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Expression of insulin-like growth factor binding protein genes and its hypoxic regulation in U87 glioma cells depends on ERN1 mediated signaling pathway of endoplasmic reticulum stress

[1,2]Minchenko DO, [1]Kharkova AP, [1]Karbovskyi LL, [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 association between the expression of insulin-like growth binding protein-1 and -2 (*IGFBP1* and *IGFBP2*), insulin-like growth factor 2 mRNA binding protein 3/KH domain containing protein over-expressed in cancer (*IGF2BP3*/*KOC1*), and HtrA serine peptidase 1/serine protease with IGF-binding domain (*HTRA1/PRSS11)* genes and function of endoplasmic reticulum stress signaling mediated by ERN1 (endoplasmic reticulum to nucleus signaling 1) as well as the regulation of these genes by hypoxia in U87glioma cells.

Methods. The expression of *IGFBP1, IGFBP2*, *IGF2BP3,* and *HTRA1* genes in U87 glioma cells and its subline with ERN1 signaling enzyme loss of function, were analyzed by qPCR. Cells underwent to hypoxia exposure (3% oxygen, 16 h).

Results. The blockade of both enzymatic activities (kinase and endoribonuclease) of ERN1 in glioma cells led to a significant down-regulation of the expression of *IGFBP1, IGFBP2,* and *IGF2BP3* genes and strong up-regulation of *HTRA1*. At the same time, the inhibition of ERN1 endoribonuclease significantly increased the expression of *IGFBP1, IGFBP2,* and *HTRA1* genes and did not affect the *IGF2BP3* gene expression. Hypoxia up-regulated the expression of *IGFBP1* and *IGFBP2* genes in control glioma cells, with more significant changes in *IGFBP1* gene. Furthermore, effect of hypoxia on these gene expressions was significantly lower in glioma cells without ERN1 signaling enzyme function.

Conclusions. Results of this study demonstrate the dependence of insulin-like growth binding proteins as well as *IGF2BP3* and *HTRA1* gene expressions in U87 glioma cells on ERN1 signaling enzyme function and hypoxia, indicating its participation in the regulation of metabolic and proliferative processes via IGF/INS receptors, because endoplasmic reticulum stress is an important component of tumor growth and metabolic diseases.

Key words: IGFBP1, IGFBP2, HTRA1/PRSS11, mRNA expression, U87 glioma cells, ERN1 knockdown, hypoxia

Receptors of insulin-like growth factor (IGF) and insulin as well as related proteins play an important role in the regulation of numerous metabolic and proliferative processes and participate in endoplasmic reticulum stress, which is an important factor of tumor growth, insulin resistance, and obesity (Ozcan et al. 2004; Pollak 2012; Cao and Kaufman 2013; Kuijjer et al. 2013; Yuzefovych et al. 2013; Han and Kaufman 2014; Lee and Ozcan 2014). It is interesting to note that there exists a cross talk between IGF and insulin receptor signaling

Corresponding author: Dmytro O. Minchenko, MD, 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.

pathways at the receptor level or downstream signaling level. This cross talk significantly changed a variety of cancers as a result of insulin receptor isoform. An over-expression and formation of hybrid receptor isoforms between IGF1 receptor and insulin receptor, which are sensitive to the stimulation of all three IGF axis ligands, as well as hybrid receptors of IGF1 receptor and insulin receptor with other tyrosine kinase potentiate the cellular transformation, tumorigenesis, and tumor vascularization (Weroha and Halushka 2012; Kuijjer et al. 2013; Singh et al. 2013).

