Article

ACTH(6-9)PGP Peptide Protects SH-SY5Y Cells from the H₂O₂, tert-Butyl Hydroperoxide, and Cyanide Cytotoxicity via Proliferation Stimulation and Antioxidant-related Genes Induction

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Abstract: Stabilized melanocortin analog peptide ACTH(6-9)PGP (FRWGPGP) possess a wide range of neuroprotective activities. However, its mechanism of action remains poorly understood. In this paper, we studied the pro-proliferative and cytoprotective activity of the adrenocorticotropic hormone fragment 6-9 (FRWG) linked with the peptide Prolyl-Glycyl-Proline on the SH-SY5Y cells in the model of oxidative stress-related toxicity. The peptide dose-dependently protected cells from H₂O₂, tert-butyl hydroperoxide, and KCN. The mechanism of its action was the modulation of proliferation-related (NF-κB and Nrf-2) and antioxidant-related (HO-1, Nqo1, Gclc) genes and apoptosis decrease.

Keywords: ACTH(6-9); Neuroprotection; MPP⁺; H₂O₂; tert-butyl hydroperoxide; cyanide; melanocortins; oxidative stress

1. Introduction

One of the actively studied classes of endogenous peptide regulators is adrenocorticotropic hormone (ACTH)/melanocyte-stimulating hormones (MSH)-like peptides, which are currently combined under the term melanocortins (MC). Studies of the effects of these peptides over the past 40 years showed that the spectrum of the physiological activity of these peptides is very wide. MC are involved in the regulation of memory and attention, emotional status, sexual and eating behavior, pain sensitivity, and some other physiological functions [1]. The MC family includes ACTH and MSH (α-, β- and γ-MSH), as well as fragments of these hormones and their synthetic analogs. The common sequence for all MC is ACTH(6-9) or FRWG, which is necessary for binding to all known types of MC receptors [2]. Of particular interest is the ability of ACTH and MSH, as well as their fragments lacking hormonal activity, to accelerate the training of animals and improve the preservation of skills. The most active of the studied peptides is ACTH(4-10). The smallest ACTH fragment retaining nootropic activity is ACTH(4-7). However, it was also shown that the ACTH(7-10) fragment has insignificant nootropic activity. Elongation of the molecule to ACTH(7-16) leads to an increase in activity to a level comparable to ACTH(4-7) and ACTH(4-10) [3,4].

The described wide range of physiological activity of melanocortins opens up opportunities for the use of drugs of this class in clinical practice in various pathological conditions. However, an obstacle to the use of these compounds in the clinic is low bioavailability and insufficient action duration. The duration of the neurotropic effects of the natural adrenocorticotropic hormone (ACTH) 4-10 fragment is 30-60 minutes [5]. The prolongation of the effects of the peptide and increase of its peptidase resistance was

found to be possible by including in the structure of the molecule sites enriched in proline residues. It is known that in the organs and tissues of most warm-blooded animals, exo- and endopeptidases with low specificity are widely represented. They do not cleave the bonds -AA-Pro-, where AA is any amino acid, as well as other sequences enriched in proline residues. Specific prolyl hydroxylases are concentrated mainly in individual organs and tissues in a small amount. Based on this, it was suggested that the addition of a proline-enriched chain to the C-terminus of the ACTH fragments would lead to both an enhancement of the effect of the peptide and its prolongation [5].

The tripeptide Proline-Glycine-Proline (PGP) is a collagen-derived matrikine that has been described as a neutrophil chemoattractant. PGP functions as a primitive and conserved damage-associated molecular pattern, which is generated during infection or injury and subsequently acts to shape ensuing inflammatory and repair processes.

PGP also possesses a profound capacity to promote proliferation, radial spreading, and prominent lamellipodia formation in human lung bronchial epithelial cells [6]. In addition to this activity, it has a   high resistance to cleavage by proteases and was successfully used for stabilization of various nootropic peptides [7], including ACTH fragments.

Animal experiments demonstrated that ACTH(6-9)PGP (FRWGPGP) exhibited nootropic and anxiolytic activities and enhanced the viability of cultured glial cells collected from the cerebral cortex of rats with ischemic stroke. Study of the effect of ACTH(6–9)PGP on the size of necrotic lesions in rats demonstrated an approximately 50% decrease in the size of the lesions in the course of ischemic stroke [8,9].

Despite a profound biological activity, the mechanisms of ACTH(6-9)PGP neuroprotective activity remains poorly understood.

In this paper, we show for the first time that a modified adrenocorticotropic hormone fragment ACTH(6-9)PGP has neuroprotective activity in the H_2O_2, tert-butyl hydroperoxide, and KCN toxicity that models cell-death inducing mechanisms in   Parkinson's disease and ischemic stroke.   We demonstrated that the neuroprotective activity of peptide is realized via proliferation increase by modulation of proliferation-related (NF-κB and Nrf2) and antioxidant-related (HO-1, Nqo1, Gclc) genes and apoptosis decrease.

2. Results

2.1. ACTH(6-9)PGP Stimulated Cell Proliferation and Increased Cell Survival after H_2O_2, Tert-Butyl Hydroperoxide, MPP+, and KCN Treatment

To study the neuroprotective activity of ACTH(6-9)PGP, four oxidative stress related models were used: oxidative stress induction by H_2O_2, tert-butyl hydroperoxide (tBH), KCN, and MPP+ cytotoxicity. The cytotoxicity of H_2O_2 and MPP+ was determined beforehand   [10], and for KCN and tBH was explicitly measured (Figure 1); EC_{50} for KCN was 906 μm (C.I. 870.5 to 941.6 μM), and for tBH 27.5 μM (C.I. 24.52 to 30.97 μM).

