

Effect of the endoplasmic reticulum to nucleus signaling 1 knockdown on the expression of *MAP3K5*, *MAP4K3*, *CIB1*, *RIPK1*, and *RIPK2* genes in U87 glioma cells and its hypoxic regulation

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ABSTRACT

Background: ERN1 (endoplasmic reticulum to nucleus signaling 1)-mediated signaling pathway plays an important role in glioma growth through changing the numerous regulatory gene expressions. We studied the possible role of protein kinases MAP4K3, MAP3K5, RIPK1, and RIPK2 as well as protein kinase interacting protein CIB1 in suppression of U87 glioma cell proliferation upon ERN1 knockdown. **Materials and Methods:** The expression of MAP4K3, RIPK1, RIPK2, and CIB1 genes was measured in human U87 glioma cells and its regulation by hypoxia in dependence of ERN1 enzyme function using quantitative PCR. **Results:** Blockade of ERN1 signaling enzyme function significantly decreases the expression level of genes encoding MAP4K3, RIPK2, and CIB1 in U87 glioma cells, but increases expression of RIPK1 gene. At the same time, the blockade of ERN1 signaling enzyme does not affect the expression of MAP3K5 gene in glioma cells. Hypoxia down-regulates the expression level of MAP3K5, MAP4K3, RIPK1, and RIPK2 mRNAs in control glioma cells. However, inhibition of ERN1 affected hypoxia-mediated changes in expression of these kinase genes and their magnitude. **Conclusion:** Results of this investigation clearly demonstrated that the expression of MAP4K3, RIPK1, RIPK2, and CIB1 genes in human U87 glioma cells relies on blockade of ERN1-mediated endoplasmic reticulum stress signaling and is mostly down-regulated by hypoxia in dependence of ERN1 enzyme function.

KEY WORDS: Endoplasmic reticulum to nucleus signaling 1 knockdown, hypoxia, mRNA expression, receptor-interacting protein kinase 1, MAP4K3, U87 glioma cells

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INTRODUCTION

Malignant gliomas are highly aggressive tumors and are characterized by a marked angiogenesis and extensive tumor cell invasion into the normal brain parenchyma. Moreover, hypoxia condition is associated to glioma development and locally induces an adaptive response which confers to tumor cells an enhanced survival and a more aggressive behavior. Hypoxia, as well as the endoplasmic reticulum stress, are important factors of malignant tumor growth and control the expression of many genes, which regulate different metabolic processes and proliferation [1-3]. The endoplasmic reticulum stress is associated with unfolded protein response and accumulation of unfolded proteins in the endoplasmic reticulum [4,5]. This adaptive response is activated upon the accumulation of misfolded proteins in the endoplasmic reticulum and is mediated by three endoplasmic reticulum-resident sensors named PRK-like ER kinase, endoplasmic reticulum to nucleus signaling 1 (ERN1) also known as inositol requiring enzyme-1 alpha and activating transcription factor 6,

however, ERN1 is the dominant component of this system [6-9]. Induction of endoplasmic reticulum stress is the early cellular response to the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum and tends to limit the de novo entry of proteins in to the endoplasmic reticulum and facilitate both the endoplasmic reticulum protein folding and degradation to adapt cells for survival [10-12].

The ERN1 has two enzymatic activities: for serine/threonine kinase and endoribonuclease, which contribute to ERN1 signaling. The ERN1-associated endoribonuclease is activated after autophosphorylation of this enzyme and is responsible for degradation of a specific subset of mRNA and pre-X-box binding protein 1 (XBP1) mRNA splicing [13-16]. This mRNA splice variant (XBPIs) encodes a transcription factor that stimulates the expression of several hundreds of unfolded protein response-specific genes [17,18]. Moreover, nuclear translocation of XBPIs is controlled by p38 mitogen-activated protein (MAP) kinase and the regulatory subunits of phosphatidyl inositol 3-kinase,

but its interaction with the Forkhead box O1 transcription factor directs it toward proteasome-mediated degradation [19-21]. The ERN1-mediated endoplasmic reticulum stress response-signaling pathway is tightly linked to cell proliferation process and tumor growth because the complete blockade of ERN1 signal transduction pathway had anti-proliferative effects both in glioma and lung cancer [22-24].