The insulin-like growth factor binding proteins (IGFBPs) bind and regulate the availability of both IGFs, prolong the half-life of the IGFs, and inhibit or stimulate the growth promoting effects of the IGFs through IGF/INS receptors. They are now understood to have many actions beyond their endocrine role in IGF transport (Azar et al. 2011; Foulstone et al. 2013; Zhu et al. 2014). IGFBPs are also functioning in the pericellular and intracellular compartments to regulate cell growth and survival. Because they interact with many proteins, IGF1 and IGF2, in addition to their canonical ligands, also play an intranuclear role in the regulation of transcription (Baxter 2014). Moreover, IGFBP2 not only modulates IGFs but also directly regulates PTEN. It has a role in the maintaining of the estrogen receptor-alpha expression (Foulstone et al. 2013). Additionally, IGFBP2 is increased in the blood of lung cancer and glioblastoma suffering patients. However its complex with alpha2 macroglobulin is significantly lower in cancer patients (Zheng et al. 2011; Hsieh et al. 2010; Ahani et al. 2014; Hu et al. 2014; Sunderic et al. 2014). Recently, it has been shown that exogenous IGFBP2 promotes proliferation and invasion in glioma cells via the integrin β1-ERK pathway and that this IGFBP with MDA9/syntenin-promotes angiogenesis in human melanoma (Han et al. 2014). Holmes et al. (2012) have shown that IGFBP2-driven glioma progression is prevented by blocking an integrin β1, integrin-linked kinase, and nuclear factor-κB (NF-κB) network, because this network serves as a physiologically active signaling pathway in vivo by driving glioma growth. Furthermore, IGFBP2 enhances VEGF gene promoter activity and consequent promotion of angiogenesis by neuroblastoma cells as well as promotes glioma tumor stem cell expansion and survival (Azar et al. 2011; Hsieh et al. 2010). It is interesting to note that both IGFBP1 and IGFBP2 are linked to insulin resistance, obesity, and the type 2 diabetes mellitus, and metabolic syndrome, because they are at the interface of growth and metabolism (Nedic et al. 2011; Sabin et al. 2011; Gokulakrishnan et al. 2012).

Insulin-like growth factor 2 mRNA binding protein 3/KH domain containing protein over-expressed in cancer (IGF2BP3/KOC1) can bind to the 5'-untranslated region of the IGF2 mRNA and may repress its translation and control its stability. Moreover, this protein contains several KH domains, which are important in RNA binding and are known to be involved in RNA synthesis and metabolism. The IGF2BP3 have been found to be re-expressed in several aggressive cancer types and is a major determinant in cancerogenesis (Findeis-Hosey and Xu 2012; Hartmann et al. 2012; Bell et al. 2013; Yamamoto et al. 2014). At the same time, *HTRA1/PRSS11* gene encoded serine protease with IGF-binding domain, which regulates the availability of IGFs by cleaving IGFBPs and has a pivotal role in both cell proliferation and differentiation (D'Angelo et al. 2014). It is down-regulated in various cancers (Zhu et al. 2010; Lehner et al. 2013; Singh et al. 2013; Wu et al. 2014). Elevated serine protease HtrA1 inhibits cell proliferation, reduces invasion, and induces apoptosis in cell carcinoma by blocking the NF-κB signaling pathway (Xia et al. 2013).

Many different factors, including chemicals, toxins, and hypoxia have been shown to induce a complex of intracellular signaling events in the endoplasmic reticulum, which is known as the unfolded protein response/endoplasmic reticulum stress (Moenner et al. 2007; Guan et al. 2010; Wang and Kaufman 2012; Minchenko et al. 2013b; Yadav et al. 2014). It participates in the early cellular response to the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum, which is a dynamic intracellular organelle with exquisite sensitivity to alterations in homeostasis, and provides stringent quality control systems to ensure that only correctly folded proteins transit to the Golgi apparatus and unfolded or misfolded proteins are retained and ultimately degraded (Lin et al. 2008). The endoplasmic reticulum stress is mediated by three sensor and signaling pathways (PERK, ATF6, and ERN1/IRE1), but ERN1 is a major pathway and its blockade leads to a decrease of tumor growth through suppression of the angiogenesis and proliferation processes (Bi et al. 2005; Fels and Koumenis 2006; Drogat et al. 2007; Auf et al. 2010). Activation of these endoplasmic reticulum stress sensors leads to transcriptional reprogramming of the cells (Maurel and Chevet 2013). The endoplasmic reticulum stress is recognized as an important determinant of cancer, obesity and type 2 diabetes and contributes to

the expression profile of many regulatory genes resulting in proliferation, apoptosis, and peripheral insulin resistance (Ozcan et al. 2004; Lin et al. 2008; Luo et al. 2010; Hetz et al. 2013; Minchenko et al. 2013a; Han and Kaufman 2014; Lee and Ozcan 2014; Manie et al. 2014). Two distinct catalytic domains of the signaling enzyme ERN1 were identified: a serine/threonine kinase and an endoribonuclease, which is responsible for degradation of a specific subset of mRNA and creation of the XBP1 (X-box binding protein 1) mRNA splice variant for control of the expression of unfolded protein response-specific genes (Acosta-Alvear et al. 2007; Aragon et al. 2009; Hollien et al. 2009; Auf et al. 2010; Arensdorf et al. 2013; Dejeans et al. 2012; Han et al. 2013; Pluquet et al. 2013; Maurel et al. 2014).

