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Original Article

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The effects of NP-POL, the nonapeptide constituent of Colostrinin, on intracellular antioxidant system

Wpływ nonapeptydu NP-POL z kompleksu PRP--kolostryniny na wewnątrzkomórkowe mechanizmy antyoksydacyjne

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Latest findings show that the proline-rich polypeptide complex (PRP), known as Colostrinin, and its component peptides may have beneficial effects on cellular response to oxidative stress, which is the main aspect of aging and an essential feature in neurodegenerative disorders. Recently isolated and described nonapeptide NP-POL (RPKHPIKHQ) was shown to have potential protective properties in neuronal cells, including the regulation of cell survival, neurite protection and intracellular ROS release. Based on those findings, further studies were performed covering the possible effects of NP-POL on molecular and enzymatic mechanisms of response to oxidative stress.

Materials/Methods: To evaluate the regulatory effects of NP-POL on oxidative stress, 6-hydroxydopamine-treated rat pheochromocytoma (PC12) cells were used as an experimental model and a series of assays were performed, including H_2O_2 release, glutathione turnover and the expression and activity of superoxide dismutases (Mn-SOD and Cu-Zn-SOD).

Results: NP-POL was shown to modify the cellular antioxidative response to reverse the toxic effects of 6-hydroxydopamine. The direct effects of NP-POL include a significant reduction of the total amount of released H_2O_2 and enhanced glutathione activation. Moreover, NP-POL enhanced the expression of superoxide dismutase in untreated cells, which confirms its contribution in secondary enzymatic response.

Discussion: The newly discovered peptide NP-POL, isolated from the colostrum-derived polypeptide complex Colostrinin, was shown to have potential antioxidant and neuroprotective properties, which makes it very promising as a natural therapeutic in neurodegenerative diseases.

Keywords: **nonapeptide NP-POL, Colostrinin, proline-rich polypeptide complex PRP, oxidative stress, antioxidant enzymes**

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INTRODUCTION

Oxidative stress has been proposed as an etiologic factor in many age-related disorders, including Parkinson's disease, Alzheimer's disease and other increasingly spreading neurodegenerative disorders [19]. The brain, compared with other organs, is especially vulnerable to free radical damage because of its high oxygen consumption rate, abundant lipid content, and relative paucity of antioxidant enzymes compared with other organs. The generation of reactive oxygen species (ROS) in normal neurons is under tight homeostatic control. Unchecked, excessive ROS generation may lead to the induction of oxidative stress, the destruction of cellular components, including lipids, proteins, and DNA, and ultimately cell death via apoptosis or necrosis [42]. Since most of the senescent conditions have oxidative and inflammatory components, studies are geared towards finding strategies to suppress these processes. It has become strongly important to understand the mechanism of action for therapeutic compounds that may influence cellular oxidation balance.

The production of ATP during oxidative phosphorylation in the mitochondria is a major metabolic source of free radicals or reactive oxygen species, such as superoxide anion•O_2–, hydrogen peroxide H_2O_2 and hydroxyl radical, •OH [34]. H_2O_2 is an important signaling molecule for synaptic and neuronal activity in the central nervous system. It also plays an important role in oxidative stress and cell degeneration [12, 29]. Low or moderate levels of ROS are essential for neuronal development and function, but excessive amounts are harmful. Antioxidant defence mechanisms provides constant protection against ROS overproduction, but when its scavenging capacity is exceeded, the extensive oxidation and peroxidation lead to damage of the macromolecules in cells. Neurotransmitter activity also generates free radicals up to auto-oxidation of dopamine and glutamatergic excitotoxicity [1, 16].

Oxidative damage by endogenous ROS is prevented by the brain antioxidant network, which includes low molecular weight antioxidants, antioxidant enzymes and also repair systems. Low molecular weight antioxidants in neurons are mainly represented by glutathione and ascorbate [35]. Additionally, uric acid, bilirubin, coenzyme Q, di- and polypeptides, and metal-binding plasma proteins play an important role [20]. Enzymatic antioxidant system includes superoxide dismutases (SODs), catalase (CAT), glutathione peroxidase (GSHPx), and glutathione reductase (GSSGR). Cellular •O_2– is managed by cytosolic (Cu/Zn-SOD) and mitochondrial (Mn-SOD) forms of superoxide dismutase, which produces H_2O_2. In turn, H_2O_2 is metabolized by GSH peroxidase, which is free in cytosol and peroxisomal catalase. Additionally, interaction of •O_2– or H_2O_2 with trace metal ions, such as iron and copper, can produce highly toxic hydroxyl radical, •OH, which can be neutralized by GSH and ascorbate. Thus, both enzymes and low-molecular weight antioxidants efficiently cooperate to prevent oxidative stress in neurons [2, 36, 37].

SOD is a very important enzyme in cellular redox mechanism, which exists in three different forms in the mitochondrial matrix (Mn-SOD, *SOD1*), cytoplasm (Cu-Zn-SOD, *SOD2*) and extracellular fluids (Cu-Zn-SOD, *SOD3*) [10]. Together with catalase and glutathione peroxidase they represent the first line defence antioxidants with an indispensable role in the entire antioxidant strategy [43]. Deficiency of any of the SOD isoforms, but notably mitochondrial Mn-SOD, may be associated with a number of pathologies, including neurodegeneration, myocardial injury and perinatal death [21]. The expression of two major SOD genes, *SOD1* and *SOD2*, has been found to be mildly up-regulated by oxidative stress and H_2O_2 [27]. Many environmental conditions, in particular stress and genetic mutations, have been shown to induce *SOD3* transcription; however, the importance of *SOD3* up-regulation for resistance to these stresses has not been well established [12].