Protein kinases, including MAP kinases (MAP3K5) and MAP4K3, receptor (TNFRSF)-interacting serine-threonine kinases receptor-interacting protein kinase 1 (RIPK1) and RIPK2 as well as NUA2, casein kinases and many others play an important role in malignant tumor growth as the key regulators of different metabolic processes [25-30]. Thus, there are data that stimulation of human aortic endothelial cells with tumor necrosis factor (TNF)-alpha led to an increased expression of p73 protein and a reduction in the levels of p53 involving MAP3K5, which is an apoptosis signal-regulating kinase (ASK) [27]. Moreover, high glucose-induced cell apoptosis and activation of MAP3K5 in mesangial cells is prevented by knockdown of thioredoxin interacting protein may be via reduction of oxidative stress [31]. It is interested to note that ASK1 and cyclin D1 compose a positive feedback loop contributing to tumor growth in gastric cancer [32]. It was also shown that SERTAD1 (SERTA domain containing 1), also known as CDK4-binding protein P34SEI or SEI-1, inhibits reactive oxygen species-induced cell death through by indirectly inducing ubiquitination of MAP3K5 [33]. Protein kinase MAP4K3 activates key effectors in cell signaling, has relation to migration and invasion of cancer cells, and is required for leucine-induced mTORC1 activation [28,34,35]. Moreover, this protein kinase orchestrates activation of BAX protein through the concerted posttranscriptional modulation of PUMA, BAD, and BIM [36].

Receptor-interacting kinase RIPK1 is a serine-threonine kinase, which transduces cell-death signals of c-FLIPL, specifically inhibits the interaction of the caspase 8 prodomain with the RIP1 death domain and, thereby, regulates caspase 8-dependent NF-kappaB activation [29]. It is interested to note that TNFSF12 (TNF [ligand] superfamily, member 12), also known as TNF-related weak inducer of apoptosis (TWEAK), induces apoptosis through a death-signaling complex, comprising RIPK1 as well as Fas-associated death domain (FADD) and caspase-8 [37]. Moreover, recently was shown that shikonin-mediated necroptosis in glioma cells is associated with the up-regulated expression of protein kinase RIPK1 and oxidative stress [38, 39]. It was also shown that knockdown of receptor-interacting serine/threonine-protein kinase 2 (RIPK2), also known as CARD-containing interleukin-1 (IL-1) beta ICE-kinase or RIP-like interacting caspase-like apoptosis regulatory protein (CLARP) kinase, down-regulated nuclear factor kappa B (NF-kB)-dependent plasminogen activator inhibitor Type 1 (PAI1), also known as serpin peptidase inhibitor, clade E, member 1, and vimentin gene expressions [30]. It is possible that protein kinase RIPK2 plays an important role in hepatic cell migration and inhibition of its tyrosine kinase activity limits NOD2-driven cytokine responses [40].

Protein kinase interacting protein calcium and integrin binding 1 (CIB1); calmyrin is a mediator of polycystic

kidney disease 2-driven carcinogenesis, may play a role in the regulation of apoptosis and angiogenesis and functions as a negative regulator of stress activated MAP kinase signaling pathways as well as polo-like kinase 3 [41-44].

The main goal of this study was an investigation the role of blockade of ERN1 signaling enzyme on the expression of MAP3K5, MAP4K3, RIPK1, RIPK2, and CIB1 genes in glioma U87 cells and its regulation by hypoxia.

MATERIALS AND METHODS

Cells

The glioma cells U-87 MG (ATCC HTB-14) was obtained from ATCC (USA) and grown in high glucose (4.5 g/l) Dulbecco's modified eagle's minimum essential medium (DMEM; Gibco, Invitrogen, USA) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., USA), penicillin (100 units/mL; Gibco) and streptomycin (0.1 mg/mL; Gibco) at 37°C in a 5% CO₂ incubator. In this study, we used two sublines of this these glioma cells. One subline was obtained by selection of stable transfected clones with overexpression of vector (pcDNA3.1), which was used for creation of dnERN1 (ERN1 dominant/negative constructs). This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of effects of hypoxia on the expression level of studied protein kinase and associated with kinase genes. Second subline was obtained by selection of stable transfected clones with overexpression of dnERN1 and has suppressed both protein kinase and endoribonuclease activities of ERN1 signaling enzyme [23].

