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Humanin Peptides Regulate Calcium Flux in the Mammalian Neuronal, Glial and Endothelial Cells under Stress Conditions

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Abstract

Humanin (HN) and humanin-like substances are short polypeptides that prevent neuronal cell death and dysfunction related to progression of Alzheimer's disease. HN activates oligomeric receptor (CNFTR/WSX-1/gp130 complex) in the extracellular environment, and it is believed to interact with intracellular proteins, such as Bcl2 family. HN stimulates JAK2/STAT3 pathway that regulates apoptosis and cell death, however exact molecular mechanism of HN mediated cytoprotection remains unknown.

The goal of our study was to evaluate effect HN (HNM) and HN-like peptides, which include HNG, HN10d and HN 10dV, on the intracellular calcium release in the cultured brain cells (LN18, C8D1A) and endothelial HUVECs under stress conditions.

We incubated the cells with low amounts of HN (4 μM) for 24 hours, generated cellular stress by addition of either 25 μM β-amyloid (neuronal and glial cells) or 5 ng/mL of TNF-α (endothelial cells); and induced calcium release with 10 μM ATP.

We demonstrated that HN regulated calcium flux in all three cell types, although it was more pronounced in the endothelial than brain cells. HNM led to decreased calcium flux in C8D1A, HUVECs and LN18; HN10d – in HUVECs, and 10dV – in the glial cells. We hypothesized that a site of HN activity and calcium release was located in the endoplasmic reticulum (ER), in which there was slightly altered expression of selected ER-stress associated proteins upon incubation with HNs. There was no effect of the exogenous HNs on the calcium release from the isolated mitochondria. We demonstrated mRNA expression of HNM, HN10d and HN10dV in the neuronal LN18 cells; however their levels were not affected by β-amyloid treatment. It might indicate that stress conditions force HN intracellular translocation instead of change in HN gene expression.

Thus it is possible that localization of HN in the ER may regulate intracellular calcium amounts, which ultimately determine fate of the cell.

Keywords: Humanins; Intracellular calcium release; Isolated mitochondria; Endoplasmic reticulum; Human umbilical vein endothelial cells; Cultured neural and glial cells

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that affects almost 2% of the population in the industrialized countries [1]. Patients suffer from progressive dementia demonstrated by deterioration of cognitive and behavioral processes [1]. Amyloid β-peptide (Aβ) is thought to initiate the cascade that leads to AD-related cell death and neuronal dysfunction. Accumulation of small soluble oligomeric or protofirbrillar assemblies of Aβ is toxic to neurons and neuronal networks [1-4]. Aβ peptide results from cleavage of amyloid precursor protein (APP). The two forms predominantly present in the brain include Aβ 1-40 and Aβ 1-42 [2].

The principal risk factor for AD is advanced age, which is characterized by increased amounts of intraneuronal calcium [5]. Typically, calcium homeostasis is regulated by number of extra- and intracellular mechanisms. ATPase pumps remove calcium from the cells, while voltage-dependent glutamate receptors such as AMPA or NMDA regulate calcium influx. Intracellular stores include ER and mitochondria, along with calcium buffering proteins, such as calbindin, calretilin, or parvalbumin. ER receptors that regulate calcium flow include Ins(1,4,5)P3 receptors (IP3R), ryanodine receptors (RyR) and sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA) [6].

There is a bidirectional relationship between calcium and AD [1,7]. Calcium facilitates Aβ generation [8], phosphorylation of APP and protein tau [9] and formation of highly toxic Aβ 1-40 soluble oligomers [1]. On the other hand, Aβ leads to disruption of calcium homeostasis, activation of enzymes, induction of apoptosis and cytoskeletal modifications [10]. Aβ oligomers associate with the NMDA receptors and increase Ca flow into the cells [11], which in turn further increases Ca release from the internal stores [12]. Mutations of presenilins, which dominate in the endoplasmic reticulum (ER) [13] enhance IP3-

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mediated calcium release from ER [14], ryanodine receptors [15] and calcium leak channels [16]. There is increased presence of calcium-dependent proteases in the AD patients in the post mortem studies, calcium overload in mouse AD models [17] and decrease in calcium binding proteins, calbindin, in AD mice [18].

Humanin (HN) is a neuroprotective peptide, which interferes with a number of AD related toxic insults [19,20]. HN and HN-like peptides were identified both in the mitochondrial and nuclear genomes. Some of the loci include mitochondrial 16S ribosomal mRNA, MT RNR2 gene, open reading frames in the chromosomes 11, 5, X, 5'UTR of WntB [20-22]. HN mRNA is found in the heart, skeletal muscles, kidneys, liver, colon, small intestine and selected brain regions, such as cerebellum, occipital lobe and others [20]. HN is also present in the blood [23]. Plasma HN decreases with age [24].