Glutathione (GSH), next to ascorbate, is the most abundant low molecular weight antioxidant in neurons, involved not only in removing peroxides, but also in regulation of the cell cycle [13]. In the presence of H_2O_2, glutathione is oxidised featuring GSHPx and forms glutathione disulphide (GSSG) [15]. The reverse reaction, GSSG-GSH, is catalyzed by GSSGR [44].

The proline-rich polypeptide complex (PRP), also known as the Colostrinin (CLN), is recognised to have diverse biological activity, affecting innate immunity, cytokine induction [17] and allergic inflammatory response [7]. Colostral proline-rich polypeptide complex PRP, at a concentration range of 1 to 100μg/ml induced the production of cytokines in human peripheral blood cells *ex vivo* and inhibited LPS--induced NO production in mice [46, 48]. Moreover, it has been shown to have a potent inhibitory effect of PRP on oxidative stress response mechanisms, mostly by decreasing intracellular levels of H_2O_2 and increasing glutathione workflow [8, 45]. In cultured neuron-like PC12 cells, it induced neuritogenesis and increased lifespan by preventing mitochondrial dysfunction [3, 4]. Observations made on human neuroblastoma SH-SY5Y cell cultures showed that pre-treatment with CLN protects cells against exogenous oxidants, such as H_2O_2, amyloid-β or ROS generated enzymatically [11]. It was confirmed to alleviate amyloid-β

induced toxicity in SH-SY5Y cells and significantly reduce elevated levels of SOD in primary hippocampal neuronal cell cultures in rats [14, 38]. CLN and its constituent nonapeptide fragment (NP) have no direct antioxidant activity, but they were shown to modulate the first-line defence against exogenous oxidants, mostly by regulating levels of intracellular antioxidant molecules and enzymes [47].

Formerly unknown nonapeptide fragment isolated from Colostrinin with the Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln (RPKHPIKHQ) sequence was recently isolated and characterised by its antioxidant, immunoregulatory and neuroprotective activity. The latest study investigating the possible therapeutic potential of NP-POL revealed that it can effectively reduce ROS generation in PC12 cell cultures treated with 6-hydroxydopamine, the neurotoxin linked to oxidative and inflammatory reactions in Parkinson's disease. Additionally, it was also shown that NP-POL is able to modulate activity of extracellular signal-regulated kinases (ERK 1/2). Treatment of PC12 cells with NP-POL nonapeptide reduced 6-OHDA-induced apoptosis and caused transient phosphorylation of extracellular signal-regulated kinases (ERK 1/2), which were shown to promote cell survival [22].

The goal of the present study is to investigate the mechanisms of the potential neuroprotective properties of NP-POL in relation to intracellular redox balance regulation and enhancement of enzymatic antioxidant response.

MATERIALS AND METHODS

Reagents

6-hydroxydopamine (6-OHDA) was provided by Tocris Bioscience (Bristol, UK). Glutathione (reduced form, GSH), glutathione disulphide (GSSH), β-NADPH, 2-vinylpirydine, triethanolamine, 5,5'-Dithiobis (2-nitrobenzoic acid, DTNB), glutathione reductase, TRIzol®, sulfosalicylic acid, Triton-X, β-mercaptoethanol were from Sigma-Aldrich (Merck Group, Darmstadt, Germany). Protease Inhibitor Cocktail Tablets were from Roche (Basel, Switzerland). High-glucose Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (pH 7.4) (PBS) were from the Laboratory of General Chemistry of the Institute of Immunology and Experimental Therapy, PAS (Wroclaw, Poland). Opti-MEM Reduced-Serum Medium was obtained from ThermoFisher Scientific (Waltham, MA, USA). Tissue culture dishes and foetal bovine serum (FBS) were obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). DNA-se I, M-MLV reverse transcriptase, and qPCR Master Mix were obtained from Promega (Madison, WI, USA). Standard oligo-DNA primers for qPCR reaction were obtained from Sigma-Aldrich (Merck Group, Darmstadt, Germany). The nonapeptide fragment of PRP (NP-POL) Arg-Pro-Lys-His-Pro-Ile- Lys-His-Gln was obtained by chemical synthesis at Lipopharm (Gdansk, Poland).

Cell culture

PC12 (Tet ON)(ATCC) rat pheochromocytoma cells were used as a model of neuronal cells. The cells were maintained under 5% CO2/95% humidified air at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% horse serum and 10% foetal bovine serum, antibiotics (penicillin and streptomycin) and 2 mM L-glutamine, the culture medium was changed once every three days. The cells were allowed to adhere and grow for 24 hours before experimental treatment. To mitigate any excessive intracellular enzymatic activity, the cells were cultured in Gibco® Opti-MEM Reduced-Serum Medium (Thermo Fisher).

Time points used in particular experimental conditions specify the time periods that are needed for 6-OHDA to penetrate the cells, transform to active metabolites and generate ROS, and simultaneously ensure that 6-OHDA does not decompose due to autoxidation.

Determination of intracellular glutathione ratio

Measurement of GSH in PC12 cells was assessed by spectrophotometric method using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), also known as Ellman's reagent [33]. One milliliter portions of PC12 cells suspended in Opti-MEM medium, (density of 10^6 cells/ml) were applied to each well of 6-well plate. Incubation with inducers lasts 6 h and 24 h, following the experimental conditions: control (untreated), 100 µg/ml NP-POL, 150 µM 6-OHDA and NP-POL added simultaneously with 6-OHDA.