The expression level of MAP3K5 and MAP4K3, receptor (TNFRSF)-interacting serine-threonine kinase RIPK1 and RIPK2 as well as CIB1 mRNAs in ERN1 knockdown glioma cells was compared with cells, transfected by vector (control 1). The glioma cells with blockade of ERN1 were also used as control 2 for investigation the effect of hypoxia on the expression of these genes upon ERN1 knockdown. Hypoxia was created in a special incubator with 3% oxygen and 5% carbon dioxide levels; culture plates were exposed to hypoxia for 16 h.

The blockade of both enzymatic activities of ERN1 in glioma cells that overexpress a dnERN1 was previously shown by analysis of ERN1 autophosphorylation and the expression of XBP1 alternative splice variant (XBP1s), a key transcription factor in ERN1 signaling, upon induction of endoplasmic reticulum stress by tunicamycin (0.01 mg/mL, 2 h) [11].

Experimental Procedure

Total RNA was extracted from glioma cells using trizol reagent according to manufacturer protocols (Invitrogen, USA). The RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water. For additional purification, RNA samples were re-precipitated with 95% ethanol and re-dissolved again in nuclease-free water. Quantitect reverse transcription kit

(QIAGEN, Germany) was used for cDNA synthesis. Polymerase chain reaction (PCR) was performed in triplicate.

The expression levels of MAP3K5, MAP4K3, RIPK1, RIPK2, and CIB1 mRNA were measured in glioma cell line U87 and its ERN1 knockdown subline (clone 1C5) by real-time quantitative PCR using “Mx 3000P QPCR” (Stratagene, USA) and Sybrgreen Mix (AB gene, Great Britain).

The amplification of MAP3K5 also known as ASK1 cDNA was performed using forward primer (5'-AAAGAGGCTTGCTGGCATAA-3') and reverse primer (5'-TCTGCAGACATGGACTCTGG-3'). These oligonucleotides correspond to sequences 2839-2858 and 3090-3071 of human MAP3K5 cDNA (GenBank accession number NM_005923). The size of amplified fragment is 252 bp. For the amplification of MAP4K3 also known as germinal center kinase-related protein kinase cDNA, we used forward (5'-GCATGGAGTTTTGTGGAGGT-3' and reverse (5'-CACTGCCAGAGATCACAGA-3') primers. The nucleotide sequences of these primers correspond to sequences 591-610 and 925-906 of human MAP4K3 cDNA (GenBank accession number NM_003618). The size of amplified fragment is 335 bp. The amplification of RIPK1 also known as cell death protein RIP cDNA for real time qPCR analysis was performed using two oligonucleotides primers: Forward -5'-TGGAAAAGCGTGATACACA-3' and reverse -5'-gacttctctgtgggtttgc-3'. The nucleotide sequences of these primers correspond to sequences 390-409 and 626-607 of human RIPK1 cDNA (GenBank accession number NM_003804). The size of amplified fragment is 237 bp. For the amplification of RIPK2 also known as CARD-containing IL-1 beta ICE-kinase or RIP-like interacting CLARP kinase cDNA, we used forward (5'-TTCCAATTTTGGGAATTTGC-3' and reverse (5'-ATGCGCCACTTTGATAAACCC-3') primers. The nucleotide sequences of these primers correspond to sequences 550-569 and 829-810 of human RIPK2 cDNA (GenBank accession number NM_003821). The size of amplified fragment is 280 bp. The amplification of (CIB1; calmyrin) also known as SNK-interacting protein 2-28 (SIP2-28) cDNA was performed using forward - 5'-CATTATGCCTTCCGCATCTT-3' and reverse - 5'-GCTGGCAAAGTCTGGAGAAC-3' primers. These primers nucleotide sequences correspond to 487-506 and 717-698 of human CIB1 cDNA (GenBank accession number NM_006384). The size of amplified fragment is 231 bp. For the amplification of beta-actin (ACTB) cDNA was used 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 size of amplified fragment is 234 bp. The expression of beta-actin mRNA was used as control of analyzed RNA quantity. The primers were received from “Sigma” (USA).