HN is a 24 amino acid long molecule, which includes a 14-aa hydrophobic core and a polar C-terminus. Its entire sequence is necessary and sufficient for HN secretion, which is released into the ER lumen in its entity [20]. HN appears to function both in the extracellular and intracellular environments [20]. HN was shown to associate with the cellular membrane and interact with a number of receptors, such as CNFTR/WSX-1/gp130, in which gp130 represents a common subunit of the interleukin 6 (IL6) receptors that regulate inflammatory and migratory responses [25,26]. HN binds and regulates a number of intracellular proteins, such as components of JAK2/STAT3 pathway [27], or an insulin-like growth factor binding protein 3 (IGFBP3) [28-30]. IGFBP3 is increased following cell exposure to tumor necrosis factor alpha (TNF-α), tumor growth factor beta (TGFβ) or p53 [31]. IGFBP3 potentiates HN neuroprotective activity following Aβ treatment [32]. HN blocks pro-apoptotic Bax by prevention of its translocation from the cytosol into mitochondria and suppression of cytochrome C release [21].

In addition to multiple receptor interactions, HN appears to affect also other components of cell homeostasis and early events of apoptosis. HN and HNG, a HN-like peptide, directly associate with Aβ 1-40, and HNG promotes Aβ change from fibrous to less toxic amorphous form. Incubation of the rat hippocampal neuronal primary cells with HNG results in decrease of the intracellular calcium rise [33]. Real time fluorescence imaging of Fura2/AM loaded cortical cells treated with Aβ 31-35 show significant decrease of calcium rise in the cells incubated with HN [34]. HN release is activated by increase of intracellular K+, and to lesser extent cAMP and Ca^{2+} [20]. The latter might constitute a calcium dependent regulation loop, in which calcium stimulates HN release that subsequently limits further calcium increase.

The goal of our study was to evaluate effects of exogenous HN (HNM) and selected HN-like peptides, such as HNG (14GlyHNM), HN10d (13ThrHN5) and HN10dV (13IleHN5) (22) on the calcium homeostasis in the cultured brain cells (LN18, C8D1A) and endothelial primary cells (HUVECs) under stress conditions. The toxic insult was carried out in a form of either Aβ in the neuronal and glial cells, or TNF-α in the endothelial cells. Calcium intracellular increase was stimulated with ATP, and the changes were monitored with live fluorescence imaging of Fura2 ratio metric dye.

Methods

Materials and reagents

All the materials and chemical reagents were obtained from Sigma, unless noted otherwise. Humanin and HN-like peptides were synthesized manually in a microwave reactor by the solid phase method using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry (with the cooperation of Lipopharm.pl, Zblewo, Poland) [22].

Cell culture

LN18, a commercial human glioma cell line (epithelial morphology) derived from the glioblastoma tumor (ATCC, CRL-2610) was cultured in Dulbecco`s Modified Eagle`s Medium (ATCC) supplemented with 5% v/v fetal bovine serum, FBS (Clonetics) at 37°C, 95% humidity, 95% air and 5% CO_2.

C8D1A, a commercial murine astrocytes cell line (ATCC, CRL-2541) (Rockville, MD, USA) was cutlured in Eagle`s Minimum Essential Medium (ATCC) supplemented with 10% v/v FBS (Clonetics) at 37°C, 95% humidity, 95% air and 5% CO_2.

Human umbilical vein endothelial cells, HUVECs, were isolated from the umbilical cords using collagenase digestion protocol according to Jaffe et al. [35]. Shortly, umbilical cord veins were digested with 0.01% v/v collagenase, washed twice with M199 medium to isolate endothelial cells, which were transferred into EBM culture medium. The medium was supplemented with 10 ng/mL human endothelial growth factor (hEGF), 1 ng/mL hydrocortisone, 12 mg/mL bovine brain extract, 50 μg/mL gentamicin, 50 ng/mL amphotercin B, and 5% v/v FBS (Clonetics) and the cells were cultured in 95% air/ 5% CO_2 incubator at 37°C. HUVECs were used for the experiments up to the 5th passage.

RT-PCR of humanins' mRNA expression

Expression of endogenous humanins' mRNA (HNM, HN10d, HN10dV) was analyzed in the neuronal LN18 cells and HUVECs. In short, the cells were seeded and cultured in their respective media for 24 hours. A subpopulation of LN18 cells was treated with 25 μM amyloid β peptide for 18 hours, while HUVECs were exposed to 10 ng/mL TNF-α for 4 hours. Control cells were not treated with any additional compounds. Next, total RNA was isolated from both control and treated cells with use of TRIzol® Plus RNAPurification System (Invitrogen). The RNA isolation quality and quantity were checked by NanoDrop®.