After incubation, the cells were harvested, transferred to 1.5 ml Eppendorf tubes, washed with 1–2 ml of cold PBS and centrifuged at 1000 xg for 5 minutes in 4°C. Supernatants were discarded, pellets suspended in 1 ml PBS, centrifuged and drained. Pellets were then re-suspended in 1 ml of extraction buffer (containing 5% sulfosalicylic acid, along with 0.1% Triton X-100 in PBS), sonicated in ultrasonic washer filled with ice water for 2 to 3 minutes with brief pulse-vortexing every 30 seconds and put under 2–3 freeze-thaw cycles to ensure proper lysis. Lysates were then centrifuged at 3000xg for 4 minutes in 4°C, supernatants were moved into new chilled Eppendorf tubes and kept on ice. Reagents were prepared according to Rahman et al. protocol [33], including 0.1 M potassium phosphate buffer with 5 mM disodium EDTA (KPE), DTNB, β-NADPH, 2-vinylpyridyne, triethanolamine, glutathione reduced (GSH) and disulphide form (GSSG) standards and glutathione reductase (GR). For total GSH assay, KPE buffer, samples and GSH standards were loaded on 96-well plate in duplicates, then freshly mixed DTNB and GR solution was added to each well. After 30 seconds, β-NADPH solution was added and absorbance was read immediately at 412 nm, every 30 seconds for 2 minutes. For GSSG assay, sulfosalicylic acid cell extracts and GSSG standards were first incubated with 2 µl 2-vinylpyridine for 1 hour to derivatize GSH, then with 6 µl triethanolamine for 10 minutes to neutralize final pH. The assay for GSSG was performed by the same method as for total GSH.

The change of absorbance in time was calculated from 5 measurements (tn-t0) and standard curve was determined to count the concentration of total GSH and GSSG in samples. Reduced GSH concentration values were calculated by subtracting the GSSG from total GSH concentration. The results were expressed as the ratio of reduced GSH to GSSG.

Determination of extracellular H_2O_2 release

A simple colorimetric method for the measurement of hydrogen peroxide was used according to the method described by Pick and Keisari [30]. One milliliter portions of PC12 cells suspended in Opti-MEM medium (density of 10^6 cells/ml), were applied to each well of 6-well plate and next incubated with NP-POL (100 µg/ml), 6-OHDA (150 µM) or NP-POL added simultaneously with 6-OHDA for several incubation times (24h, 9h, 6h, 3h, 90 min and 45 min). At the start of each incubation time the inducers were added simultaneously with phenol red solution (0.28 M in PBS) mixed with horseradish peroxidase solution (20 U/ml in PBS). The reactions were stopped by adding 10 µl 1M NaOH. Cells were centrifuged and supernatants were assayed on 96-well plates on microplate reader and absorbance was measured at 610 nm. H_2O_2 concentration was calculated from standard curve and expressed as µM/10^6 cells.

Determination of intracellular SOD activity

SOD activity was assayed, using Cayman Superoxide Dismutase Assay Kit (which is based on xanthine oxidase and water soluble tetrazolium method) for colorimetric determination of SOD activity in cell extracts. All reagents and assay scheme with two blanks (one without sample and one without converting enzyme) were prepared according to manufacturer's instructions. PC12 cells cultured on 6-well plates in 1 ml Opti-MEM medium at a density of 10^6 cells/ml were incubated with inducers (100 µg/ml NP-POL, 150 µM 6-OHDA or NP-POL added simultaneously with 6-OHDA) for 6 and 24 hours. After incubation, the cells were centrifuged, washed with cold PBS, and lysed with 100 µl lysis buffer (0.1 M Tris-HCl pH 7.4 + 0.5% Triton X-100 + 5 mM β-mercaptoethanol, 0.1 mg/ml protease inhibitor). Cell lysates were centrifuged at 14000xg for 5 minutes (4°C). 96-well reaction plates with cell extract samples and standard curve were incubated in 37°C for 20 minutes and absorbance was measured at 450 nm. Total SOD activity (% of inhibition) was calculated from the following formula: (Ablank1 -Ablank2) -Asample/(Ablank1 – Ablank2) * 100%. The results were then expressed by amount of SOD units per ml per 106 cells.

Cu-Zn-SOD and Mn-SOD expression analysis

PC12 cells cultured in 1 ml of Opti-MEM medium on 6-well plates at a density of 10^6 cells/ml were incubated with NP-POL (100 µg/ml), 6-OHDA (150 µM) or NP-POL added simultaneously with 6-OHDA for 90 minutes. After incubation, the medium was aspirated, cells were washed with 1 ml of ice cold PBS, suspended in TRIzol reagent (1ml per 10^6 cells) and transferred to 2 ml Eppendorf tubes.

RNA was extracted from TRIzol with chloroform-isopropanol extraction method [35]. Half a millliliter of chloroform per 1ml of TRIzol suspension was added and the tubes were shaken vigorously for about 15 seconds, left at room temperature for 7 minutes and centrifuged at 12000 xg for 15 minutes. The clear aqueous phase was removed carefully and placed in another Eppendorf tube. Half a milliliter of isopropanol was added to the aqueous phase and mixed gently, left at room temperature for 5 minutes and centrifuged at 14000 xg for 10 minutes. RNA pellets were washed with 75% ethanol, air-dried and resolved in DEPC-treated water (30–50 µl). RNA quality and yield were measured on BioPhotometer plus (Eppendorf, Germany). Before performing PCR analysis, the remaining genomic DNA was degraded by enzymatic reaction with DNAse I. The reverse transcription step using M-MLV reverse transcriptase was then performed to obtain the total DNA from the 1 ng of extracted RNA.

cDNA was subjected to qPCR with GoTaq qPCR Master Mix with BRYT Green dye (Promega) on CFX Connect Real-Time PCR Detection System (Bio-Rad). For the amplification of the specific genes the following primers were used:

rat *Cu-Zn*SOD, forward: 5'-AGGGCATCATCAATTTCGAGC-3' and reverse: 5'-ACATTGCCCAAGTCTCCAAC-3';

rat *Mn*SOD, forward: 5'-GGAAGCCATCAAACGTGACT-3' and reverse: 5'-CCTTGCAGTGGATCCTGATT-3'.