In the experiments with the peptide, the added toxic agent concentration was chosen to induce 50 to 70% cell death in 24 h; the peptide was added together with the toxin.
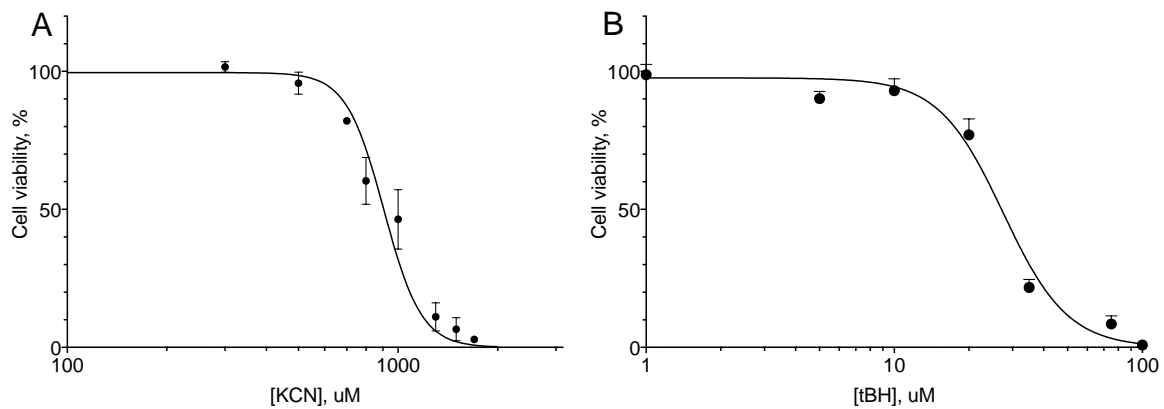
Figure 1. Cytotoxicity of KCN (**A**) and tBH (**B**) for the SH-SY5Y cells. Incubation time 24 h, MTT assay data, mean±standard error, N=5 experiments.

ACTH(6-9)PGP dose-dependently increased cell viability under the treatment with all of the toxic agents except MPP+ (Figure 2A-D). For H_2O_2 and tBH, the protective activity maximum war observed at the concentration around 1-10 μM (Figure 2 A, B), and for KCN, the maximum increase of cell survival was at 100 μM (Figure 2C).
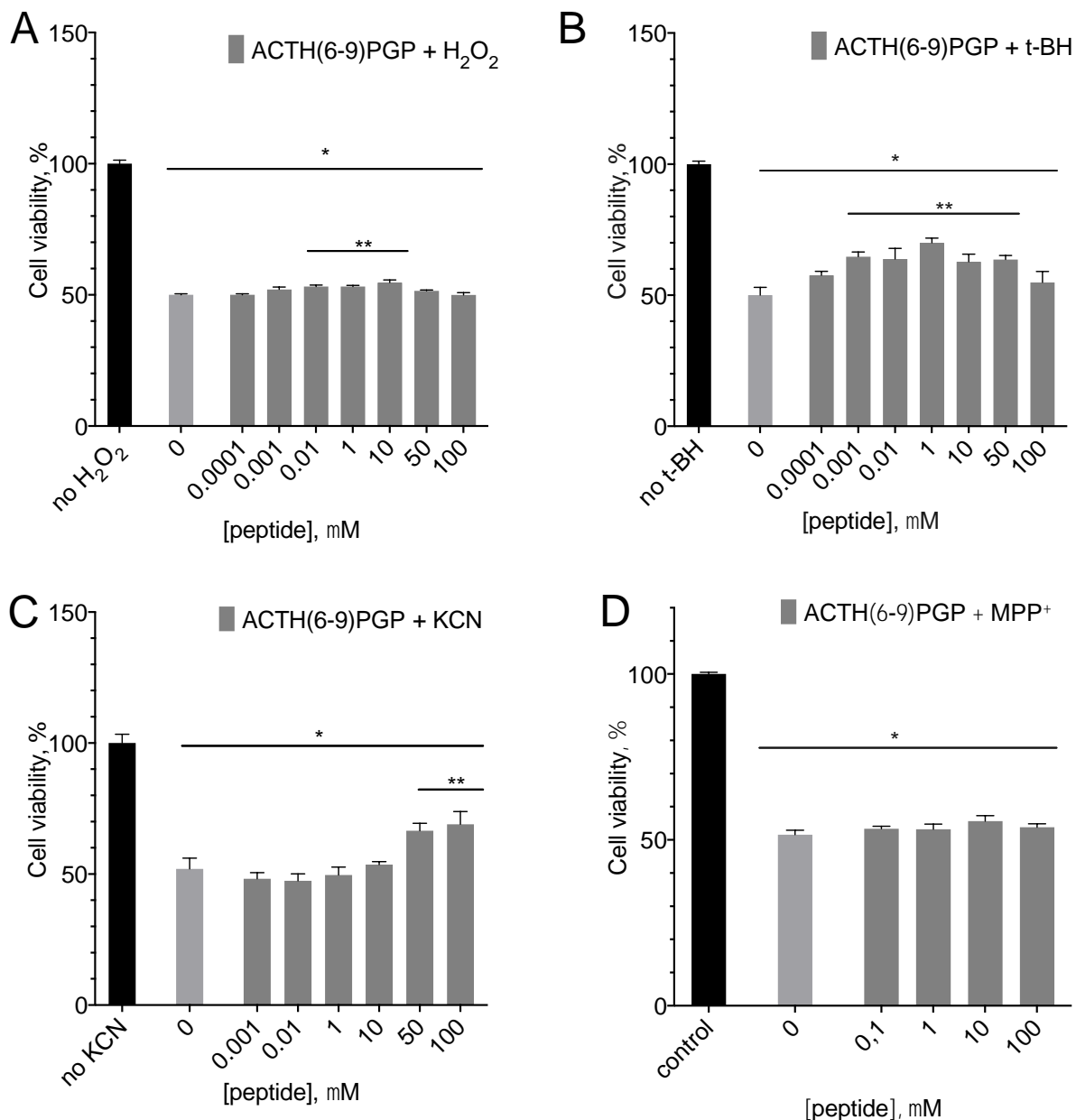
Figure 2. ACTH(6-9)PGP effect on SH-SY5Y cell viability after H_2O_2, tBH, MPP^+, and KCN treatment. The cells were treated with 475 μM H_2O_2 (**A**), 15 μM tBH (**B**), 850 μM KCN (**C**), or 1.3 mM MPP^+ with various peptide concentrations for 24 h or 48 h in the case of MPP^+ Untreated cells were used as control. MTT test, mean±standard error, N=7 experiments. *, a statistically significant difference from the untreated control; **, a statistically significant difference from the control without peptide, ANOVA with the Holm-Sidak post-test, p≤0.05.