An analysis of qPCR was performed using special computer program “differential expression calculator” and statistical analysis using program OriginPro 7.5. The values of MAP3K5, MAP4K3, RIPK1, RIPK2, and CIB1 mRNA expressions were

normalized to the expression of beta-actin mRNA and represent as percent of control (100%). All values are expressed as mean $\pm$ standard error of the mean. From triplicate measurements performed in four independent experiments.

RESULTS

In this work, we studied the expression of genes encoded two MAP kinases (MAP kinase 3 and 5), two RIP kinases (receptor [TNFRSF]-interacting serine-threonine kinase 1 and 2) protein kinase and kinase interacting protein CIB1 in U87 glioma cells with knockdown of ERN1, the major component of endoplasmic reticulum stress signaling, as well as effect of hypoxia on these gene expressions. As shown in Figure 1, the suppression of both enzymatic activities of sensor and signaling enzyme ERN1 did not change significantly the expression level of MAP3K5 gene in U87 glioma cells but significantly decreased MAP4K3, RIPK2, and CIB1 gene expressions. At the same time, the expression of RIPK1 gene is up-regulated (almost to 2 fold) in glioma cells with suppressed function of ERN1 signaling enzyme. Thus, blockade of ERN1 signaling enzyme function affects the expression level most of the studied protein kinase genes as well as kinase interacting protein CIB1 gene.

As shown in Figure 2, the expression level of MAP3K5 mRNA is significantly decreased under hypoxia both in control glioma cells (–43%) and cells with blockade of ERN1 signaling enzyme function (–50%). The expression level of MAP4K3 gene is also suppressed under hypoxic condition in control glioma cells (–37%), but in cells with blockade of ERN1 signaling enzyme effect of hypoxia on the expression level of this mRNA was significantly less (–13%) [Figure 3]. Thus, blockade of ERN1 signaling enzyme function does not change significantly the

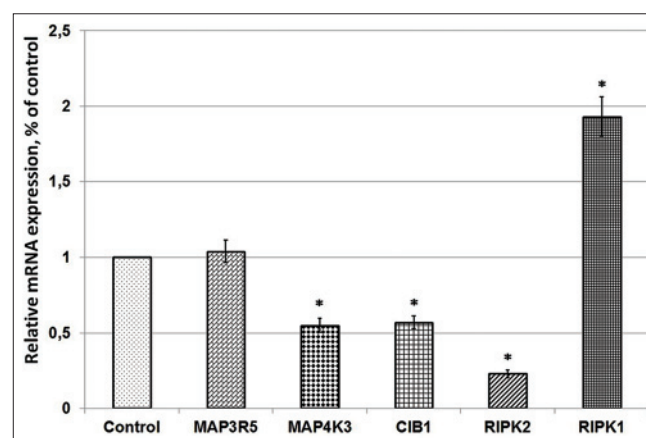


Figure 1: Effect of the blockade of signaling enzyme endoplasmic reticulum to nucleus signaling 1 (ERN1) by its dominant/negative constructs (dnERN1) on the expression level of mitogen-activated protein kinase (MAP3K5), MAP4K3, receptor-interacting protein kinase 1 (RIPK1), RIPK2, and calcium and integrin binding 1 mRNA in U87 glioma cells, measured by quantitative polymerase chain reaction. The level of this mRNA expression was normalized to the expression of beta-actin. The changes in the expression of different mRNA in ERN1 knockdown glioma cells were compared with control (cells, transfected with vector pcDNA3.1; 100%); $n = 4$; values are mean $\pm$ standard error of mean; * $P < 0.05$