For each mRNA quantification, the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a reference point using following primers: GAPDH, forward: 5'-GCAAGTTCAACGGCACAG-3' and reverse: 5'-CGCCAGTAGACTCCACGAC-3'. Real-time PCR data were analysed using $2^{-\Delta\Delta C}$T method. The results were presented as the normalized expression rate taking control group expression as the reference and equals one.

Statistical analysis

Each experimental procedure was performed in at least three independent cell preparations with two or three replicates of each measure, according to the assay. One-way or two-way ANOVA was used respectively to the analysed data with post-hoc multiple comparisons tests (with Sidak or Dunnett's algorithms used when either was recommended) using GraphPad Prism v.8.0.1 software. Results were expressed as mean of the independent measures±SEM and p-values were considered statistically significant at <0.05.

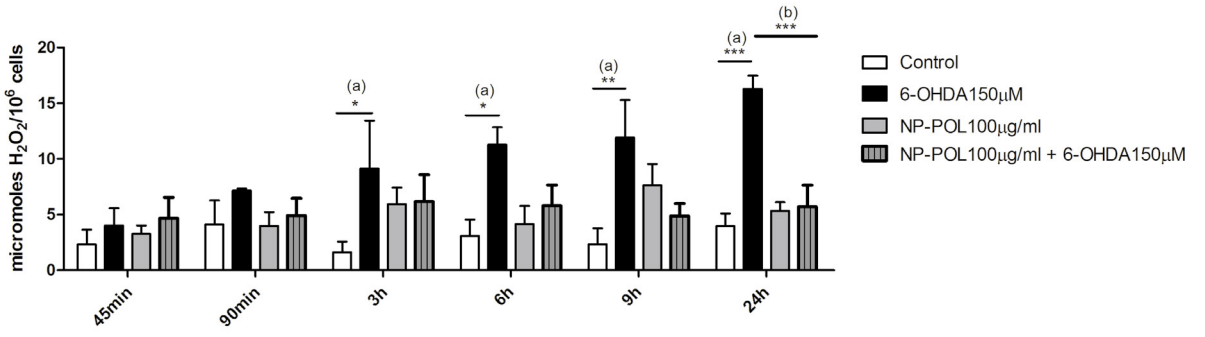

Fig. 1. Changes in extracellular H_2O_2 concentration in PC12 cells: after incubation in time intervals (45 min, 90 min, 3h, 6h, 9h, 24h) with NP-POL or 6-OHDA compared to untreated control (a) and after co-incubation with NP-POL and 6-OHDA compared to 6-OHDA alone (b). Data are expressed as mean ± SEM (n = 3). Significant results were indicated by asterisks: *p<0.05, **p<0.01 and ***p<0.001

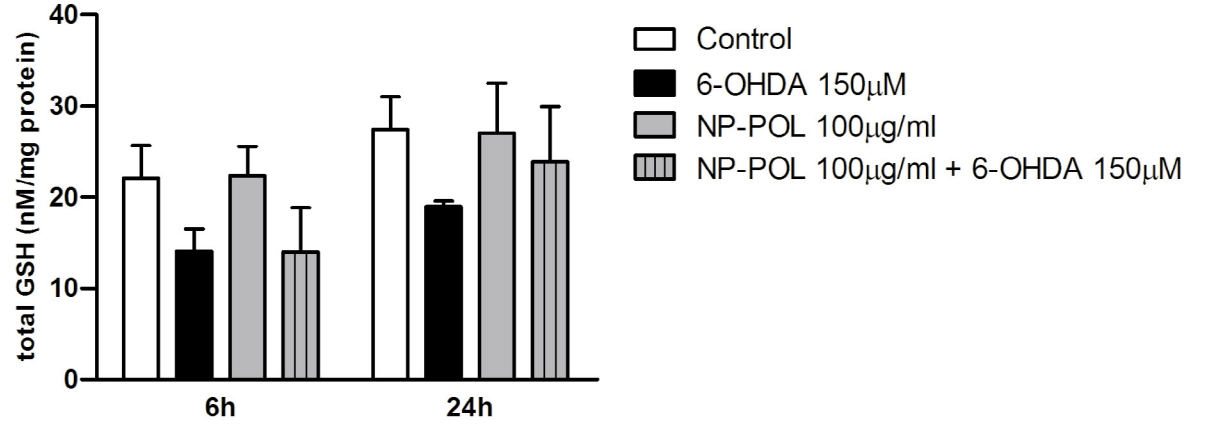

Fig. 2. Total GSH in PC12 cells after 6 and 24 hours of incubation with NP-POL, 6-OHDA and co-incubation with NP-POL and 6-OHDA. Data were presented as mean ± SEM (n = 4)

