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100 %); n=4.

control glioma cells and in 1.8 times in cells without IRE1 signaling enzyme function in comparison with corresponding controls (control 1 and control 2). Investigation of *STC2* gene expression showed high sensitivity to hypoxia (Figure 6). Thus, in control glioma cells the expression level of *STC2* gene was significantly up-regulated (7.0 times) in comparison with corresponding control (control 1) and slightly lesser induction of this gene expression (4.1 times) was observed in cells without IRE1 signaling enzyme function (Figure 6).

Thus, the expression of *IGF1* and *IGF1R* genes was resistant to hypoxia both in glioma cells with and without IRE1 signaling enzyme function, but *IGFBP4, STC2,* and *IGF2* genes expression was sensitive to treatment cells by hypoxia: significant up-regulation of *IGFBP4* and *STC2* genes and down-regulation of *IGF2* gene, but only in cells without IRE1 function. Moreover, effect of hypoxia on *IGFBP4* gene expression did not depend on the inhibition of IRE1 signaling enzyme, but the expression of *STC2* gene increased by hypoxia more significantly in control glioma cells in comparison with cells without IRE1 function.

Discussion

The growing tumor requires the ER stress and hypoxia for own neovascularization and growth as well as for inhibition of apoptosis (Drogat et al. 2007; Auf et al. 2010, 2013; Minchenko et al. 2015a). The ER has an important position as a signal integrator both in normal and malignant cells because its stress signaling pathways is involved in numerous metabolic pathways (Bravo et al. 2013; Minchenko et al. 2013b; Manie et al. 2014; Pluquet et al. 2014). It is known that the inhibition of the activity of IRE1 signaling

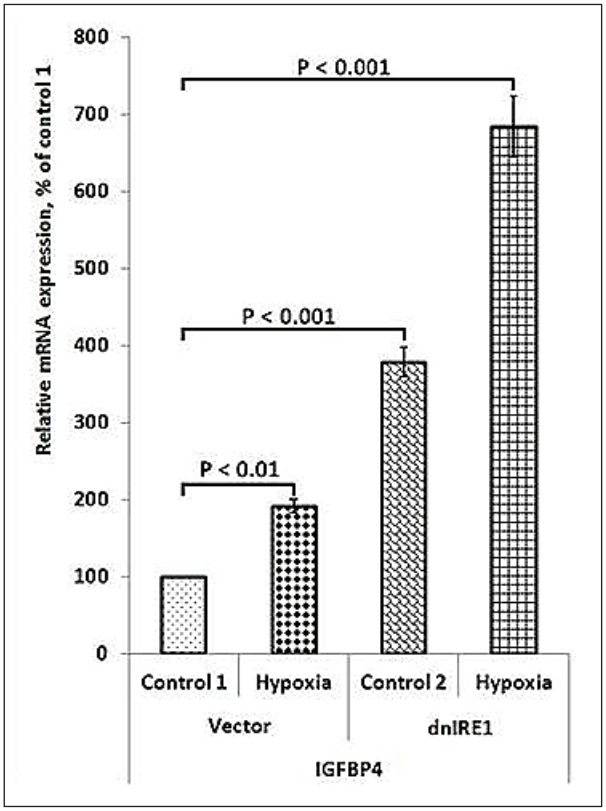

Figure 5. Effect of hypoxia on the expression level of insulin-like growth factor binding protein 4 (IGFBP4) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of IGFBP4 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100 %); n=4.

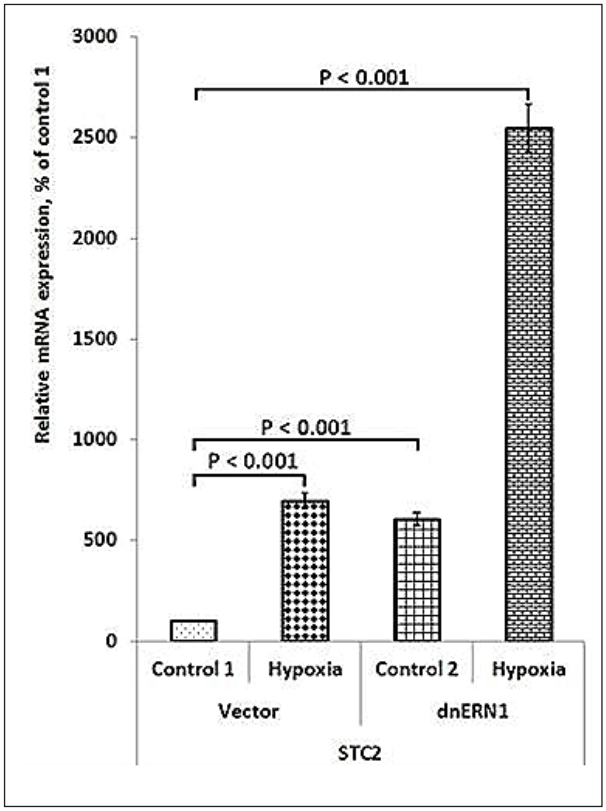

Figure 6. Effect of hypoxia on the expression level of stanniocalcin 2 (STC2) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dnIRE1 measured by qPCR. Values of IGFBP4 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100 %); n=4.

enzyme in glioma cells had anti-tumor effects (Auf et al. 2010, 2013; Minchenko et al. 2014, 2015c). In this work, we studied the expression of genes encoded different insulin-like growth factors, their receptor, and binding protein-4 as well as stanniocalcin-2, which responsible for stability of IFGBP4 and bioavailability of IGFs, in glioma cells with inhibition of IRE1 signaling enzyme function upon hypoxia. It is needed for evaluation of possible significance of these genes in the control of glioma growth through ER stress signaling mediated by IRE1 and hypoxia.