The endoplasmic reticulum stress response-signaling pathway is associated with hypoxia and linked to the neovascularization process, 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 (Denko et al. 2008; Guan et al. 2010; Langlais et al. 2011; Lenihan and Taylor 2013; Minchenko et al. 2013b; Minchenko et al. 2014). The endoplasmic reticulum has an essential position as a signal integrator in the cell and is instrumental in the different phases of tumor progression because the signaling pathways elicited by endoplasmic reticulum stress sensors have connections with metabolic pathways and with other plasma membrane receptor signaling networks (Manie et al. 2014).

The main goal of this work was to study the role of the genes encoding the IGFBP1 and IGFBP2, IGF2BP3, and HtrA serine peptidase 1/serine protease with IGF-binding domain in glioma cell line U87 and its subline with ERN1 loss of function for evaluation of its possible significance in the control of tumor growth through ERN1 mediated endoplasmic reticulum 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 three sublines of this glioma cells. One subline was obtained by selection of stable transfected clones with over-expression of vector pcDNA3.1, which was used for creation of dnERN1. This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of the effect of hypoxia on the expression level of *IGFBP* and related genes. Second subline was obtained by selection of stable transfected clone with over-expression of ERN1 dominant/negative construct (dnERN1) and has suppressed both protein kinase and endoribonuclease activities of this signaling enzyme (clone 1C5) (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 ERN1. Third subline is represented a stable transfected clone with over-expression of dnrERN1, a dominant/negative construct with suppressed only endoribonuclease activity of this signaling enzyme (Auf et al. 2013). The efficiency of ERN1 suppression in these glioma cell sublines was estimated previously (Minchenko et al. 2013a; Auf et al. 2013) by determining the expression level of the XBP1 alternative splice variant, a key transcription factor in the ERN1 signaling, and the level of the phosphorylated isoform ERN1 using cells treated by tunicamycin (0.01 mg/ml during 2 h).

To test if endoplasmic reticulum stress regulates the tested IGFBP genes through kinase activity of ERN1 or through other branches of this stress, we investigated the effect of tunicamycin on the expression level of these genesin glioma cells deficient in endoribonuclease activity of ERN1.

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.

Reverse transcription and quantitative PCR analysis. The expression levels of IGFBP1 and IGFBP2, IGF2BP3/KOC1, and HtrA serine peptidase 1/serine protease with IGF-binding domain (HTRA1/PRSS11) mRNAs were measured in control U87 glioma cells and cells with a deficiency of ERN1 by quantitative polymer-

ase chain reaction in real-time using „Mx300P QPCR" (Stratagene, La Jolla, CA, U.S.A.) and SYBRGreen Mix (ABgene, Thermo Fisher Scientific, Epsom, Surrey, UK). QuaniTectReverseTranscription 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 *IGFBP1* cDNA we used forward (5'-tatgatggctcgaaggctct-3') and reverse (5'-gagacccaggatcctcttc-3') primers**.** The nucleotide sequences of these primers correspond to sequences 765-784 and 1020-1001 of human *IGFBP1*cDNA (GenBank accession number NM_000596). The amplification of *IGFBP2* cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward-5'-cctcaagtcgggtatgaaggag-3' and reverse-5'-caacaggaactggaccaggt-3'. The nucleotide sequences of these primers correspond to sequences 669-688 and 830-811 of human *IGFBP2* cDNA (GenBank accession number NM_000597). The amplification of *IGF2BP3/KOC1* cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward-5'-ggcaaaacggtgaatgaact-3' and reverse-5'-gtccactttgcagagccttc-3'. The nucleotide sequences of these primers correspond to sequences 1785-1804and 1983-1964 of human *IGF2BP3* cDNA (GenBank accession number NM_006547). For amplification of *HTRA1/PRSS11* cDNA we used forward (5'-tggaatctcctttgcaatcc-3') and reverse (5'-acgctcctgagatcacgtct-3') primers. The nucleotide sequences of these primers correspond to sequences 1175-1194 and 1365-1346 of human *HTRA1* cDNA (GenBank accession number NM_002775). The amplification of *ACTB* (beta-actin) cDNA 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.).