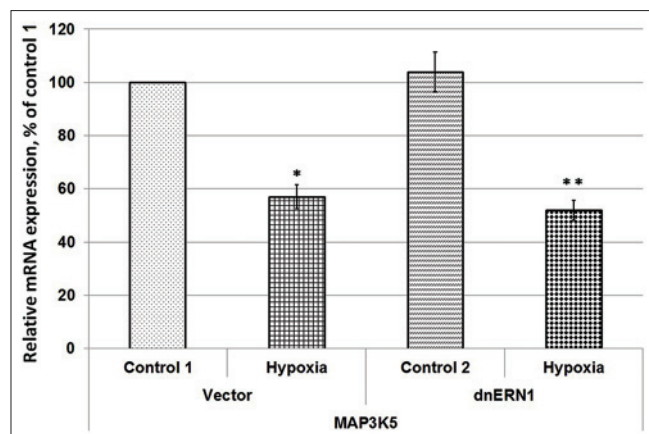


Figure 2: Effect of hypoxia on the expression level of mitogen-activated protein kinase kinase kinase and 5 mRNA, also known as apoptosis signal-regulating kinase 1, in U87 glioma cells, transfected with vector pcDNA3.1 (vector) and cells, transfected with dominant endoplasmic reticulum to nucleus signaling, measured by quantitative polymerase chain reaction. The level of this mRNA expression was normalized to the expression of beta-actin; $n = 4$; * $P < 0.05$ as compared to control 1, ** $P < 0.05$ as compared with control 2. In Figure 2-6: The changes in the expression of different mRNA in hypoxia in both types of glioma cells were compared with control 1 (cells, transfected with vector pcDNA 3.1; 100%), but in cells, transfected with dnERN1, – with control 2 (cells, transfected with dominant endoplasmic reticulum to nucleus signaling 1)

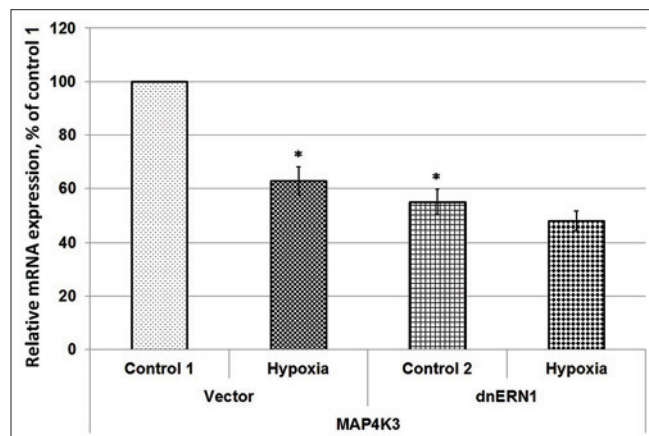


Figure 3: Effect of hypoxia on the expression level of mitogen-activated protein kinase kinase kinase and 3 mRNA, also known as the germinal center kinase-like kinase (GLK), in U87 glioma cells, transfected with vector or dominant endoplasmic reticulum to nucleus signaling 1, measured by quantitative polymerase chain reaction. The level of this mRNA expression was normalized to the expression of beta-actin; $n = 4$; * $P < 0.05$ as compared to control 1

effect of hypoxia on the expression level of MAP3K5 gene in U87 glioma cells modifies its effect on MAP4K3 gene expression.

Control glioma cells show slight but statistically significant decrease in the level of RIPK1 mRNA (–21%) under hypoxic condition [Figure 4]. At the same time, no significant changes were found under hypoxic condition in dnERN1 glioma cells (stable transfected by dnERN1). As shown in Figure 4, the