2.2. ACTH(6-9)PGP Decreased Apoptosis and Increased Cell Viability, but Did Not Affect Acute ROS Level

Three possible mechanisms of the protective action of ACTH(6-9)PGP could be proposed: proliferation stimulation, apoptosis inhibition, and ROS level decrease either as a direct ROS scavenging effect or via the appropriate cellular enzyme systems activation. The latter hypothesis is possible, as far as all the toxic agents used are linked to reactive oxygen species generation [11,12].

To test the first two hypotheses, we studied SH-SY5Y proliferation increase after ACTH(6-9)PGP treatment and apoptosis levels after the combined application of the peptide and H_2O_2. The peptide stimulated cell proliferation (Figure 3 A, B). On the other

hand, it decreased apoptosis, but the effect was statistically significant only at 100 μM of the peptide (Figure 3A). No ROS scavenging effect was observed after a 1 h incubation (Figure 3C).
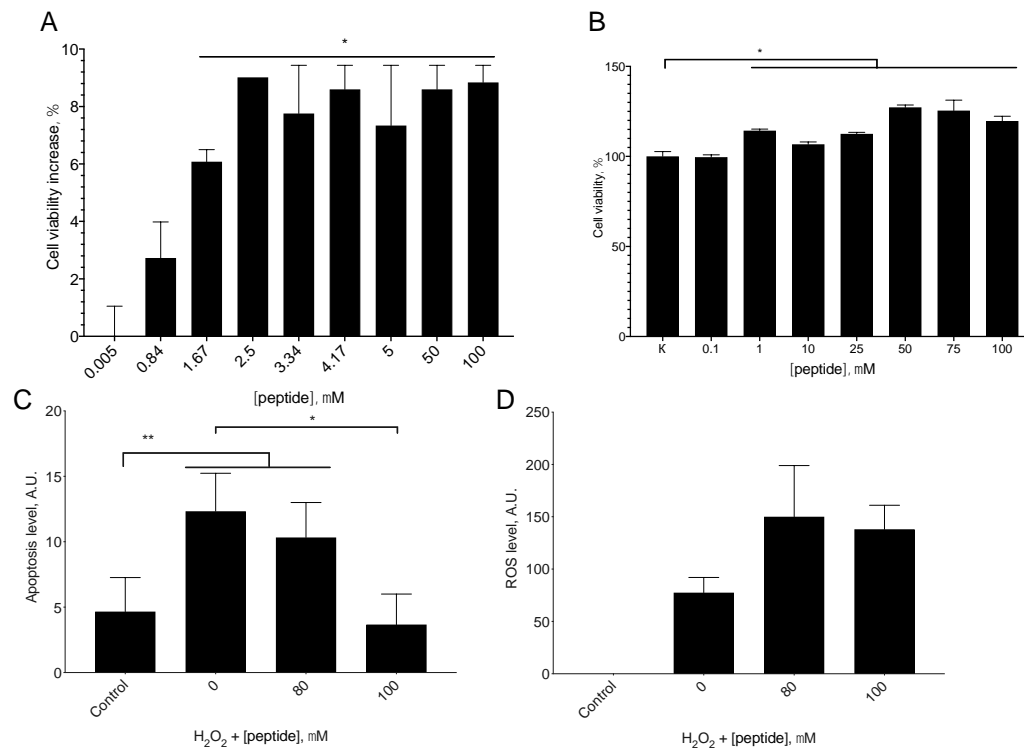
Figure 3. ACTH(6-9)PGP influence on cell proliferation (**A, B**) and H_2O_2 induced apoptosis (**C**) and ROS level (**D**). For the proliferation studies, the cells were incubated with the peptide for 7 days and analyzed using the MTT (**A**) or BrdU (**B**) assay. SH-SY5Y cells were treated with with 475 μM H_2O_2 either alone, or together with the peptide for 1 h, after which ROS level and apoptotic cell counts were determined. Untreated cells were used as control. Mean±standard error, N=3 experiments, *, statistically significant difference from H_2O_2 alone for ROS and apoptosis, and from the untreated control for the proliferation, ANOVA with the Holm-Sidak post-test, $p \leq 0.05$.