Synthesis of cDNA was performed using 1,5 μg of total RNA, which was reversely transcribed with the High Capacity cDNA RT kit with random primers according to the manufacturer's guidelines (Applied Biosystems). Next, cDNA was subjected to real-time PCR. The primer sets for each humanin were as follows: HNM, F: 5' CTGTATGAATGGCTCCACGA 3', R: 5' GCAGGTCAATTTCACTGGTTA 3', HN10d, F: 5' TGTATGAATGGCCACACCA 3', R: 5' CATAGGTAGGTCAATTTCACTGG 3', HN10dV, F 5' TGTATGAATGGCCACACCA 3', R: 5' TCATAGGTAGGTCAATTTCACTGATT 3'. Expression of GAPDH served as a reference. Expression rate of each probe was calculated as a difference between averages of the sample C_T and their controls, adjusted for the amplification efficiency as indicated by the housekeeping gene, GAPDH.

Calcium intracellular release assay

Total calcium secretion was monitored in the three cell lines in order to compare effect of either humanins or L-arginine on the cellular metabolism. The cells, LN18, CD81A and HUVECs were maintained under basic culture conditions as described above. The cells were seeded and cultured on the 96-well flat bottom black plates (BD Falcon). Both neuronal and endothelial cell lines were incubated with either 4 μM

humanins, HNM, HNG, HN10d, HN10dV or 0.1M L-arginine (0.1 L-Arg) for 24 hours at 37°C, 95 % humidity, 95 % air and 5% CO_2. In addition, HUVECs were treated with 5 ng/mL TNFα for 4 hours and the neuronal cell lines were treated with 25 μM β-amyloid for 1 minute prior to the induction of calcium release. The calcium changes were analyzed with Ratiomeric Calcium Assay kit (BD Biosciences) in which increase of Fura2 ratio (excitation wavelengths 340/380 nm) reflected release of calcium ions from the intracellular calcium storage into the cytosol. Both neuronal and endothelial live cells were stimulated with 10 μM ATP and the calcium release was recorded for 40 or 60 seconds. The extent of calcium release was defined as area (pixels) under an averaged Fura 2 signal ratio recorded from multiple cells within a monitored well.

Calcium retention capacity in isolated mitochondria and mitochondrial swelling

Mitochondria were analyzed as potential organelles affected by humanins to regulate calcium metabolism under stress conditions. Mitochondria were isolated from rat livers by differential centrifugation in a medium that contained 225 mM mannitol, 75 mM sucrose, 3 mM Tris–HCl (pH 7.4) and 0.1 mM EGTA. The mitochondria were incubated with 4 μM humanins (HNM, HNG, HN10d, or HN10dV) for 15 minutes at 37°C or left untreated under the same conditions. The incubation medium (225 mM mannitol, 75 mM sucrose, 1 mM KH_2PO_4, 1mM $MgCl_2$, 0.5 μM EGTA, 5 mM Tris, pH 7.4) included glutamate (5 mM) and malate (5 mM) as respiratory substrates. The amount of mitochondrial protein ranged from 0.3 to 1.2 mg, as estimated by Lowry method. The results were presented as Fura2 ratio of emission spectra at 540 nm generated by the excitation wavelengths at 340 nm and 380 nm. Calcium was supplied in 10 μM-doses. Accumulation of calcium in the mitochondrial matrix was reflected by a drop in the Fura2 fluorescence ratio. Release of the accumulated calcium into the measurement medium (increase in Fura 2 fluorescence ratio) was an indication of mitochondrial megachannels (MMC) opening. Calcium retention capacity (CRC) of the mitochondria was calculated as amount of calcium needed to activate the MMC, normalized for amount (mg) of the mitochondrial protein.

Mitochondrial swelling was analyzed in the isolated rat mitochondria incubated with 4 μM humanins (HNM, HNG, HN10d, HN10dV) for 15 minutes prior to the analysis, and supplemented with glutamate (5 mM) and malate (5 mM). The EGTA content in the incubation medium was increased to 0.5 mM. Changes in the mitochondrial matrix volume were monitored by measuring absorbance at 540 nm. The MMC activation, resulting in mitochondrial swelling, was induced by addition of 0.5 mM $CaCl_2$. Cyclosporine A (CsA), which is a potent MMC blocking agent, served as a positive control.