RESULTS

Extracellular H2O2 release in 6-OHDA-induced PC12 cells

NP-POL alone did not significantly change the extracellular release of H_2O_2 over the whole time of incubation (Fig. 1). Two-way ANOVA showed significant effects of incubation time ($F_{(5,36)}$ = 3.046, p = 0.022) and treatment ($F_{(2, 36)}$ = 23.54, p<0.001). The H_2O_2 concentration in PC12 cells milieu treated with 6-hydroxydopamine increased significantly after 3 hours of incubation compared to untreated cells and this effect progressed until 24 hours of incubation (mean difference within groups: Control vs 6-OHDA, 3 h: 7.54 ±2.59; p = 0.012; 6 h: 8.19 ±2.59, p = 0.006; 9 h: 9.59 ±2.59, p = 0.001; 24 h: 12.31 ±2.59, p<0.001, Sidak's test, Fig. 1). When NP-POL was co-administered to 6-OHDA-treated cells, the trend of hampering the release H_2O_2 began from 90 minutes of incubation and after 24 hours of incubation the amount of extracellular H_2O_2 remained significantly reduced (two-way ANOVA showed significant effect of treatment: F(1,24) = 13.63, p = 0.001; mean difference within groups: 6-OHDA vs NP-POL+6-OHDA, 24 h: 10.6 ±3.065, p = 0.012, Sidak's test).

Intracellular glutathione turnover in 6-OHDA--induced PC12 cells

Firstly, the total level of GSH was determined. It was shown that incubation of the PC12 cells with NP-POL (100 μg/mL) applied alone did not change the level of total GSH after 6 and 24 h of incubation, similarly to control (untreated) cells. In the presence of 6-OHDA total GSH level was reduced; however, the results were not statistically significant (Fig. 2). This can be the result of ROS generation in response to 6-OHDA oxidation.

Next, the intracellular glutathione transformations in PC12 cells due to oxidative stress response were analysed as changes of the ratio of reduced form of glutathione (GSH) to its oxidation product – glutathione disulphide (GSSG). The GSH/GSSG ratio did not change significantly after 6 hours incubation with NP-POL at the dose of 100 μg/mL, but the significant drop was observed after 6 hours of

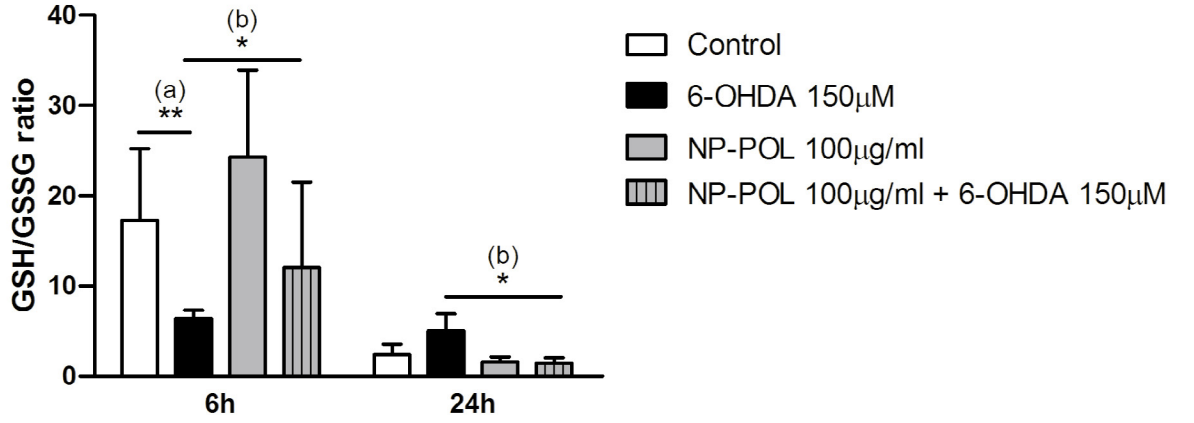

Fig. 3. Changes in GSH/GSSG ratio in PC12 cells: after 6 and 24 hours of incubation with NP-POL or 6-OHDA compared to untreated control (a); after 6 and 24 hours of co-incubation with NP-POL and 6-OHDA compared to 6-OHDA alone (b). Data were presented as mean ± SEM (n = 4). Significant results were indicated by asterisks *p<0.05 and **p<0.01

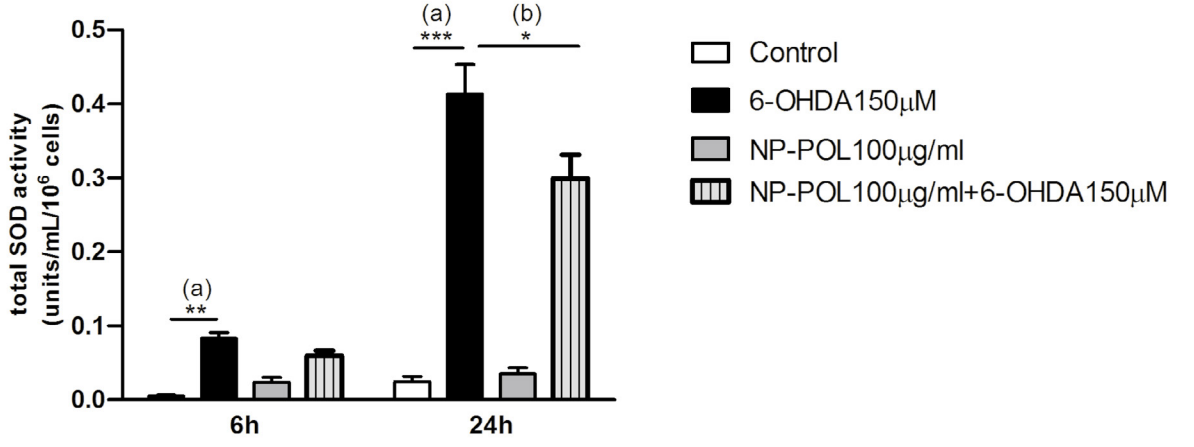

Fig. 4. Intracellular superoxide dismutase activity in PC12 cells: after 6 and 24 hours of incubation with NP-POL or 6-OHDA compared to untreated control(a); afterco-incubation with NP-POL and 6-OHDA compared to 6-OHDA alone (b). Data are expressed as mean ± SEM (n = 5). Significant results were indicated by asterisks: **p<0.01 and ***p<0.001