2.3. ACTH(6-9)PGP Protection from the KCN Cytotoxicity is Inhibited by the MEK, PKC, PLC, and Ras Inhibitors

To evaluate the signaling pathways involved in the peptide protective activity, we used a panel of receptor and intracellular signal transduction components inhibitors. As far as extracellular oxidative stress inducers like H_2O_2 could oxidize these inhibitors, we used the KCN based toxicity model. Of the inhibitors tested, only those for the protein kinases C and A (PKC+PKA), phospholipase C (PLC), mitogen-activated protein kinase 1 and 2 (MEK1,2), and Ras removed the protective effect of the peptide (Figure 4), and thus these components participate in the peptide action. Overall, the observed inhibitor response points to the activation of the PLC->PKC->Ras->MEK signal transduction pathway [13–15].
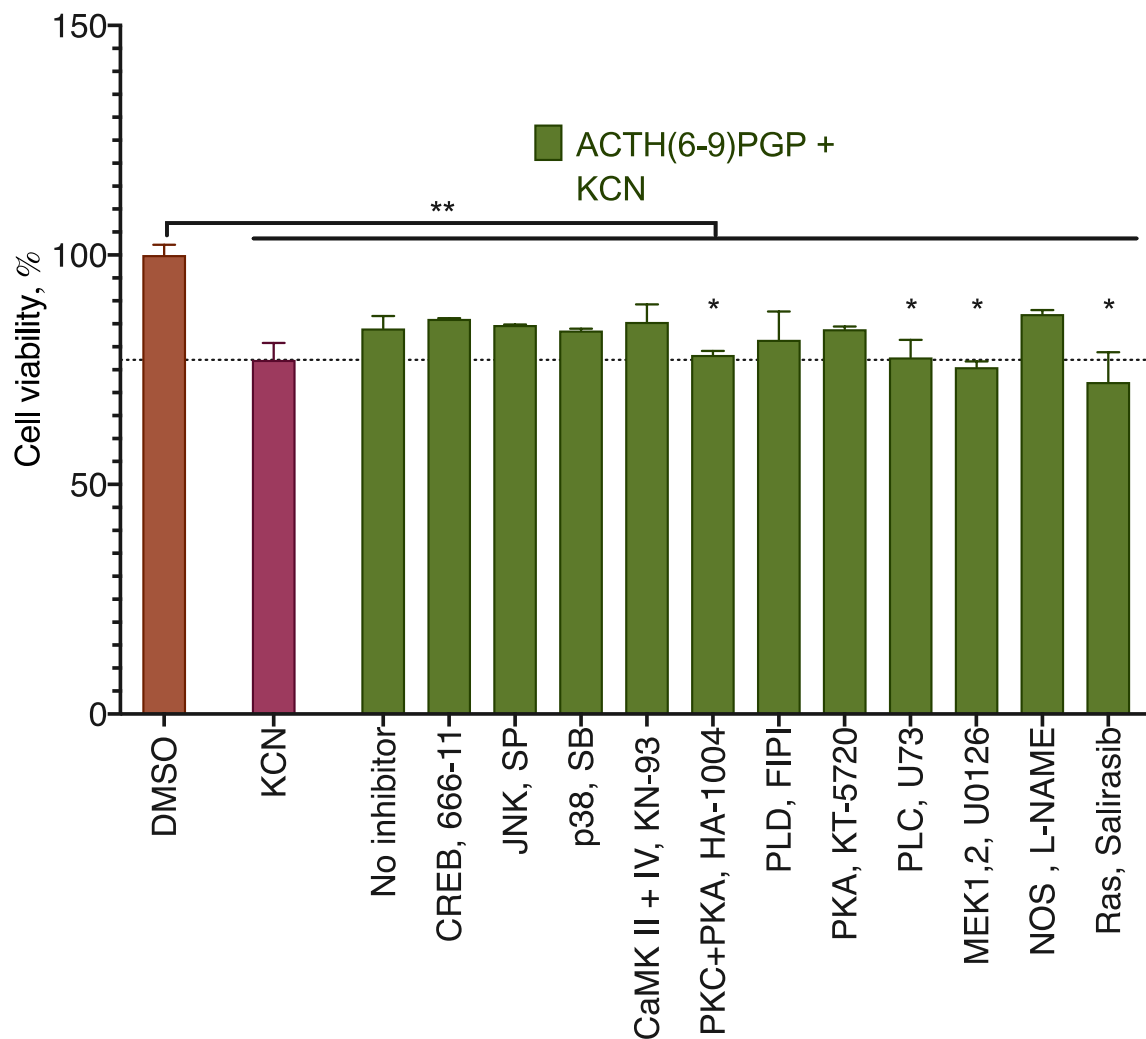
Figure 4. Intracellular signal transduction components participation in ACTH(6-9)PGP protection against the KCN cytotoxicity for the SH-SY5Y cells. Inhibitors 666-11 (1 μM), SP 600125 ("SP", 1 μM), SB 202190 ("SB", 1 μM), KN-93 (4 μM), HA-1004 (10 μM), FIPI (0.5 μM), KT-5720 (0.5 μM), U-73122 ("U73", 10 μM), U-0126 (0.2 μM), L-NAME (25 μM), Salirasib (10 μM) were added 1 h before the KCN, and then together with KCN (950 μM) and peptide (50 μM). The cells were incubated with the inhibitors, KCN, and peptide for 24 h. MTT assay data, mean±standard error. *, statistically significant difference from the KCN+peptide without any inhibitor; **, statistically significant difference from the untreated control, p≤0.05, ANOVA with the Tukey post-test, N=3 experiments.

2.4. ACTH(6-9)PGP did not Alter Cellular cAMP Content

As far as the receptor for the full-length ACTH is a GPCR coupled to a $G_{\alpha s}$ subunit, which in turn activates cAMP synthesis, and that one of the active inhibitors was the mixed PKA/PKC one, we decided to check whether the action of ACTH(6-9)PGP affects the concentration of this second messenger. We analyzed cellular cAMP content after a 20-minute incubation with the peptide, while prostaglandin PGE2 was used as a positive control (Figure 5). We did not detect neither increase nor drop of the cAMP concentration, and thus the participating kinase is PKC, and not a cAMP-dependent PKA.
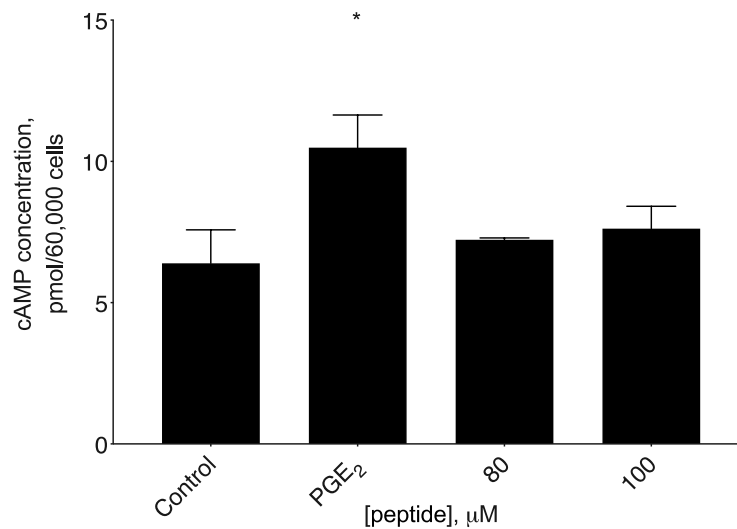
Figure 5. cAMP production after ACTH(6-9)PGP treatment. SH-SY5Y cells were treated with 80 and 100 μM of the peptide or with 40 μM of PGE$_2$ (positive control) for 20 min, after which the cAMP concentration was measured using a competitive ELISA kit. Untreated cells were used as control. Mean±standard error, N=3 experiments. *, a statistically significant difference from the control, ANOVA with the Holm-Sidak post-test, p≤0.05.