Protein expression

Expression levels of calregulin and Grp 94, selected markers of endothelial reticulum (ER) stress, were analyzed in the neuronal cells, LN18 and control endothelial HUVECs in order to evaluate effect of HN on this organelle. The cells were incubated under conditions described above in presence of 4 μM humanins for 24 hours, and either 25 μM β-amyloid for 18 hours (neuronal cells) or 5 ng/mL TNF-α (HUVECs). The cells were fixed with 4% v/v paraformaldehyde in PBS for 30 minutes, washed twice with PBS, and blocked with 0.2M glycine, 0.1% v/v saponin and 0.2% v/v BSA diluted in PBS for 30 minutes at ambient room temperature. The cells were washed with binding buffer (0.1% v/v saponin, 0.2% v/v BSA in PBS) between each step of the staining procedure. Primary antibodies selected for the study included: mouse monoclonal IgG1 specific for calregulin (sc-70488, Santa Cruz) and goat polyclonal IgG specific for Grp94 (sc-1794, Santa Cruz). The cells were incubated for 18 hours at 4°C, then they were exposed to either MFP488 donkey anti-goat IgG (H+L) (MoBiTec, Germany) or Cy3-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch) secondary antibodies for 1 hour at ambient temperature. The cellular nuclei were counterstained with Hoechst (Invitrogen). The images were obtained and analyzed with BD Pathway 855 Bioimager (BD Applied Biosystems).

Statistical analysis

Results of continuous independent variables were expressed as means ± standard deviation (SD) or variance (indicated in the figure legends). The results were calculated from three to four experiments. Statistical comparisons were carried out with use of ANOVA, and further verified with student t-Test for comparison of quantitative variables. Level of statistical significance was set at $p < 0.05$. All the statistical analyses were completed with Microsoft Office Excel.

Results

Endogenous expression of humanins M, 10d and 10dV in LN18

We showed recently that endogenous HNs, that included HNM, HN10d, and HN10dV, were present in the endothelial cells, HUVECs (material submitted for publication). There, the amount of detected mRNA was the highest for HNM, and relatively much lower for the other two HN-like peptides. Exposure of HUVECs to stress conditions (5 ng/mL TNF-α) for 4 hours resulted in a significant increase of both HN10d and HN10dV, but not HNM. In the presented study, we tried to verify whether there was endogenous HN expression in the neuronal cancer cells and whether β-amyloid could lead to any changes in HN expression. We detected presence of all the above humanins, HNM, HN10d and HN10dV. HNM demonstrated the highest expression relative to a housekeeping gene, GAPDH, and the other two HN-like peptides were present in much smaller amounts (Figure 1A). In contrast to HUVECs, incubation with a low, physiologically relevant concentration of Aβ (25 μM) did not affect expression of any of the tested HNs (Figure 1B).

Humanins regulate calcium release in the brain and endothelial cells

In search of a potential mechanism, which would explain HN mediated neuroprotective activity, we analyzed effect of the extracellular HN on the calcium release in the cells. The neuronal (LN18) and glial cells (C8D1A) cells were incubated with HNM, HNG, HN10d or HN10dV for 24 hours under basic culture conditions. In a subpopulation of cells, incubation medium was supplemented with a minimal concentration of L-Arginine (0.1M) to verify whether a substrate for NO generation demonstrated any effect on the calcium metabolism in those cells [36]. In order to test calcium release, the cells were stimulated with 25 μM Aβ for 1 minute followed by addition of 10 μM ATP. Changes in the Fura2 fluorescence ratio corresponded proportionally to the intracellular calcium flux (Figure 1, supplemental material).

There were some noticeable changes in the calcium release in C8D1A glial cells upon incubation with humanins. Two endogenous humanins, HNM and HN10dV decreased significantly calcium flux in

those cells, while the other humanins and L-Arg did not bear any effect in this regard (Figure 2A).

The results in LN18 neuronal cells were not as easy to interpret as the ones in the other cells. The cells demonstrated resistance to ATP stimulation, and typically barely 20-50% of the cells underwent calcium flux (data not shown). Cumulated data indicated that potentially HNG resulted in reduced calcium release, however it could not be verified by employed statistics (Figure 2B).

The control cells, HUVECs, were incubated with selected four HNs for 24 hours as the other cells above, however it was followed by their incubation with TNF-α, as a more physiological and relevant stressor in this type of cells. The endothelial cells were incubated with TNF-α for four hours; then stimulated with 10 μM ATP. Three humanins demonstrated beneficial effects in HUVECs. These were HNM, HNG and HN10d to the highest degree. HN10dV did not show any effect under applied conditions, while 0.1 L-Arg slightly reduced calcium release (Figure 2C).