incubation with 6-OHDA (two-way ANOVA showed significant effect of time: $F_{(1,18)}$= 9.374, p = 0.007, mean-difference within groups: 6 h, Control vs 6-OHDA 10.89 ±3.99, p = 0.005, Dunnett's test, Fig. 3). The decrease in GSH/GSSG ratio was observed when NP-POL was co-administered with 6-OHDA and this effect was significant after 6 hours, but it significantly dropped after 24 hours of incubation ($F_{(2,9)}$= 1.557, p = 0.27, mean difference within groups: 6 h, 6-OHDA vs NP-POL+6-OHDA: 5.66 ±2.41, p = 0.024; 24h, 6-OHDA vs NP-POL+6-OHDA: 3.56 ±0.94, p = 0.011, Dunnett's test, Fig. 3).

Intracellular SOD activity in 6-OHDA-induced PC12 cells

The display of distinct inhibitory activity of NP-POL on harmful implications of the 6-OHDA presence in the PC12 cells milieu was contributed by the total intracellular SOD activity assay. Two-way ANOVA showed significant effects of time ($F_{(1,22)}$= 66.37, p<0.001) and treatment ($F_{(2,22)}$= 105.5, p<0.001) and significant interaction between time and treatment ($F_{(2,22)}$= 51.61, p<0.001). NP-POL alone did not change the total SOD activity, but it significantly increased in exposed cells after 6 and 24 hours of incubation with 6-OHDA (mean difference within groups: 6 h, Control vs 6-OHDA: 0.078 ±0.025, p = 0.009; 24 h, Control vs 6-OHDA: 0.389 ±0.025, p<0.001, Dunnett's test, Fig. 4). When NP-POL was co-administered to 6-OHDA-treated cells it significantly reduced total SOD activity after 24 hours of incubation (mean difference within groups: 24h, 6-OHDA vs NP-POL+6-OHDA: 0.115 ±0.037, p = 0.014, Dunnett's test, Fig. 4). Two-way ANOVA shows significant effect of time (F(1,16) = 119.3, p<0.001).

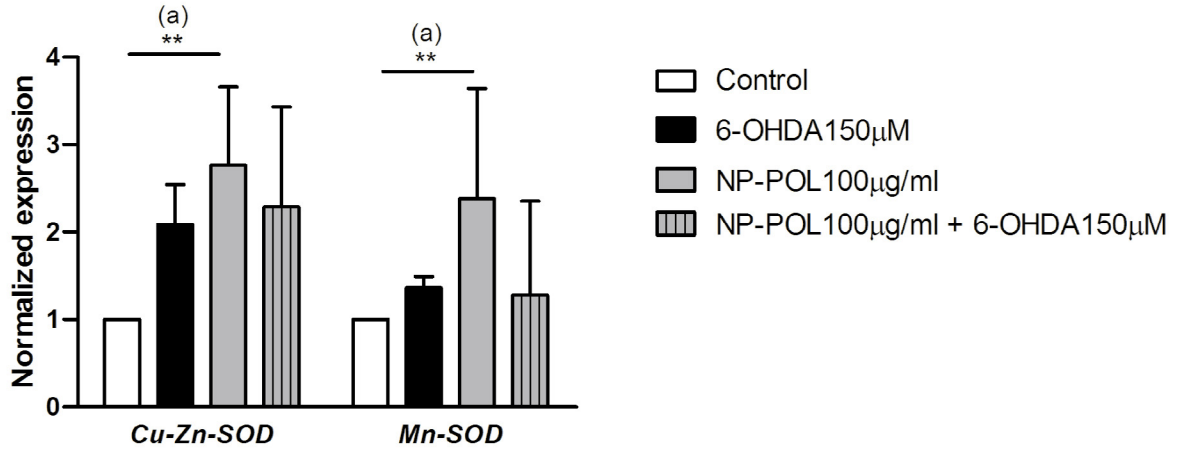

Fig. 5. Normalized expression of Cu-Zn-SOD and Mn-SOD analysed by real-time PCR in PC12 cells: after 90 minutes of incubation with NP-POL or 6-OHDA compared to untreated control (a); after 90 minutes of co-incubation with NP-POL and 6-OHDA compared to 6-OHDA alone (no significant difference).Data are expressed as mean ± SEM (n = 3). Significant results were indicated by asterisks: **p<0.01

Intracellular Cu-Zn-SOD and Mn-SOD expression profiles in 6-OHDA-induced PC12 cells

To determine the basis of altered SOD activity observed in colorimetric assay, another investigation was performed, comprising mRNA expression of Cu-Zn-SOD and Mn-SOD in PC12 cells treated with either NP-POL, 6-OHDA or with NP-POL applied to the cells simultaneously with6-OHDA for 90 minutes (Fig. 5). Two-way ANOVA showed significant effect of treatment with NP-POL or 6-OHDA alone ($F_{(2, 12)}$= 14.68, p<0.001). NP-POL alone significantly enhanced expression of both Cu-Zn SOD and Mn--SOD in PC12 cells (mean differences within groups: Cu-Zn-SOD, NP-POL vs Control: 1.77 ±0.51, p = 0.002; Mn-SOD, NP-POL vs Control: 1.38 ±0.73, Dunnett's test, p = 0.007, Fig. 5). 6-OHDA considerably enhanced expression of Cu-Zn-SOD, but not Mn-SOD in exposed cells (mean differences within groups: Cu-Zn-SOD, 6-OHDA vs Control: 1.08 ±0.27, p = 0.057; Mn-SOD, 6-OHDA vs Control: 0.37 ±0.07, p = 0.828, Dunnett's test, Fig. 5). The expression of neither Cu-Zn-SOD nor Mn- SOD isoforms changed when NP-POL was added simultaneously with 6-OHDA (Fig. 5).