2.5. ACTH(6-9)PGP Decreased the Expression of the NF-κB and Nrf-2 Related Genes, but not of the Antioxidant Enzymes

The downstream mechanism for the protection against the ROS cytotoxicity could be the induction of the cellular antioxidant genes.

To check this possibility and to further elaborate the pro-proliferative activity of the peptide, we analyzed the mRNA levels of a set of signaling pathways after ACTH(6-9)PGP application both alone and in combination with H$_2$O$_2$ for 24 h [16]:

- *NF-κB pathway*: AKT3, IκB, and NF-κB
- *Nrf-2 pathway*: Nrf2, HO-1, GST, NQO1, GCL, SOD1, SOD2, and catalase
- *MAPK pathway*: JNK, P38, MKP1, PP2A, PP5, and Ki-67
- *DNA damage related*: P53

The mRNA levels of the Akt3, Catalase, SOD1 and Pp2a genes were below the non-specific amplification (no template control) threshold (data not illustrated). Peptide treatment alone activated NF-κB pathway expression, stimulated the p38 protein kinase, and activated the expression of several Nrf-2 pathway components (Figure 6). In the combination with H$_2$O$_2$, the stimulation of the NF-κB pathway was somewhat decreased, and also a small decrease in the Ki-67 gene expression was observed. P38 expression was further enhanced. Two specific gene expression changes were observed for the combination of the peptide with H$_2$O$_2$: first, the addition of ACTH(6-9)PGP restored the levels of p53 and pp5 to the control level, and second, the expression of the Gclc gene was increased. These changes agree with the pro-survival and pro-proliferative action of the peptide.
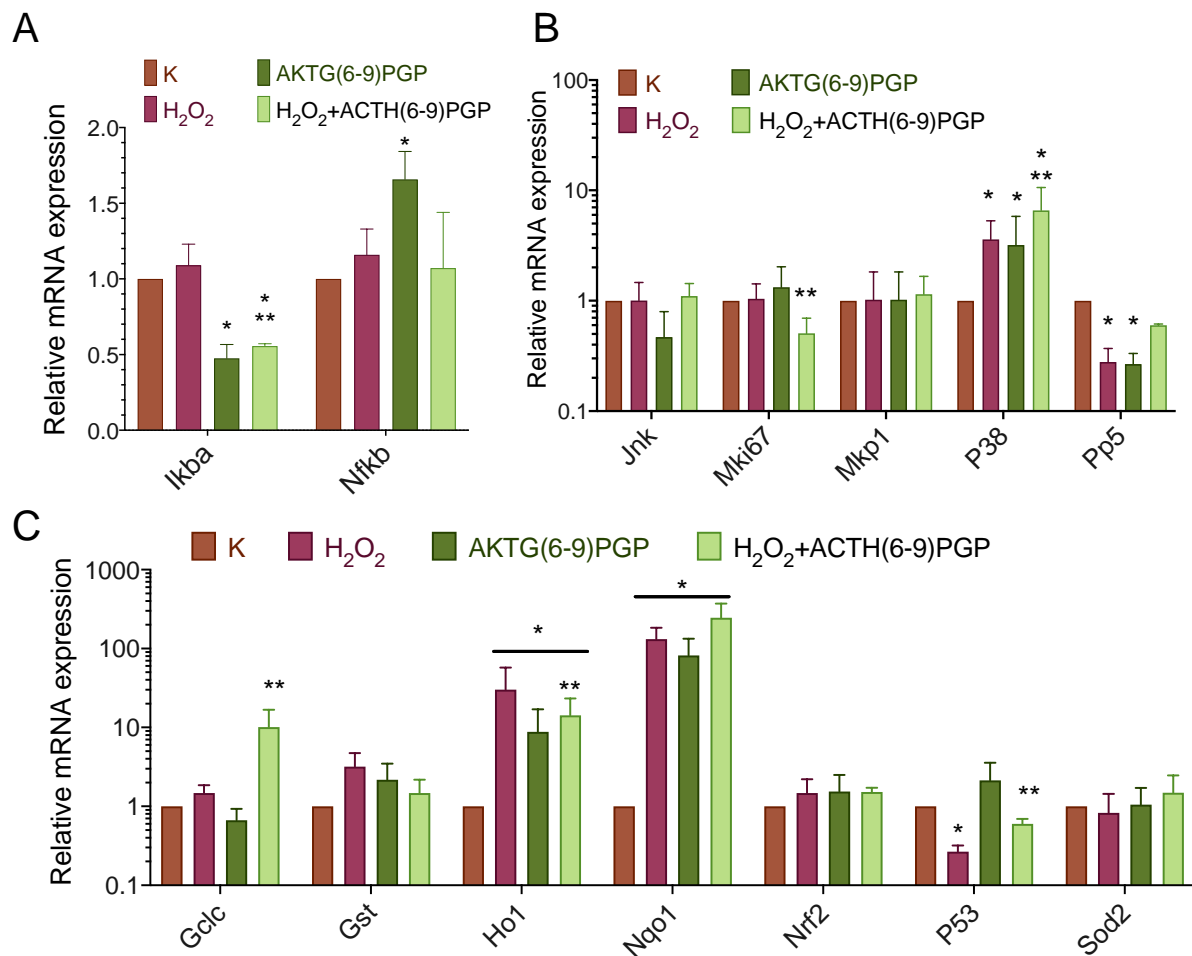
Figure 6. mRNA level changes after ACTH(6-9)PGP treatment with and without H_2O_2. SH-SY5Y cells were treated with 475 μM of H_2O_2 either alone, or with the 50 μM of the peptide for 24 h, after which mRNA levels were determined. Untreated cells were used as control. Data are normalized to beta-2 microglobulin, RPII, and GPDH. The results are grouped into genes from the Nk-κB (**A**), MAPK (**B**), and Nrf-2 (**C**) pathways. Mean±standard error, N=3 experiments. *, a statistically significant difference from untreated control; **, a statistically significant difference from the H_2O_2 alone, ANOVA with the Holm-Sidak post-test, p≤0.05.