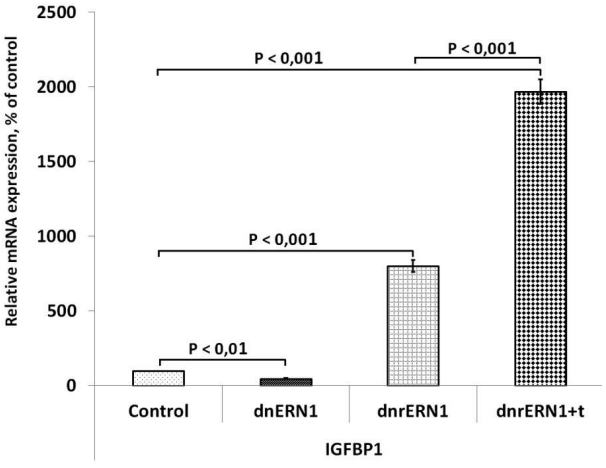

Fig. 1. Expression of insulin-like growth factor binding protein 1 (IGFBP1) mRNA in control U87 glioma cells (Control) and cells with a blockade of both kinase and endoribonuclease activities of the ERN1 (endoplasmic reticulum to nucleus signaling 1) by dnERN1 (dnERN1) or with a blockade of only endoribonuclease activity by dnrERN1 (dnrERN1) as well as in dnrERN1 cells treated by tunicamycin (dnrERN1 + t) measured by qPCR. Values of IGFBP1 mRNA expression were normalized to the expression of beta-actin mRNA and represent as percent of control (100%); n = 4.

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 *IGFBP1, IGFBP2, IGF2BP3,* and *HTRA1* 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 4 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

Expression of IGFBP genes in glioma cells with ERN1 knockdown. We found that IGFBP genes (*IGFBP1, IGFBP2, IGF2BP3,* and *HTRA1/PRSS11)*, which have relation to the control of many physiological processes, including angiogenesis, cell proliferation, and apoptosis, are expressed in U87 glioma cells. The levels of the expression of these genes are significantly dependent on the ERN1 signaling enzyme function. Thus, the expression levels of *IGFBP1* mRNA in glioma cells, deficient in both enzymatic activities (kinase and endoribonuclease) of signaling enzyme ERN1 (dnERN1), were decreased more than 2 times in comparison with the control cells (Fig. 1). At the same time, inhibition of only ERN1 endoribonuclease activity by dnrERN1 led to an increase of *IGFBP1* mRNA expression levels 8 times in comparison with the control glioma cells (Fig. 1). Thereafter, we tested the effect of endoplasmic reticulum stress induced by tunicamycin 2 h treatment on this gene expression in glioma cells deficient in en-

doribonuclease activity of ERN1. Thus, the expression of *IGFBP1* mRNA induced by tunicamycin increased 2.5 times in cells without endoribonuclease activity of ERN1 (Fig. 1). These results demonstrate that the inhibition of ERN1 endoribonuclease activity does not completely eliminate the regulation of this gene expression by endoplasmic reticulum stress.

We next examined *IGFBP2* gene expression in glioma cells with inhibition both kinase and endoribonuclease activities as well as only endoribonuclease activity. As shown in Fig. 2, the changes in the expression levels of this mRNA were at these experimental conditions similar to that described above for *IGFBP1* gene: more than in 2 times down-regulation in cells without both enzymatic activities of ERN1 and strong up-regulation (12 times) in cells without ERN1 activity. At the same time, no significant changes were found in *IGFBP2* gene expression in tunicamycin-treated glioma cells with a deficiency of ERN1 endoribonuclease (Fig. 2).