DISCUSSION

Current work focused on investigating the potential protective effects of NP-POL, the recently characterised component peptide of proline-rich polypeptide complex (PRP), also known as Colostrinin [31]. The activity of NP-POL was studied through in vitro oxidative stress paradigm using rat pheochromocytoma PC12 cells as a model of neuronal cells and 6-hydroxydopamine as essential intracellular pro-oxidant, which is also the common by-product of dopamine metabolism in the brain [26].

Previous results indicated that NP-POL neither displayed cytotoxicity nor showed any adverse effect on PC12 cell viability [22]. Moreover, we revealed no antioxidant capacity of NP-POL itself. However, PC12 cells treated with NP-POL applied alone showed a significant decrease in intracellular level of ROS in a dose-dependent manner [22].

Considering the NP-POL peptide as a modulatory agent of the first line of antioxidant defence, its impact on reduced GSH pool was studied. It was observed that NP-POL is able to up-regulate short-time GSH/GSSG ratio in PC12 cells after 6 hours of incubation; however, after 24 hours it returned to control level. Additionally, the impact of NP--POL on up-regulation of SOD1 and SOD2 mRNA expression was observed. Therefore, the obtained results suggest that NP-POL peptide may enhance the antioxidant potential of neurons, making them less sensitive to free oxygen radicals.

6-hydroxydopamine is an oxidant compound that mimics the oxidative stress and dopaminergic damage underlying PD via ROS, such as H_2O_2, O_2-• and OH• [6]. Amongst a number of studies on 6-OHDA effects on intracellular metabolism, some of them have shown that endogenous reduced glutathione (GSH) protected from 6-OHDA-induced apoptosis in cultured human neuroblastoma cells and astrocytes [39, 51]. Recently, it has been shown that 6-OHDA causes the time-dependent increase of intracellular ROS levels in PC12 cells. We observed that in the cells treated with6-OHDA, H_2O_2 levels increased from 90 minutes of incubation and the GSH/GSSG ratio noticeably decreased after 6 and 24 hours of incubation. This indicates the activation of the small molecular defence against 6-OHDA metabolites. 6-OHDA also triggered Cu-Zn-SOD mRNA expression and increase SOD enzymatic activity, but mainly after 24 hours of incubation, which showed that generation of ROS by 6-OHDA degradation happen gradually and is long lasting.

Testing the hypothesis that NP-POL is able to modify toxic effect of 6-OHDA on PC12 cells, it was co-administered to 6-OHDA-treated cells. Our results revealed that NP-POL down-regulates6-OHDA-dependent H_2O_2 production, which can be associated with its impact

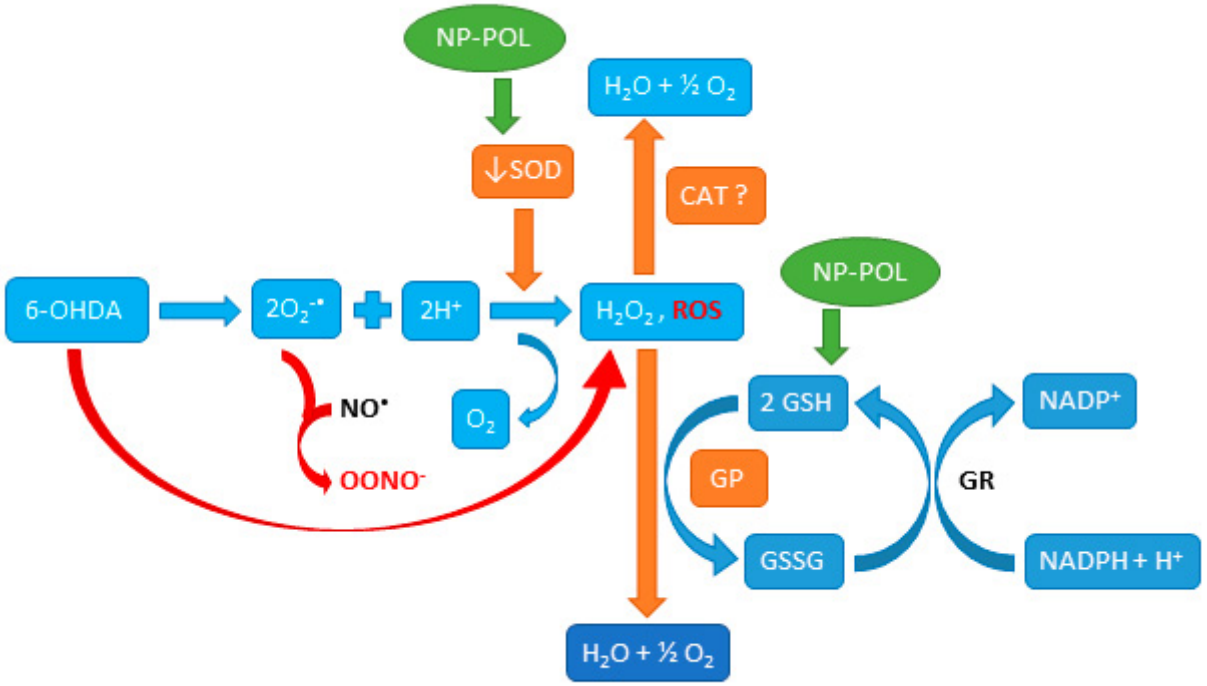

Fig. 6. Possible contribution of NP-POL in molecular and enzymatic antioxidative response mechanisms. SOD – superoxide dismutase, CAT – catalase, GP – glutathione peroxidase, GR – glutathione reductase, GSH – glutathione, GSSG – glutathione disulphide, 6-OHDA – 6-hydroxydopamine