3. Discussion

The aim of this work was to identify the intracellular systems, which could participate in the neuroprotective activity of the ACTH(6-9)PGP peptide. Such data could give an insight into the possible interactions with other neuroprotective drugs to produce a drug combination with an enhanced activity in the future. The obtained results point to the following two peptide properties: 1) the peptide evoked pro-proliferative effect; 2) the peptide stimulates the NF-κB and Nrf-2 signaling pathway.

The peptide enhanced cell viability in the H_2O_2, tBH, and KCN cytotoxicity models, but not in the MPP+ cytotoxicity model. The ability of the peptide to enhance cell viability increase in the sequence H_2O_2 < tBH ≤ KCN.

The increase of cell survival in the H_2O_2 model after the ACTH(6-9)PGP treatment was comparable to the peptide's pro-proliferative effect. To induce cell death, H_2O_2 is added to the cells in vast quantities. Therefore, the antioxidant systems of the cell are hardly possible to cope with the ROS from H_2O_2, even if they are stimulated by the peptide, and the net increase of the cell viability after ACTH(6-9)PGP treatment should be due to the proliferation increase. The cytotoxic concentration of tBH was more than 20 times lower, and thus it is quite expectable that the peptide was able to protect much more cells in this model.

The effect of the peptide in the KCN cytotoxicity model was somewhat different from H_2O_2 and tBH, as it was active only at quite high concentrations. This discrepancy could be explained by the fact that tBH and H_2O_2 due to their short lifetime in the culture medium produce a rather short stress [17], while KCN, which targets mitochondria [18], produces a long-term ROS production and thus may require a much higher activation of the antioxidant systems.

MPP[+], a neurotoxin which plays dominant neurotoxic roles in selectively damaging catecholaminergic neurons including dopaminergic neurons, has widely been used in the experimental models of PD, and it can operate in extracellular or intracellular oxidation, yielding ROS that lead to toxic downstream molecules and result in neuronal damage [19]. It was demonstrated that the treatment of SH-SY5Y cells with MPP[+] results in a significant increase of ROS [11]. However, MPP[+] is quite unstable, and thus it causes only a temporary ROS concentration increase. In this condition, the antioxidant activity of a substance should play a substantial role, as it was shown, for example, for α-lipoic acid [20]. ACTH(6-9)PGP failed to decrease the ROS concentration after an hour and thus does not act as a direct ROS scavenger. Its structure (His-Phe-Arg-Trp-Pro-Gly-Pro) does not entirely preclude such activity, as phenylalanine and tryptophan residues may react with ROS [21]. It could be proposed that the concentration of the peptide is not enough for this activity to manifest. The vast quantities of MPP[+] required to induce cytotoxicity also make it hardly possible for the cellular antioxidant machinery to be able to cope with such stress, and this is the most probable explanation for the lack of the peptide activity in this model.

The signaling behind the activity of ACTH(6-9)PGP requires further investigation; however, several hypotheses could be put forward to explain it. As far as the active peptide concentrations were much smaller than the toxic agent's ones, ACTH(6-9)PGP should interact with some cellular signaling machinery, rather than inactivate the added toxins or produced ROS directly. This agrees with the observed disappearance of the peptide's protective action after the inhibition of the PLC->PKC->Ras->MEK signal transduction pathway.

Downstream of these signaling components, after the peptide treatment, the mRNA expression changed according to several patterns.

Peptide treatment alone activated NF-κB expression and decreased its contra partner IκB expression; the latter change was also observed in the presence of H_2O_2. This change should activate the pro-proliferative NF-κB pathway [22,23] and agrees with the observed pro-proliferative action of the peptide. The increase of the NF-κB expression after the peptide treatment also agrees with the detected inhibitor activity, as this gene activity is activated by Ras [24].

ACTH(6-9)PGP treatment also stimulated the expression of the p38 protein kinase, and of several Nrf-2 pathway components. In the presence of H_2O_2, p38 expression was further enhanced. PKC, which was one of the components of the detected peptide signaling, is also known to stimulate the activity of the Nrf-2 transcription factor [25], and thus the observed activation of the expression of the Nrf-2 targets Nqo1 and Ho-1 after the peptide treatment seems quite logical.

Three specific gene expression changes were observed for the combination of the peptide with H_2O_2: first, the addition of ACTH(6-9)PGP restored the levels of p53 and pp5 to the control level, second, the expression of the Gclc gene was increased, and finally, a small decrease in the Ki-67 gene expression was observed. These changes mostly agree with the pro-survival and pro-proliferative action of the peptide with some shift toward the former one in the presence of cytotoxic agents. The activation of the gene of the rate-limiting enzyme in the glutathione biosynthesis Gclc [26] is of particular interest, as it presents an interesting way of the long-term defense against the oxidative stress, induced by the peptide.

The direct target of the peptide is not clear. Based on the fact that the peptide represents a part of the adrenocorticotropic hormone, its receptor could be the first candidate. ACTHR is coupled to a $G_{\alpha s}$ subunit [27], and so its activation should lead to an in-

crease of cAMP concentration. However, cAMP levels did not change after ACTH(6-9)PGP application (Figure 5), and thus ACTHR could be excluded from the list of potential targets.

Recently deorphanized cannabinoid receptors GPR18 and GPR55 are known to stimulate proliferation [28,29]; recently, a possibility of GPR55 peptide modulation was demonstrated [30]. In our experimental setting, the inhibitors of these two receptors (ML-193 at 2 μM and PSB CB5 at 0.5 μM) prevented ACTH(6-9)PGP protection against KCN cytotoxicity (Supplementary figure S1). Thus, these receptors could be at least allosteric targets for this peptide; however, a more detailed study of this interaction is out of the scope of this paper.