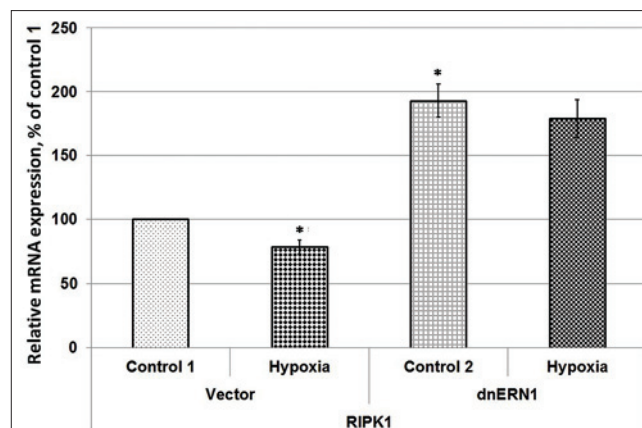


Figure 4: Effect of hypoxia on the expression level of receptor-interacting protein kinase 1 (RIP) (receptor (TNFRSF)-interacting serine-threonine kinase (1), also known as cell death protein RIP, mRNA in U87 glioma cells, transfected with vector or dominant endoplasmic reticulum to nucleus signaling 1, measured by quantitative polymerase chain reaction. The level of this mRNA expression was normalized to the expression of beta-actin; $n = 4$; * $P < 0.05$ as compared to control 1

expression level of RIPK2 mRNA is significantly decreased in control glioma cells at hypoxic condition; however, in cells with ERN1 knockdown effect of hypoxia on the expression of this gene was much less robust (–17%) [Figure 5]. Thus, down-regulation of ERN1 signaling enzyme eliminates the effect of hypoxia on RIPK1 mRNA level and significantly decreases hypoxic regulation of RIPK2 gene expression in glioma cells.

We also investigated the effect of hypoxia on the expression level of protein kinase interacting protein CIB1 mRNA [Figure 6]. We found that hypoxia does not affect the expression level of gene encoding CIB1 protein in control glioma cells, but in cells with ERN1 knockdown hypoxia causes slight statistically significant decrease (–18%) of this mRNA level. We, therefore, concluded that hypoxia down-regulates the expression level of MAP3K5, MAP4K3, RIPK1, and RIPK2 mRNA, but has no effect on CIB1 gene expression and that ERN1 knockdown modifies hypoxic regulation most of these genes in glioma cells.

DISCUSSION

In this study, we have shown that the inhibition of the ERN1 gene function in U87 glioma cells decreases the level of mRNA of MAP4K3, RIPK2, and CIB1 [Figure 1], which coordinates diverse cellular functions through the regulation of cell signaling, the expression of target genes, thereby inducing cell proliferation, migration, and invasion [28,34,35]. Protein kinase MAP4K3 is multifunctional pro-proliferative kinase and decrease of its expression after knockdown of ERN1 signaling enzyme can contribute to anti-proliferative effect of ERN1 knockdown [23,28,36]. Moreover, protein kinase RIPK2 as well as protein kinase interacting protein CIB1 may play a role in the regulation of apoptosis and angiogenesis and CIB1 also functions as a negative regulator of stress activated MAP kinase signaling pathways as well as Polo-like kinase 3 [30,41-44]. Thus, down-regulation of CIB1

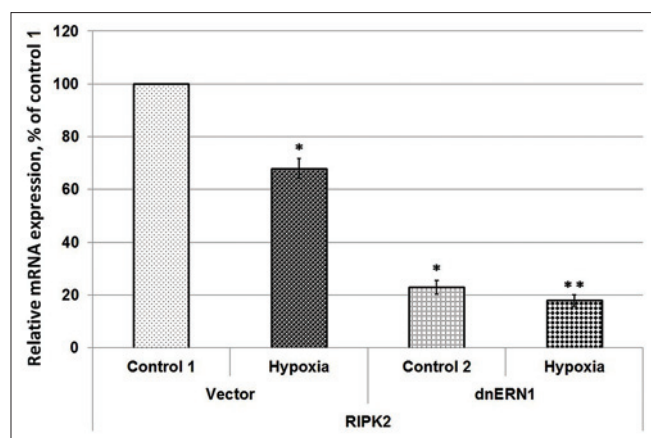


Figure 5: Effect of hypoxia on the expression level of receptor-interacting protein kinase 2 (RIPK2) (receptor-interacting serine-threonine kinase 2), also known as RIP-like interacting caspase-like apoptosis regulatory protein kinase, mRNA in U87 glioma cells, transfected with vector or dominant endoplasmic reticulum to nucleus signaling 1, measured by quantitative polymerase chain reaction. The level of this mRNA expression was normalized to the expression of beta-actin; $n = 4$; * $P < 0.05$ as compared to control 1