Results of this study demonstrate that inhibition of IRE1 signaling enzyme function significantly decreased the expression level of both *IGF1* and *IGF2* genes, indicating its participation in IRE1 mediated network of unfolded protein response. It is possible that decreased expression of *IGF1* and *IGF2* genes in glioma cells without enzymatic activity of IRE1 contributes to the suppression of proliferation and glioma growth from cells with IRE1 knockdown (Auf et al. 2010, 2013; Minchenko et al. 2015c). These results conform the view that *IGF1* and *IGF2* have mainly pro-proliferative functions through interaction with different proteins and signaling pathways and are overexpressed in various malignant tumors (Pollak 2008; Halje et al. 2012; Panda et al. 2013; Baxter 2014). We also showed that the expression level of *IGF1R* gene slightly increases upon inhibition of IRE1. It is possible that this increase in *IGF1R* gene expression level is a result of IGFs down-regulation as an emerging paradigm of insulin-like growth factor type 1 receptor signaling regulation (Girnita et al. 2014). At the same time, IGF signaling system is over expressed in variety of cancer, especially in prostate and breast cancer, and inhibitors of IGF signaling have been subjected to clinical cancer trials with the

main objective to confirm the effectiveness of these receptors as a therapeutic target (Singh et al. 2014). However, the results, which these trials produced, proved to be disappointing as the role played by the cross-talk between IGF and insulin receptor signaling pathways at the receptor level or at downstream signaling level became more lucid (Singh et al. 2014).

The insulin-like growth factor binding proteins bind and regulate the availability of both IGFs with different affinity and inhibit or stimulate the growth promoting effects of the IGFs through IGF/insulin receptors and through many other signaling pathways and regulate cell proliferation and survival as well as cancer cell migration (Weroha and Haluska 2012; Baxter 2014; Pan et al. 2014; Singh et al. 2014). In this study, we are showing that inhibition of IRE1 signaling, which is a central mediator of the unfolded protein response and an important component of malignant tumor growth, is strongly up-regulated the expression level of *IGFBP4* gene in U87 glioma cells and that induction of this gene expression possibly contributes to suppression of IRE1 knockdown cell proliferation, because this protein negatively regulating IGF signaling and preferentially acts as a tumor