Humanins did not affect calcium release from isolated mitochondria

Our calcium release data indicated that humanins, in particular HNM in both brain and endothelial cells, HN10d and HNG in HUVECs, and HN10dV in C8D1A glial cells, prevented increase of the intracellular calcium ions following incubation with agents that induced cellular stress, such as Aβ or TNF-α. Therefore, we decided to evaluate two major storage sites for calcium ions in the cells, which were mitochondria and endoplasmic reticulum, ER [6].

We examined calcium retention capacity (CRC) and calcium - induced mitochondrial swelling to verify potential HN-induced effect onto the mitochondria. Mitochondria that had been isolated from rodent livers were incubated with 4 μM humanins for 15 minutes prior to the analysis. Calcium ions ($CaCl_2$) were titrated in 10 μM doses until opening of a calcium megachannel (MMC). The extra-mitochondrial calcium was visualized with Fura2, which fluorescent signal ratio increased proportionally to the calcium concentration in the measurement medium, and decreased when calcium ions were re-absorbed into the mitochondria. None of the humanins had any statistically significant effect on the calcium retention capacity, which was indicated by the threshold calcium concentration that activated MMC (Figure 3).

Another test used to evaluate mitochondrial calcium metabolism was an analysis of calcium-induced mitochondrial swelling. The mitochondria that had been pre-incubated with selected HNs for 15 minutes were blasted with very high amounts of calcium ions (0.5 mM, 1 mM) in order to verify whether an analyzed compound bore any protective effect upon opening of the MMC. None of the four analyzed humanins showed any effect under analyzed conditions (Figure 4).

Humanins altered expression of selected ER-associated proteins

The next step of our study included expression analysis of ER unfolded protein response (UPR) markers in the cells incubated with one of four humanins under stress conditions, either Aβ or TNF-α in the brain cells or control endothelial cells, respectively. We selected four proteins, which expression reflected UPR in the endoplasmic reticulum, out which two are presented in this study. These were calregulin and Grp94. The proteins were visualized with fluorescent microscopy, BD Bioimager 855.

In the neuronal LN18 cells, expression of Grp94 was slightly decreased following their incubation with Aβ. Decline in Grp94