Other possibilities for the ACTH(6-9)PGP target include receptors like GLP1R, FGFR, and heat shock proteins. GLP1R is the receptor for neuroprotective action of the peptide Glp-1 [31]. This receptor signaling is biased [32], so it possibly could interact with ACTH(6-9)PGP. However, it is also coupled to $G_{\alpha s}$, and thus does not fit the observed data. FGFR activation by a peptide representing the receptor-binding domain of bFGF was shown to elicit a neuroprotective response in SH-SY5Y cells [33]. FGFR is a receptor tyrosine kinase, and it is linked to the Akt signal transduction pathway [34]. Therefore, this or a similar receptor could be the ACTH(6-9)PGP target. Heat shock proteins were found to interact with other bioactive peptides [35] and increase cell proliferation [36], and this pathway should be kept in mind. A more detailed investigation of discussed pathways is necessary to elucidate mechanisms of cytoprotective activity of modified ACTH(6-9) peptide.

4. Materials and Methods

4.1. Materials

L-glutamine, fetal bovine serum, penicillin, streptomycin, amphotericin B, Hanks' salts, trypsin, DMEM, and (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from PanEco, Moscow, Russia. 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), HEPES, DMSO, D-glucose, MPP+, KCN, MPP+, L-NAME, tert-butyl hydroperoxide, and bovine serum albumin were from Sigma-Aldrich, St. Louis, MO USA. 666-11, SB 202190, SP 600125, Salirasib, FIPI, KN-93, ML-193, PSB C5, HA-1004, U-0126, KT-5720, U-73 were from Tocris Bioscience, Bristol, UK. Total RNA Purification kit was from Jena Biosciences, Jena, Germany. MMLV reverse transcription kit and qPCR master mix qPCRmix-HS SYBR were from Evrogen, Moscow, Russia. cAMP determination kit and BrdU cell proliferation assay kit were from Abcam, Cambridge, MA, USA. DNase I was from Thermo Fisher Scientific, Walthon, MA USA.

The peptide was synthesized, as described earlier [8].

4.2. Cell Culture

SH-SY5Y cells (ATCC CRL-2266) were maintained in the 1:1 MEM: F12 medium supplemented with 10% of fetal bovine serum, 2 mM of L-glutamine, 0.5 mM of sodium pyruvate, 0.5% of non-essential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B in a CO_2 incubator with the atmosphere of 5% CO_2 and 95% humidity at 37°C. Cells were passaged every 72 to 96 h by washing with Versene's solution and treatment with 0.25% trypsin with 0.53 mM EDTA in Hank's balanced salt solution.

4.3. Oxidative Stress Induction

For cell viability experiments, the cells were seeded at a density of 15,000 per well of 96-well plate in 100 μL of culture medium and incubated for 12 h. After that, the substance solution with or without the toxic agent in 100 μL fresh cell culture medium was added to the medium present in the wells and incubated for 24 h. Cytotoxicity was induced by either 475 μM of H_2O_2, 850 μM KCN, 1.3 mM MPP+ (freshly prepared1) or 15 μM tert-butyl hydroperoxide (all with the addition of 10 mM of HEPES, pH 7.4).

4.4. Cell Viability Assay

Cell viability was analyzed using the MTT test [37]. In short, the culture medium was removed from the wells, and 75 µl of the 0.5 mg/ml solution of MTT with 1 g/L D-glucose in Earle's salts was added to each well and incubated for 90 min in the CO_2 incubator at 37°C. After that, 75 µL of 0.04 M HCl in isopropanol was added to the MTT solution in each well and incubated on a plate shaker at 37°C for 30 min. The optical density of the solution was determined using a plate reader (Efos 9226, MZ Sapphir, Russia) at the wavelength of 570 nm with a reference wavelength of 620 nm.

4.5. cAMP Assay

cAMP levels were measured using a cAMP determination kit according to the manufacturer's instructions. The cells were seeded at a density of 60,000 per well of 96-well plate and grown for 12 h. The substances were added in 100 µL fresh cell culture medium to the medium present in the wells and incubated for 20 min. After that, the cell culture medium was removed, and the cells were subjected to cAMP determination.

4.6. ROS Assay

ROS generation was measured using the DCFH-DA dye. The cells were seeded at a density of 60,000 per well of 96-well plate and grown for 12 h. After that, the medium was replaced with a fresh one with 25 µM of the dye, and the cells were incubated in the CO_2 incubator at 37°C for 1 h. After the incubation, the cells were washed twice with the culture medium and treated with the substances in the culture medium for 1 h at 25°C. Cells treated with medium without H_2O_2 and substances were used as a control. After the incubation, the cells were washed twice with Hank's balanced salts solution with 25 mM HEPES and 1 mg/ml fatty acid-free bovine serum albumin, pH 7.4, and the fluorescence was measured using the plate reader Hidex Sense Beta Plus (Hidex, Turku, Finland), λ_{ex}=490 nm, λ_{em}=535 nm.

4.7. Apoptosis Assay

Apoptosis level was analyzed using the Apoptosis/Necrosis detection kit. The cells were seeded at a density of 15,000 per well of 96-well plate and grown for 12 h. After that, 475 µM of H_2O_2 alone or with the peptide was added in 100 µL of the fresh medium to 100 µL of the old medium in the wells and incubated for 1 h at 37°C in a CO_2 incubator. After that, the medium was removed, and the cells were stained according to the manufacturer's instructions. The stained cells were photographed using an inverted fluorescent microscope Nikon Ti-S using a Semrock GFP-3035D filter cube with magnification 100x. For each well, 5 non-intersecting view fields were captured, and apoptotic cells were counted.