on up-regulation of antioxidant system both non-enzymatic and also enzymatic. Firstly, the significant increase in GSH/GSSG ratio was observed in the presence of NP-POL, which resulted in a significant decrease in the level of hydrogen peroxide released as a product of 6-OHDA autoxidation. Significant recovery of reduced glutathione pool was observed after 6 h of incubation of PC12 cells in the presence of NP-POL. 6-OHDA-treated cells response to increasing ROS levels was based on activity of small molecular antioxidant defence, namely glutathione activity. Enhanced expression of SOD genes and increased activity of antioxidant enzymes appear as the additional defence mechanisms against oxidative stress triggered by high ROS concentration.

Incubation of PC12 cells with of NP-POL and 6-OHDA applied to the cells simultaneously resulted also in up-regulation of SOD mRNA expression compared to the control cells (Fig. 5); however, reduced activity of SOD was observed (Fig. 4). We speculate that NP-POL could down-regulate autoxidation of 6-OHDA and O_2-• formation and subsequently diminish generation of H_2O_2 by superoxide dismutase. Another explanation is that NP-POL peptide could modulate 6-OHDA-dependent over-activation of superoxide dismutase and stabilize the process of O_2-• neutralisation by SOD. Both these suggestions lead to the conclusion that NP-POL may act against oxidative stress either by indirect modification of the enzymatic and non-enzymatic response mechanisms and direct hampering of ROS workflow.

The enzymatic response in the presence of NP-POL and 6-OHDA is possibly linked with the modulation of gene expression mediated by transcription factor NF-κB, which handles SOD enzymes as the crucial antioxidant targets [28]. The study on PRP regarding modulation of inflammatory response revealed its potential role in the regulation of protein transcription. As it was previously shown, PRP/NP complex affected the activity of transcriptional factor NF-κB, and SOD expression and activity suggesting that it can prevent oxidative damage by regulating the initial steps of antioxidant response [49]. NF-κB activation is dependent on ROS and H_2O_2 levels, thus lowering them would most likely suppress the initiation of antioxidative enzymatic defence line. It has been observed that PRP/Colostrinin complex can regulate the activity of antioxidant enzymes, glutathione metabolism and mitochondrial function [9, 47].

Collecting the above evidence together, it appears that NP-POL may have the potential to modulate and support the endogenous mechanism of oxidative stress reduction, mostly by regulating the expression of antioxidant enzymes and maintaining glutathione supply restoration (for possible contributions of NP-POL in those mechanisms see Fig. 6).

Taking into account the economic issues and undesirable side effects of synthetic drugs, natural therapeutics give promising options for the treatment of the neurodegenerative diseases, such as Parkinson's disease, whose

pathomechanism includes free radicals-induced oxidative stress. Regarding the family of colostrum-derived preparations, recent studies generally demonstrate that their antioxidant activity is based on the activation and modulation of the first and second-line mechanisms of intracellular antioxidant defence [9, 45, 50]. This is the hallmark of the majority of natural compounds, such as polyphenols, steroidal lactones, alkaloids, catechins, glycosides, anthocyanins and many others. Specific bioactive molecules, present in a wide range of herbs, fruits, grains, nuts and spices and their relationship to neuropathological features associated with PD, were assessed in a number of studies. They include neuroprotective and anti-apoptotic properties of soy isoflavone, genistein, in the mouse model of cerebral ischemia, and flavonoids such as rutin and isoquercetin, which have been found to activate the endogenous mechanisms of antioxidant defence, upregulating the expression of SOD, GP, CAT and glutathione in 6-OHDA-induced PC12 cells [24, 25, 32].

Based on this evidence, the antioxidant potential of PRP complex, their components and NP-POL, which we have examined here, may have an advantage over common phytochemical or nutraceutical products used in the treatment of PD and other neurodegenerative disorders, also due to the fact that they act as cognitive enhancers [40, 41]. Neuroprotective activity of PRP/Colostrinin, its ability to improve learning, memory, and cognitive function in rats [31] was confirmed in clinical studies on Alzheimer's disease patients [5, 18, 23]. Hence, PRP/Colostrinin was found to have potential use in Alzheimer's disease therapy.

CONCLUSIONS

The role of oxidative stress in the pathogenesis of neurodegenerative diseases, also in Parkinson's disease, has been well demonstrated in many preclinical and clinical studies. Additionally, the benefit of antioxidant therapy has shown promising results. Therefore, looking for the exact therapeutic substances able to minimalize or eliminate adverse effects of oxidative stress is very important. The use of the compounds of natural origin with neuroprotective properties is very promising, especially showing multidirectional action. An example of such a compound may be the newly discovered peptide NP-POL (RPKHPIKHQ), isolated from the colostrum-derived polypeptide complex Colostrinin and subsequently obtained by chemical synthesis. The results of the study so far have shown the potential of its use in the therapy of neurodegenerative diseases.

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