Thereafter, we tested how ERN1 inhibition modulates the expression of *IGF2BP3* gene, which encode the IGF2BP3 for regulation of its translation and stability. Thus, the expression levels of *IGF2BP3* mRNA in glioma cells without both kinase and endoribonuclease activities of ERN1 signaling enzyme decreased 2.5 times in comparison with the control cells (Fig. 3). However, inhibition of only ERN1 endoribonuclease activity by dnrERN1 does not affect significantly the expression of this mRNA in comparison with the control glioma cells (Fig. 3). At the same time, induction of endoplasmic reticulum stress by tunicamycin led to a strong down-regulation of *IGF2BP3* gene expression in glioma cells without ERN1 endoribonuclease activity (Fig. 3).

Additionally, we studied the effect of ERN1 inhibition on the expression of gene, encoded a serine protease with IGF-binding domain, which regulate the availability of IGFs by cleaving IGFBPs. As shown in Fig. 4, the expression levels of *HTRA1/PRSS11* mRNA are strongly up-regulated in both types of glioma cells with modified ERN1: in cells without both ERN1 enzymatic activities (kinase and endoribonuclease) 5.5 times and 4.2 times in cells with suppressed endoribonuclease activity of ERN1 in comparison with the control cells. At the same time, no significant changes were found in *HTRA1* gene expression in glioma cells with a deficiency of ERN1 endoribonuclease treated by tunicamycin (Fig. 4).

Effect of hypoxia on the expression of IGFBP genes in glioma cells with and without ERN1 function. To reveal a possible role of endoplasmic reticulum stress signaling mediated by ERN1 in hypoxic regulation of the expression of different IGFBP genes, we studied the effect of hypoxia on these genes expression in glioma cells with normal ERN1 function (control cells) and cells without both enzymatic activities of this signaling enzyme (dnERN1). As shown in Fig. 5, the expression levels of *IGFBP1* mRNA are strongly up-regulated in

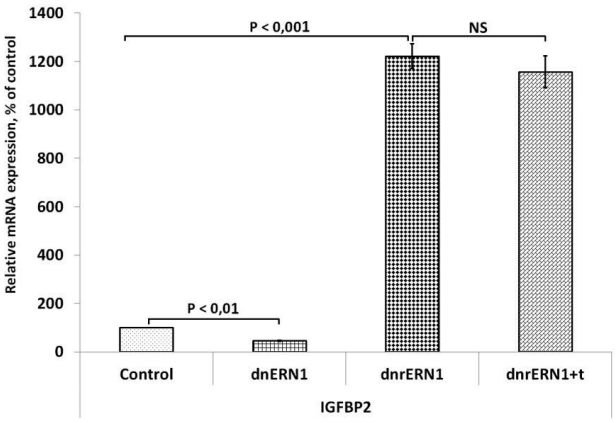

Fig. 2. Expression of insulin-like growth factor binding protein 2 (IGFBP2) mRNA in control U87 glioma cells (Control) and cells with a blockade of both kinase and endoribonuclease activities of the ERN1 (dnERN1) or with a blockade of only endoribonuclease activity (dnrERN1) as well as in dnrERN1 cells treated by tunicamycin (dnrERN1 + t) measured by qPCR. Values of IGFBP2 mRNA expression were normalized to the expression of beta-actin mRNA and represent as percent of control (100%); n = 4.

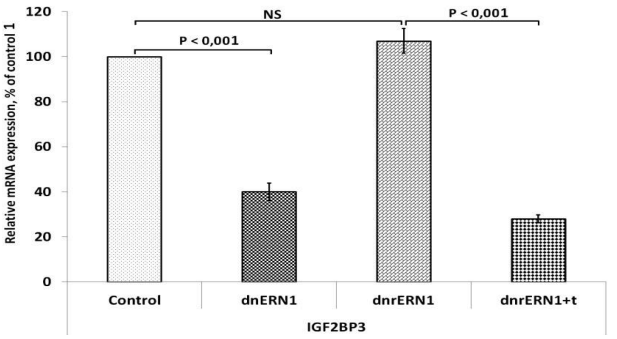

Fig. 3. Expression of insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3) mRNA in control U87 glioma cells (Control) and cells with a blockade of both kinase and endoribonuclease activities of the ERN1 (dnERN1) or with a blockade of only endoribonuclease activity (dnrERN1) as well as in dnrERN1 cells treated by tunicamycin (dnrERN1 + t) measured by qPCR. Values of IGF2BP3 mRNA expression were normalized to the expression of beta-actin mRNA and represent as percent of control (100%); n = 4.