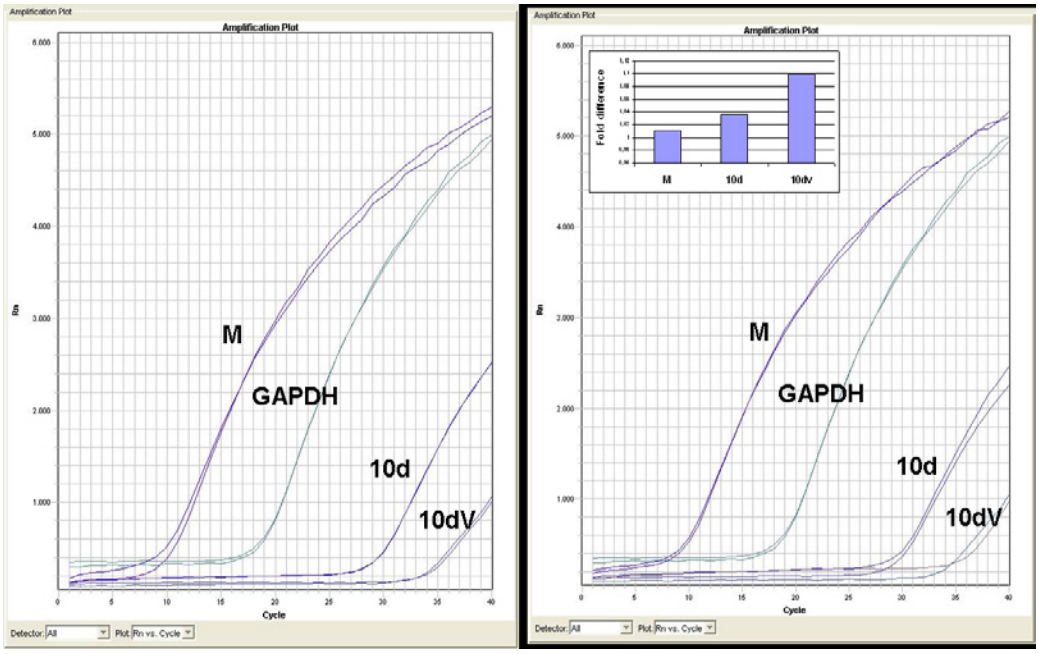

Figure 1: RT-PCR analysis of endogenous expression of humanins HNM, HN10d and HN10dV in LN18. The cells were lysed with TRIzol® Plus RNA Purification System (Invitrogen). Obtained total RNA was reversely transcribed into cDNA, then subjected to PCR with the primers corresponding to the humanins listed above. **A.** Amplification plot of HN M, 10d and 10dV in LN18 cells. **B.** Amplification plot of HN M, 10d and 10dV in the LN18 cells incubated with 25 mM β-amyloid (βA) for 18 hours prior to their harvest. Insert, fold difference in threshold values for HN M, 10d and 10dV in the Ab-treated cells compared to their controls. Expression rate of each probe was adjusted for the amplification efficiency of the housekeeping gene, GAPDH.

expression was statistically significant in the control cells and in the presence of HNM, HNG and HN10d. It was also interesting to note an increase of this protein expression in the presence of HNM, HNG and HN10d, that might have indicated some interaction of those humanins and ER (Figure 5A). There was also increase of calregulin expression in the brain cells following their incubation with selected exogenous humanins, HNM, HNG, HN10d, HN10dV, and L-Arginine. Introduction of the stress conditions, 25 μM Aβ, resulted in a further increase of calregulin expression, in particular in the presence of HNG, HN10d and HNM. There was no increase in calregulin expression in the cells incubated with HN10dV and L-Arg when stimulated with Aβ (Figure 5B). There was no effect on Erp72 expression observed (not shown).

The changes in the UPR expression in HUVECs were much less pronounced compared to the LN18 cells (Figure 6). Inclusion of exogenous humanins in the cell medium did not appear to significantly alter Grp94 expression. Introduction of Ab-mediated stress conditions resulted in a definite decrease of Grp94 in the control cells. It might appear that addition of humanins prevented such a decline in the other cells (Figure 6A). Expression of calregulin was not affected by incubation with any of the humanins. In contrast to the neuronal cells, there was some decrease in calregulin expression in the endothelial cells incubated with HNG, HN10d and slightly in 10dV following exposure to Aβ. HNM did not appear to affect calregulin expression either under normal or stress conditions (Figure 6B).

Discussion

Neuroprotective activities of humanins, in particular HNM, were demonstrated in a variety of cell and/or tissue types in degenerative diseases, such Alzheimer's Disease and others [37]. It is still not well understood how humanins could shield the cells from the pro-apoptotic insults. HN blocks pro-apoptotic Bax protein [21], it promotes JAK2/STAT3 anti-apoptotic pathway, it appears to promote cell proliferation and differentiation via their interactions with IGFBP3 [32] or FPRL-1 [25]. A possible mechanism that links all the HN-mediated events might HN effect on the calcium and calcium-dependent processes in the cell.

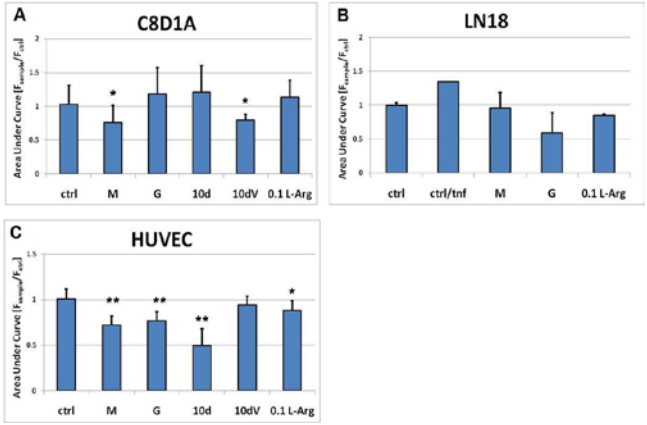

Figure 2: Effects of exogenous humanins on the intracellular calcium release. The cells were incubated with selected HNM, HNG, HN10d, HN10dV for 24 hours, followed by either stimulation with 25 μM βA for one minute (brain cells) or 5 ng/mL TNFα (endothelial cells). The calcium release was induced by 10 mM ATP, and changes in Fura2 fluorescent ratios were recorded for 40 seconds. The results were calculated as average area under curve derived from continuous recording of Fura2 ratios. The results were normalized to the signal generated by the control cells; the experiments were done in triplicates at minimum. **A)** C8D1A cells, **B)** LN18 cells, **C)** HUVECs.