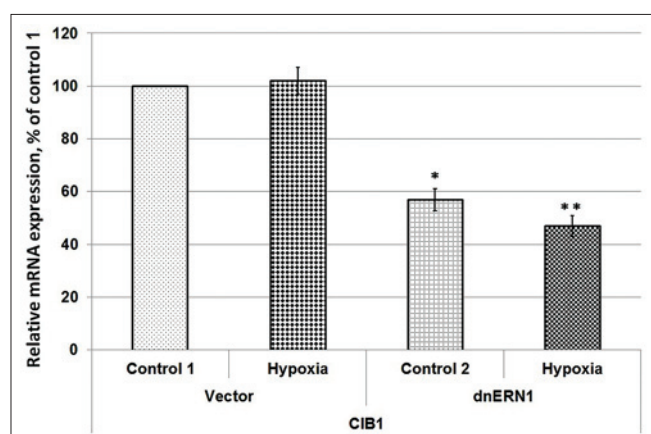


Figure 6: Effect of hypoxia on the expression level of calcium and integrin binding 1, also known as SNK-interacting protein 2-28 (SIP2-28), mRNA in U87 glioma cells, transfected with vector or dominant endoplasmic reticulum to nucleus signaling 1, measured by quantitative polymerase chain reaction. The level of this mRNA expression was normalized to the expression of beta-actin; $n = 4$; * $P < 0.05$ as compared to control 1, ** $P < 0.05$ as compared with control 2

gene expression in glioma cells with ERN1 knockdown possibly also contributes to suppression of tumor growth from these cells [23].

We have also demonstrated that ERN1 inhibition did not changes mRNA expression level of protein kinase MAP3K5, which is an ASK [Figure 1]; however, this protein kinase and cyclin D1 compose a positive feedback loop contributing to tumor growth in cancer [32] and decreased expression of cyclin D1 in these cells [45] possibly contributes to suppression of cell proliferation after blockade of ERN1 [23].

We have also shown that RIPK1 gene expression is strongly up-regulated in glioma cells when ERN1 function is inhibited

[Figure 4]. These results correlate with data that ERN1 knockdown suppressed proliferation rate of these cells [23] because TWEAK induces apoptosis through a death-signaling complex comprising RIPK1 as well as FADD and caspase-8 [37]. Moreover, strong down-regulation of RIPK2 in glioma cells after blockade of ERN1 [Figure 5] is argued with anti-proliferative effect of ERN1 knockdown, because there are data that protein kinase RIPK2 plays an important role in hepatic cell migration and inhibition of its tyrosine kinase activity limits NOD2-driven cytokine responses and down-regulated NF- κ B-dependent PAI1 gene expression, which may promote tumor growth through inhibition of apoptosis [30,40,46,47].

Results of this investigation clearly demonstrated that the expression of MAP4K3, RIPK1, RIPK2, and CIB1 genes in U87 glioma cells is dependent from blockade of ERN1-mediated endoplasmic reticulum stress and is mostly regulated by hypoxia in dependence from ERN1 signaling enzyme function. Significant increase of the expression of RIPK1 as well as decrease of MAP4K3, RIPK2, and CIB1 genes in glioma cells with knockdown of signaling enzyme ERN1 correlates with suppressed proliferation rate of these cells and possibly contributes in this effect, because proteins, encoded by these genes, are multifunctional and play an important role in the regulation of proliferation and apoptosis [11,32,34,36,38,41,42,44]. Thus, the expression of MAP4K3, RIPK1, RIPK2, and CIB1 genes in U87 glioma cells is dependent from endoplasmic reticulum stress mediated by ERN1 and hypoxia mostly affect these gene expressions in dependence of ERN1 signaling enzyme function.

CONCLUSION

Results of this study shown that suppression of ERN1, the major component of endoplasmic reticulum stress signaling, decreases the expression level of genes encoding MAP4K3, RIPK2, and CIB1 in U87 glioma cells, but increases expression of RIPK1 gene. Hypoxia leads to decrease the expression level of MAP3K5, MAP4K3, RIPK1, and RIPK2 genes in control glioma cells, but ERN1 knockdown modifies the effect of hypoxia on these gene expressions.

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