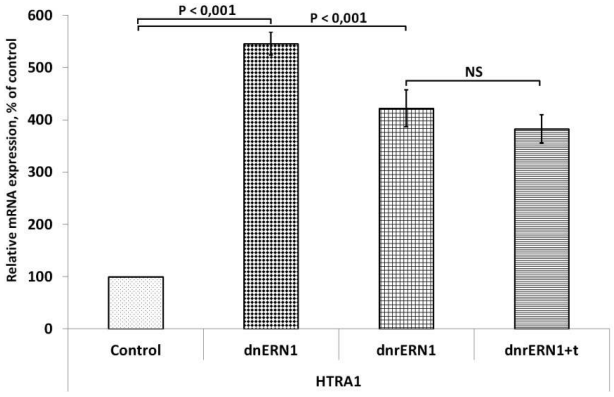

Fig. 4. Expression of HtrA serine peptidase 1/serine protease with IGF-binding domain (HTRA1/PRSS11) mRNA in control U87 glioma cells (Control) and cells with a blockade of both kinase and endoribonuclease activities of the ERN1 (dnERN1) or with a blockade of only endoribonuclease activity (dnrERN1) as well as in dnrERN1 cells treated by tunicamycin (dnrERN1 + t) measured by qPCR. Values of HTRA1 mRNA expression were normalized to the expression of beta-actin mRNA and represent as percent of control (100%); n = 4.

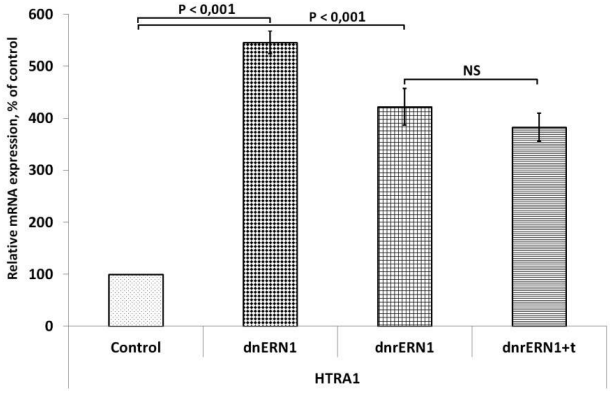

Fig. 5. Effect of hypoxia on the expression of insulin-like growth factor binding protein 1 (IGFBP1) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the ERN1 by dnERN1 (dnERN1) measured by qPCR. Values of IGFBP1 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control1 (100%); n = 4.

control glioma cells (5.6 times) in comparison with the control cells (control 1), but in cells with modified ERN1 (without both kinase and endoribonuclease activities) effect of hypoxia was significantly lower in comparison with the dnERN1 cells (control 2). At the same time, significantly lower changes were found in *IGFBP2* gene expression in by hypoxia treated glioma cells with normal function of signaling enzyme ERN1 (+23 %; Fig. 6). Moreover, inhibition of both enzymatic activities of ERN1 completely eliminated the effect of hypoxia on this gene expression (Fig. 6).

Investigation of *IGF2BP3* and *HTRA1* genes showed that both gene expressions in control glioma cells are resistant to hypoxia. However, the blockade of ERN1 enzyme function by dnERN1 in glioma cells significantly (2 times) down-regulated the expression of *IGF2BP3* gene, but did not change the expression of *IHTRA1* gene in comparison with the dnERN1 cells non-treated with hypoxia (Fig. 7, Fig. 8).

Discussion

The endoplasmic reticulum has an essential position as a signal integrator in both normal and malignant cells, because the endoplasmic reticulum stress signaling pathways have connections with various metabolic pathways and with other plasma membrane receptor signaling networks (Manie et al. 2014). Moreover, the growing tumor requires the endoplasmic reticulum stress as well as hypoxia, which initiate the endoplasmic reticulum stress for own neovascularization and growth, for apoptosis inhibition (Drogat et al. 2007; Auf et al. 2010). It is known that the complete blockade of the activity of ERN1 signaling as well as ERN1 endoribonuclease enzyme in glioma cells has an anti-tumor effect (Auf et al. 2010, 2013; Minchenko et al. 2014). In this work we studied the expression of genes encoding different IGFBPs in glioma cells with complete suppression of ERN1 function or only its endoribonuclease activity for evaluation of possible significance of these genes in the control of glioma growth through endoplasmic reticulum stress signaling mediated by ERN1.