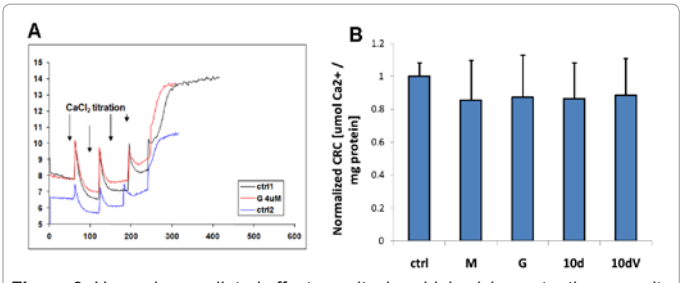

Figure 3: Humanins-mediated effect on mitochondrial calcium retention capacity (CRC). The mitochondria were isolated from the rat livers, suspended in glutamate/malate-containing solution and incubated with a selected HN for 15 minutes prior to calcium titration. Calcium ions ($CaCl_2$) were added in 10 μM doses until opening of calcium megachannel (MMC) and complete calcium release from the mitochondria. **A)** Example of calcium titration in the control mitochondria (ctrl1, ctrl2) and mitochondria incubated with HN G. **B)** The CRC were adjusted for protein content, and normalized to the control (mitochondria incubated in absence of humanin) from the respective experiments. CRC in the control measurements were at the level of 0.12 ± 0.06 μmol Ca^{2+} / mg protein. Each experiment was repeated 3-5 times.

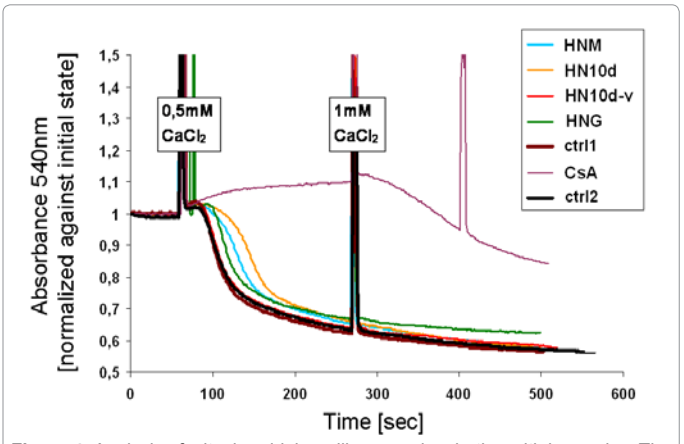

Figure 4: Analysis of mitochondrial swelling upon incubation with humanins. The mitochondria were isolated from the rat livers, suspended in glutamate/malate-containing solution, and incubated with selected HN for 15 minutes prior calcium addition. Calcium ions were titrated in two doses, 0.5 mM and 1 mM, and decrease of the absorbance signal at 540 nm marked mitochondrial swelling due to opening of the calcium megachannel (MMC) was recorded. Positive control was Cyclosporine A (CsA), which represented a potent MMC blocker.

The presented study was undertaken to improve our understanding of roles that humanins play in the cells. We selected two commercially available brain cell lines, neuronal LN18 cells and glial C8D1A cells and exposed them to AD-derived toxic amyloid, Aβ 1-42. The control included endothelial cells, HUVECs, incubated with their respective toxin, TNF-α. We demonstrated that exogenous HN and HN-like peptides, HNM, HNG, HN10d and HN10dV, affected ATP-stimulated intracellular calcium increase in the tested cells (Figure 2). HNM led to decreased calcium flux both in the glial (C8D1A) and endothelial (HUVEC) cells, while HN10dV decreased calcium release in HUVECs, and HN10d resulted in decline of calcium flux in the brain cells. There was no significant effect of any HN on the isolated mitochondria (Figure 3 & 4). Even though, there was significant increase in the mitochondrial membrane potential in the HUVECs under similar conditions (material to be submitted). We demonstrated that humanins affected expression of the proteins associated with ER, in particular

Grp94 and calregulin (Figure 5 & 6). RT-PCR analysis of endogenous humanins demonstrated that Aβ treatment did not affect HN mRNA amounts in LN18 cells (Figure 1). Hence, we assumed that expression of endogenous humanins did not alter calcium changes induced by their exogenous counterparts.

There were a number of potential obstacles that could affect our results. First, both C8D1A and LN18 cells were cell lineages derived from the brain tumors. These cells are commercial products, which were previously characterized by their resistance to different chemotherapeutics and other treatments. It was then possible that amounts of Aβ and/or exogenous humanin peptides used in the study were not sufficient to provide physiologically relevant protection for those cells. It was exemplified by LN18 particular resistance to ATP-induced calcium release, when only some cells responded to this otherwise powerful stimulus. Next, we had no previous knowledge of the endogenous amounts of any humanins, HNM or other HN-like peptides. Even though we confirmed presence of those peptides in LN18 cells, we could not verify how exogenous HNs affected their intracellular endogenous amounts. It was reported that humanins were located both inside and outside the cells, however it was not verified what synergistic, antagonistic or neutral effects they could generate together.