4.8. mRNA Assay

mRNA levels were analyzed using RT-qPCR. The cells were seeded at the density 240,000 per well of a 24-well plate in 200 µL of culture medium and incubated for 12 h. The substances were added in 200 µL of fresh culture medium to the medium in the wells and incubated for 24 h. Total RNA was extracted using a Total RNA isolation kit according to the manufacturer's protocol. The isolated RNA was treated with DNase I according to the manufacturer's protocol. cDNA was synthesized using an oligo-dT primer using the MMLV reverse transcription kit. qPCR was performed using a SYBR Green containing master mix qPCRmix-HS SYBR with the following amplification protocol: 95°C for 2 min, cyclic 95°C for 10 s, 57°C for 20 s, 72°C for 15 s for 40 cycles using a Bio-Rad C1000 thermal cycler (Bio-Rad, Hercules, CA, USA). After the amplification, PCR product melting curve was recorded in the range from 65°C to 95°C. Primer sequences were generated using the IDT PrimerQuest service (https://eu.idtdna.com/PrimerQuest) and validated using the NCBI PrimerBLAST service [38], or taken from the paper by Jaafru et al. [16]. The primer sequences were as follows (5'-3'):

AKT3 forward AGGTGACACTATAGAATAAGACATTAAATTTCCTCGAA, reverse GTACGACTCACTATAGGGAATCCTCATCATATTTTTCAGGT,

Beta-2 microglobulin forward CAGCAAGGACTGGTCTTTCTAT, reverse ACATGTCTCGATCCCACTTAAC,

Catalase forward AGGTGACACTATAGAATAAGAAATCCTCAGACACATCT, reverse GTACGACTCACTATAGGGAATGTCATGACCTGGATGTAA,

GST forward AGGTGACACTATAGAATAATACATGGCAAATGACTTAAA, reverse GTACGACTCACTATAGGGATGATGTCTTCATTCCTTGAC,

GCLC forward AGGTGACACTATAGAATAATGAAGCAATAAACAAGCAC, reverse GTACGACTCACTATAGGGATGGAATGTCACCTGGAG,

GAPDH forward GAATGGGAAGCTGGTCATCAA, reverse CCAG-TAGACTCCACGACATACT,

HO-1 forward AGGTGACACTATAGAATAACTGCGTTCCTGCTCAACAT, reverse GTACGACTCACTATAGGGAGGGCAGAATCTTGCACTTTGT,

IKBA forward AGGTGACACTATAGAATACTGCAGCAGACTCCAC, reverse GTACGACTCACTATAGGGAGGGTATTTCCTCGAAAGT,

JNK forward AGGTGACACTATAGAATAAAGGAAAACGTGGATTTATG, reverse GTACGACTCACTATAGGGACCAGCATATTTAGGTCTGTT,

MKP1 forward AGGTGACACTATAGAATAAGAAGAACCAAATACCTCAA, reverse GTACGACTCACTATAGGGACAGGTCATAAATAATCAGCA,

MKI67 forward GCTGAGAACTCCTAAGGGAAAG, reverse GCTGTGAA-GCTCTGTAGGATAC,

NFkB forward AGGTGACACTATAGAATACGTTTTAGATACAAATGTGAAG, reverse GTACGACTCACTATAGGGACACTTTTCCTTTTCCATAAT,

NQO1 forward AGGTGACACTATAGAATACTGCGAACTTTCAGTATCC, reverse GTACGACTCACTATAGGGAGAAGGGTCCTTTGTCATAC,

NRF2 forward AGGTGACACTATAGAATATCGCAAACAACTCTTTATCT, reverse GTACGACTCACTATAGGGAAGAGGAGGTCTCCGTTA,

P38 forward AGGTGACACTATAGAATATGAGCTGAAGATTCTGGA, reverse GTACGACTCACTATAGGGATGTCAGACGCATAATCTG,

P53 forward AGGTGACACTATAGAATAATGGAAACTACTTCCTGAAA, reverse GTACGACTCACTATAGGGAATTCTGGGAGCTTCATCT,

PP5 forward AGGTGACACTATAGAATACAAGGACTACGAGAACGCCA, reverse GTACGACTCACTATAGGGAGCTTCACCTTGACCACCGTC,

PP2A forward AGGTGACACTATAGAATACCGCCATTACAGAGAG, reverse GTACGACTCACTATAGGGAAGGATTTCTTTAGCCTTCT,

RPII forward CCCAGCTCCGTTGTACATAAA, reverse TCTAACAG-CACAAGTGGAGAAC,

SOD1 forward AGGTGACACTATAGAATAAAGTACAAAGACAGGAAACG, reverse GTACGACTCACTATAGGGATGACAAGTTTAATACCCATCT,

SOD2 forward AGGTAGCATGGACCGAATTTAC, reverse GA-TAGCCAGGTGTTTGCTTCT.

4.9. Proliferation Assay via the BrdU Incorporation

The stimulation of the cell proliferation by the ACTH(6-9)PGP peptide was validated using the BrdU cell proliferation kit (Abcam, Cambridge, MA, USA). The cells were seeded in 96-well plates at the density of 4000 per well and grown overnight. After that, peptide solution in the fresh culture medium was added to the cells with full medium replacement; the peptide addition was performed on days 1 and 4 after the seeding. On day 6, BrdU reagent was added to the cells for 24 h, and assayed according to the manufacturer's protocol.

4.10. Statistics

All experiments were performed at least in triplicate. Statistical analysis was performed with the GraphPad Prism 6.0 software using ANOVA with the Holm-Sidak post-test; $p < 0.05$ was considered a statistically significant difference.

5. Conclusions

For the first time, we showed that the peptide combining fragment of ACTH and tripeptide PGP (FRWGPGP) protects SH-SY5Y cells against the H_2O_2, tBH, KCN, but not the MPP^+ cytotoxicity. The mechanism of its action was the modulation of proliferation-related (NF-κB and Nrf-2) and antioxidant-related (HO-1, Nqo1, Gclc) genes and apoptosis decrease.

Supplementary Materials: Supplementary Figure S1: GPR55 and GPR18 participation in ACTH(6-9)PGP protection against the KCN cytotoxicity for the SH-SY5Y cells.

Author Contributions: Conceptualization, M.G.A. and V.V.B.; methodology, M.G.A., V.V.B., and L.A.A.; software, M.G.A.; formal analysis, M.G.A. and V.V.B..; investigation, M.G.A., E.V.F., L.A.A., and P.V.D.; resources, N.F.M. and V.V.B.; data curation, M.G.A. and V.V.B.; writing—original draft preparation, M.G.A.; writing—review and editing, V.V.B.; visualization, M.G.A.; supervision, V.V.B..; project administration, V.V.B.; funding acquisition, N.F.M. and V.V.B. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to legal issues.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Sample Availability: Samples of the compounds ACTH(6-9)PGP are available from the authors.

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