Results of this study demonstrated that the blockade of ERN1 enzyme function significantly decreased the expression of IGFBP1 and IGFBP2 as well as IGF2BP3, but strongly up-regulated the expression of HtrA serine peptidase 1/serine protease with IGF-binding domain indicating its participation in ERN1 mediated network of endoplasmic reticulum stress. It is possible that decreased expression of *IGFBP, IGFBP2,* and *IGF2BP3* genes in glioma cells without both enzymatic activities of ERN1 contributes to the suppression of glioma growth from cells with ERN1 knockdown (Drogat et al. 2007; Auf et al. 2010, 2013). These results conform numerous data that *IGFBP1, IGFBP2,* and *IGF2BP3* have mainly pro-proliferative functions through interaction with different proteins and signaling pathways and are

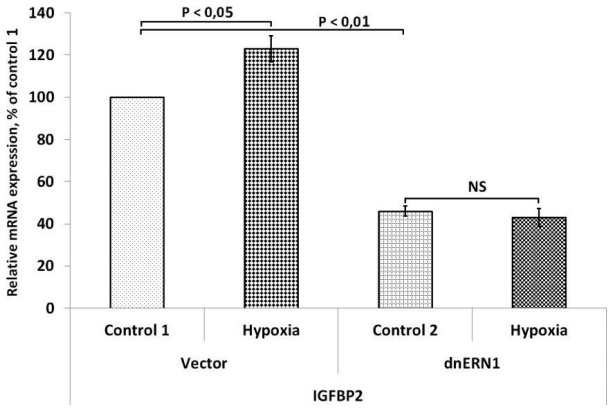

Fig. 6. Effect of hypoxia on the expression of insulin-like growth factor binding protein 2 (IGFBP2) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the ERN1 by dnERN1 (dnERN1) measured by qPCR. Values of IGFBP2 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control1 (100%); n = 4.

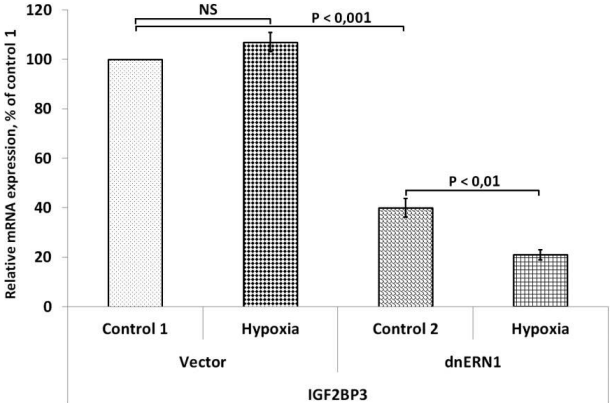

Fig. 7. Effect of hypoxia on the expression of insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the ERN1 by dnERN1 (dnERN1) measured by qPCR. Values of IGF2BP3 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control1 (100%); n = 4.

overexpressed in various malignant tumors (Hsieh et al. 2010; Foulstone et al. 2013; Guo et al. 2013; Ahani et al. 2014; Han et al. 2014; Hu et al. 2014; Sunderic et al. 2014; Zhu et al. 2014). Moreover, Marchand et al. (2006) have shown that IGFBP1 is potently induced during endoplasmic reticulum stress in human hepatocytes through ATF4 transcription factor, because mutation of the ATF4 site led to the loss of IGFBP1 regulation. At the same time, we observed strong up-regulation of *IGFBP1* and *IGFBP2* gene expressions in glioma cells with inhibition only endoribonuclease, without significant changes in the expression of *IGF2BP3* gene. It is possible that the expression of *IGFBP1* and *IGFBP2* genes is synergistically controlled by both kinase and endoribonuclease of bifunctional enzyme ERN1 and elimination only one enzymatic activity may destroy this regulation. Similar results have been previously shown for *FOXF1* (forkhead box F1) gene in the same experimental conditions (Minchenko et al. 2015).