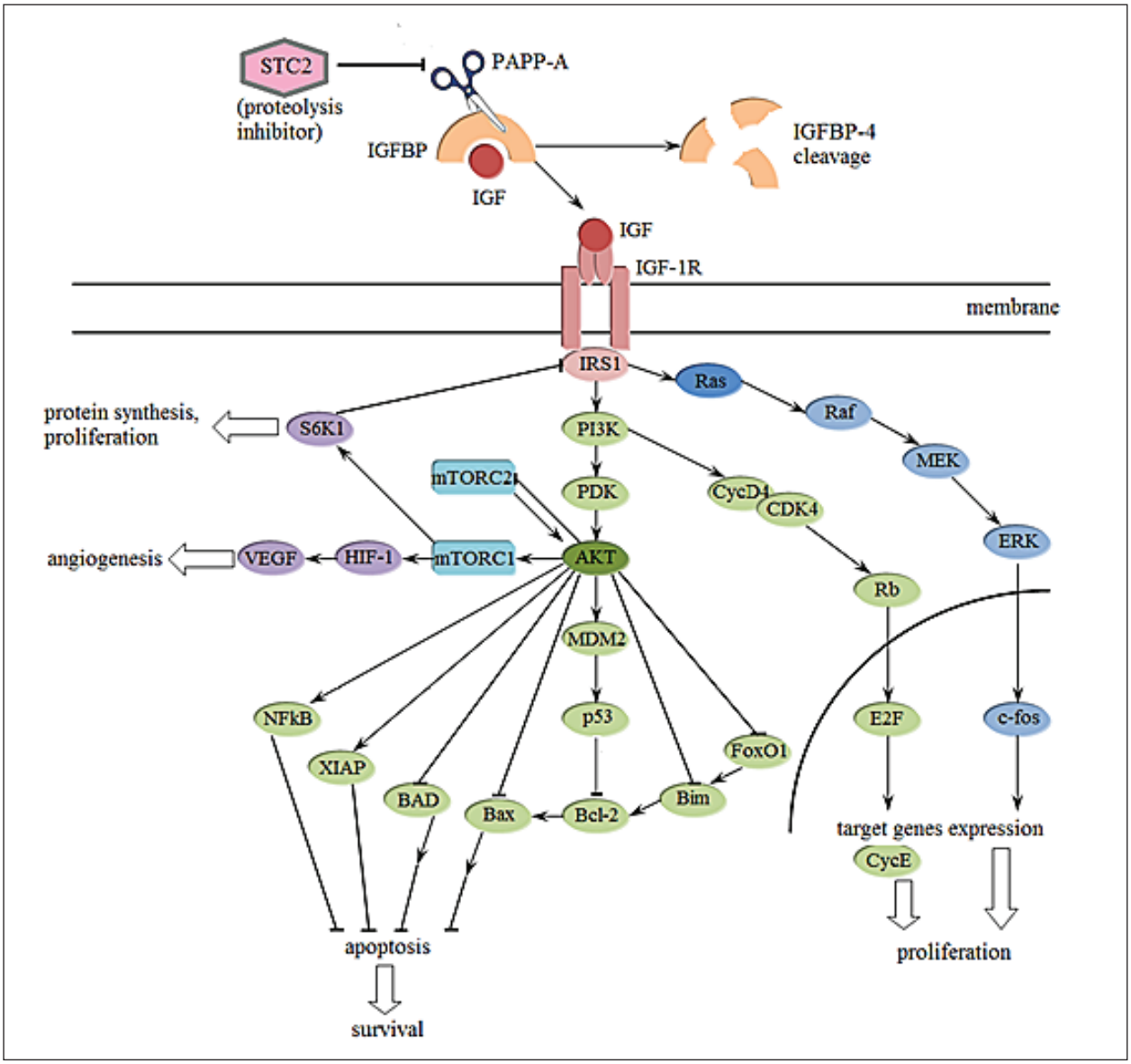

Figure 7. Schematic representation of mechanisms of regulation of IGF activity by IGFBP4.

suppressor (Praveen Kumar et al. 2014). Recently it has been shown that inhibition of tumor-associated αvβ3 integrin regulates the angiogenic switch by enhancing expression of *IGFBP4*, an important negative regulator of IGF-1 signaling, leading to reduced angiogenesis and melanoma growth (Contois et al. 2015). It is interesting to note that adipose tissue expandability in response to high fat diet is associated with the IGFBP4 down-regulation (Gealekman et al. 2014).

We also observed significant up-regulation of *STC2* gene expression in glioma cells after inhibition of IRE1 signaling, which protects IGFBP4 protein from degradation by proteolytic inhibition of the growth-promoting metalloproteinase, pregnancy-associated plasma protein-A (PAPP-A) (Jepsen et al. 2015). Thus, up-regulation of the expression of these multifunctional proteins (STC2 and IGFBP4) in glioma cells after inhibition of IRE1 can contribute to the suppression of these glioma cells proliferation, because IGFBP4 protein negatively regulating IGF signaling (Praveen Kumar et al. 2014; Contois et al. 2015).

Our data demonstrate that all of the genes studied are ER stress responsive and consequently play a pivotal role in the control of cell proliferation as well as metastasis. However, the mechanisms of activation or suppression of these genes expression upon inhibition of IRE1 differs and warrant further studies.

The investigation of hypoxic regulation of the expression of different IGFs and related genes in glioma cells in respect to inhibition of IRE1 signaling is very important for understanding of malignant tumor growth mechanisms, because low oxygen has essential role in the control of proliferation as that tumor progression (Lenihan and Taylor 2013; Minchenko et al. 2013a, 2015a). We showed that hypoxia leads to up-regulation of the expression of *IGFBP4* and *STC2* genes in both types of used glioma cells and down-regulation of *IGF2* gene but only in glioma cells with inhibited IRE1 activity. This down-regulation of IGF2 gene expression is correlated well with anti-proliferative effect of IRE1 inhibition; however, the functional significance of up-regulation of *IGFBP4* as well as *STC2* gene expression is not clear yet (Figure 7) and warrants further investigation. At the same time, the expression of *IGF1* and *IGF1R* genes was resistant to hypoxic exposure in both types of glioma cells (control and IRE1 knockdown). Thus, inhibition of IRE1 modifies the sensitivity of some studied genes expression to hypoxia and induces negative effect of IRE1 inhibition on *IGF2* gene expression as well as reduces effect of hypoxia on the expression of *STC2* gene in glioma cells. At the same time, effect of hypoxia on the expression of *IGFBP4* gene is not modified by inhibition of IRE1 enzyme in U87 glioma cells.

This study provides unique insights into the molecular mechanisms regulating the expression of genes encoded insulin-like growth factors and some related proteins and their correlation with slower cell proliferation in cells harboring dnIRE1, attesting to the fact that endoplasmic reticulum stress is a necessary component of malignant tumor growth, cell survival and metastasis. Moreover, the expression of some studied genes upon hypoxia is significantly depended on the IRE1 signaling enzyme function; however, the detailed molecular mechanisms of this regulation are complex yet and warrants further studies.

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