We demonstrated a small, but clear effect of humanins on the intracellular calcium release in three types of cell lines. Both Aβ and TNF-α resulted in transient increase of the intracellular calcium. It was shown that amyloid β-peptide activates NMDA calcium channels, and presenilins mutations promote calcium release via all ER-associated

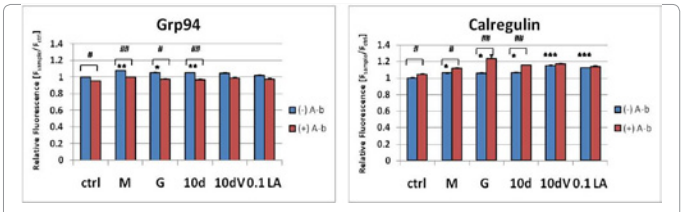

Figure 5: Humanins regulated UPR expression in LN18 cells. The cells were incubated with 4 μM humanin M, HNG, HN10d or HN10dV for 24 hours, out of which the last 18 hours they were exposed to Aβ in the sample wells. At the end, the cells were fixed with 4% paraformaldehde, and relative protein expression was determined using appropriate primary and secondary antibodies. All the results were normalized to the control cells without HNs. The protein expression is presented as a mean of relative fluorescence of a particular sample normalized to the control cells +/- variance. The statistical significance was set at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). **A)** Grp94 expression, **B)** Calregulin expression.

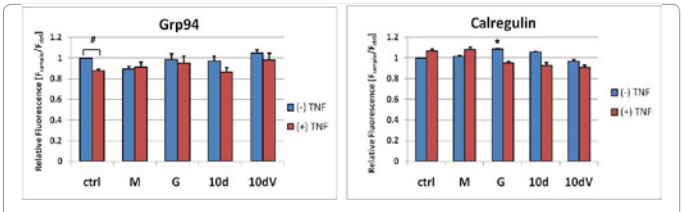

Figure 6: Humanins slightly altered UPR expression in HUVECs. The cells were incubated with 4μM humanin M, HNG, HN10d or HN10dV for 24 hours; they were exposed to 5 ng/mL TNFα in the sample wells during last 4 hours. At the end, the cells were fixed with 4% PFA, and protein expression was determined using specific primary and secondary antibodies. All the results were normalized to the control cells without HNs. The protein expression is presented as a mean of relative fluorescence of a particular sample normalized to the control cells +/- variance. The statistical significance was set at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). **A)** Grp94 expression, **B)** Calregulin expression.

receptors [11,14-16]. TNFα stimulates IP3R and augments calcium release from the ER [38]. Since both Aβ and TNF-α affect ER calcium pool and we did not observe any significant effect of HN on the other calcium storage site, namely mitochondria, we hypothesize that humanins interfere with calcium flux from the ER. One of the events initiated by intracellular calcium increase is apoptosis. Bcl-2 protein family and caspases are intimately associated with the mitochondrial function and progression of the apoptotic events. HN were shown to block pro-apoptotic activities of Bax and Bak proteins [21,39]. It is interesting to note that Bax is not only mitochondrial, but also ER-associated protein [40]. Bax increases amount of calcium stored in the ER, and it magnifies calcium ER release upon stimulation. Since HN controls Bax translocation to the mitochondria, it would be important to test whether HN prevents Bax transfer into the ER thus reducing calcium flux after Aβ or TNF-α treatment. Next, analysis of humanin intracellular location under control and stress conditions might explain how humanins act inside the cell. Direct visualization of HN location in the cells, and HN potential transfer into the mitochondria or/and ER would provide better understanding of HN role under harmful conditions. Analysis of humanin's potential binding factors would greatly facilitate further studies of these peptides. Even though a number of receptors and proteins were already identified, any other HN interactions, in particular endogenous HN, with proteins, membranes, nucleic acids or combination of the above were not truly addressed.

To summarize, we demonstrated that humanin mediated cytoprotection might at least partially result from humanin ability to decrease intracellular calcium release under stress. The calcium release was impeded more significantly in the endothelial than neuronal or glial cells. The potential site of the humanin activity might be endoplasmic reticulum, since there was no effect of any of the four exogenous humanins on the isolated mitochondria. Ultimate verification of the mechanism how humanins regulate calcium metabolism might greatly improve our understanding of humanin function in the cell, and prove humanin usefulness in design of new drugs and treatments in the future.

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