Furthermore, significant up-regulation of *HTRA1/PRSS11* can also contribute to the suppression of ERN1 knockdown glioma cells proliferation, because there are data indicating that elevated expression of this protease inhibits cell proliferation and induces apoptosis in carcinoma cells by blocking the NF-κB signaling pathway (Xia et al. 2013). It is possible that *HTRA1/PRSS11* serine protease regulates the availability of IGFs by cleaving IGFBPs, because it has IGF-binding domain, and consequently has a pivotal role in the control of cell proliferation (D'Angelo et al. 2014). Thus, our data

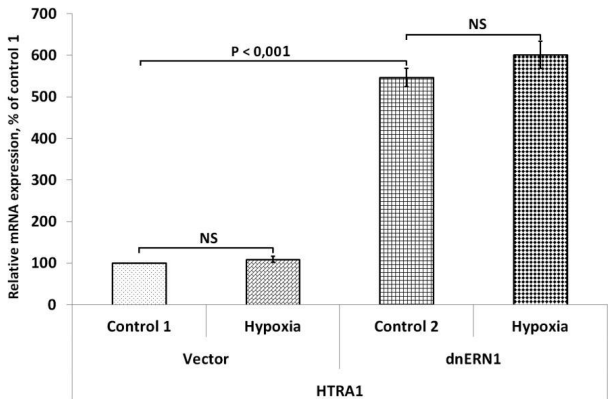

Fig. 8. Effect of hypoxia on the expression of HtrA serine peptidase 1/serine protease with IGF-binding domain (HTRA1/PRSS11) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the ERN1 by dnERN1 (dnERN1) measured by qPCR. Values of HTRA1 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100%); n = 4.

concerning the over-expression of *HTRA1/PRSS11* protease in ERN1 knockdown glioma cells completely agree with down-regulation of this gene expression in various cancers (Zhu et al. 2010; Lehner et al. 2013; Singh et al. 2013; Wu et al. 2014). We have also shown that the expression of *HTRA1* gene in glioma cells with inhibition only endoribonuclease is strongly up-regulated like in dnERN1 cells. Due to this fact the regulation of this gene expression is possibly controlled only by

ERN1 endoribonuclease, because in both dnERN1 and dnrERN1 glioma cells we have similar changes in *HTRA1* gene expression.

Our results demonstrate that all of the genes studied are endoplasmic reticulum stress responsive, but the mechanisms of activation or suppression of these gene expressions upon inhibition of ERN1 may differ. Tunicamycin experiments helped to clarify of some aspects of these regulatory mechanisms. It is possible that up-regulation of *IGFBP1* as well as down-regulation of *IGF2BP3* mRNA expression in response to endoplasmic reticulum stress induced by tunicamycins likely mediated through other signaling pathways of this stress. At the same time, the inhibition of only endoribonuclease of ERN1 eliminates the regulation of *IGFBP1* and *HTRA1* gene expressions by tunicamycin-induced endoplasmic reticulum stress, which supports the ERN1-mediated mechanisms of the expression of both these genes.

The investigation of the hypoxic regulation of the suppression of genes encoded different IGFBPs in glioma cells with and without ERN1 enzyme function shown that hypoxia is induced of *IGFBP1, IGFBP2,* and *IGF2BP3* gene expressions in control glioma cells and that this effect strongly depends on ERN1 signaling enzyme function, because blockade of both enzymatic activities of ERN1 significantly suppresses (*IGFBP1)*, introduces (*IGF2BP3)* or eliminates (*IGFBP2*) hypoxic regulation. Previously, it has been also shown the ERN1-dependent character on hypoxic regulation of *VEGF* and many other genes in human glioma and other carcinoma cells (Drogat et al. 2007; Minchenko et al. 2012, 2013a, 2014). It is possible that two other signaling pathways of endoplasmic reticulum stress also participate in the regulation of *IGFBP1* and *IGF2BP3* gene expressions by hypoxia, but not *IGFBP2*.

Finally, the changes observed in the most of the above studied IGFBPs gene expressions correlate well with slower cell proliferation in cells harboring dn-ERN1 or dnr-ERN1, attesting to the fact that endoplasmic reticulum stress is a necessary component of the malignant tumor growth and cell survival. Moreover, the expression of IGFBP1 and IGFBP2 genes is regulated by hypoxia in glioma cells and this regulation significantly depends on the ERN1 signaling enzyme function. However, the detailed molecular mechanisms of this regulation are complex and require further studies.

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