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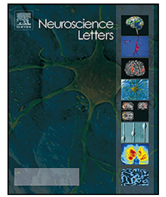

Inhibition of prolyl oligopeptidase increases the survival of alpha-synuclein overexpressing cells after rotenone exposure by reducing alpha-synuclein oligomers

Lana Dokleja[1], Mirva J. Hannula[1], Timo T. Myöhänen*

Division of Pharmacology and Toxicology, Faculty of Pharmacy, , Viikinkaari 5E, PO Box 56, FIN-00014 University of Helsinki, Finland

HIGHLIGHTS

- Rotenone induced α-synuclein (aSyn) aggregation in [A53T]aSyn overexpressing cells.
- Prolyl oligopeptidase (PREP) inhibition reduced the levels of aSyn oligomers.
- PREP inhibition decreased ROS production by reducing aSyn oligomers.
- These effects of PREP inhibition led to increased cell survival.

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ABSTRACT

α-Synuclein (aSyn) aggregation, mitochondrial dysfunction and oxidative damage has been shown to be related to the death of dopaminergic neurons in Parkinson's disease (PD). Prolyl oligopeptidase (PREP) is proposed to increase aSyn aggregation, and PREP inhibition has been shown to inhibit the aggregation process in vitro and in vivo. In this study, we investigated the effects of a specific PREP inhibitor, KYP-2047, on rotenone induced aSyn aggregation and increased the production of reactive oxygen species (ROS) in cells overexpressing A53T mutation of aSyn. Rotenone, a mitochondrial toxin that induces oxidative damage and aSyn aggregation, associated with PD pathology, was selected as a model for this study. The results showed that rotenone induced the formation of high-molecular-weight aSyn oligomers, and this was countered by simultaneous incubation with KYP-2047. Inhibition of PREP also decreased the production of ROS in [A53T]aSyn overexpressing cells, leading to improved cell viability.

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1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder where dopaminergic neurons of the nigrostriatal pathway degenerate for unknown reasons. This causes instability in neuronal circuits leading to clinical symptoms including tremor, rigor and bradykinesia [1]. One of the key players in neuronal death may be the aggregation of α-synuclein (aSyn), a brain protein with functions in neurotransmitter packing and release [2]. In 1998, Spillantini et al. [3] found that Lewy bodies, histopathological hallmarks of PD, consisted mostly of aggregated aSyn. However, recent studies are proposing that fibrillar aggregates in Lewy bodies may be protective for cells, and aSyn oligomers are actually the toxic species [4]. In addition, mutations in the aSyn gene are shown to be risk factors for PD to further support the role of aSyn in this disease [2].

Post mortem studies in patients with PD have shown systemic defects in the mitochondrial respiratory complex I, especially in the substantia nigra (SN) [5]. Mitochondrial damage causes a reduction in ATP production and increases the levels of reactive oxygen species (ROS), and dopaminergic neurons in the SN seem to be particularly sensitive to oxidative damage [5–7]. In addition, the aggregation of aSyn increases in the presence of ROS, leading to further ROS overproduction [8]. aSyn oligomers can interact with the mitochondrial membrane and cause fragmentation of mitochondria, followed by cell death [9]. Rotenone is a toxin used as a

Abbreviations: aSyn, α-synuclein; DMSO, dimethylsulfoxide; DMEM, Dulbecco's modified Eagle medium; HMW, high-molecular-weight; LC3BI-II, microtubule-associated protein light chain 3B I-II; OD, optical density; PD, Parkinson's disease; PBS, phosphate-buffered saline; PREP, prolyl oligopeptidase; ROS, reactive oxygen species; WB, Western blot; WT, wild type.

* Corresponding author. Tel.: +358 2941 59459; fax: +358 02941 59138.
E-mail addresses: lana.dokleja@helsinki.fi (L. Dokleja), mirva.hannula@helsinki.fi (M.J. Hannula), timo.myohanen@helsinki.fi (T.T. Myöhänen).

[1] Equal contribution.

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pesticide, acting as a high-affinity inhibitor of the mitochondrial complex I and causing an increase in intracellular ROS [7]. Therefore, rotenone has been used as a toxin model for PD, and systemic administration of rotenone causes PD-like symptoms with aSyn aggregation and dopaminergic cell death in vivo [7].

Brandt et al. [10] showed in an in vitro turbidity assay that the aggregation rate of aSyn is increased in the presence of prolyl oligopeptidase (PREP), and this action can be blocked by a specific PREP inhibitor. PREP is a serine protease that hydrolyzes proline containing peptides shorter than 30-mer at least in vitro, and several functions in cell proliferation, intracellular signaling and memory functions has been proposed [11]. In the CNS, PREP activity has been reported to increase during aging and neurodegenerative disorders, supporting the possible role in neuropeptide catabolism, and this has led to PREP inhibitor development. However, the results of PREP inhibition on neuropeptide levels in vivo are inconsistent [12]. Moreover, Tenorio-Laranga et al. [13] showed that 4-day PREP inhibition changed the amount of cytosolic peptides but they were not derived from traditional PREP substrates such as substance P etc., and most of them were not cleaved after proline specifically. Recently, studies with knock-out models have shown that lack of PREP leads to decreased neuronal growth and impaired memory but these functions seem to be independent of its hydrolytic activity [14,15], and the true physiological role of PREP remains largely unknown.

In our current studies, we have shown that PREP colocalizes with aSyn in brain of PD patients [16], and that PREP inhibition decreases aSyn aggregation and enhances the clearance of aggregates via autophagy in aSyn overexpressing cells and animal models [17,18]. However, the mechanism has remained unclear. By using a cell line overexpressing A53T mutated aSyn with rotenone-induced mitochondrial damage, we were able to study not only the effect of PREP inhibition on aSyn aggregation but also the effect of PREP inhibition on ROS and autophagy markers. This would further explain the mechanisms how PREP inhibition can reduce aSyn aggregation in cells.

2. Materials and methods

2.1. Chemicals

Chemicals used were purchased form Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified. Ethanol was purchased from Altia (Helsinki, Finland). The PREP inhibitor, KYP-2047 (4-phenylbutanoyl-l-prolyl-2(*S*)-cyanopyrrolidine), was obtained from Dr. Elina Jarho (University of Eastern Finland), and its synthesis was described in [19]. KYP-2047 has been shown to be potent, selective and enter the cells in culture [18,20,21]. KYP-2047 was dissolved in DMSO at a concentration of 100 mM and then further diluted.

2.2. Cell lines

WT SH-SY5Y human neuroblastoma cell line was cultured as described earlier [20]. The stable cell line expressing A53T aSyn ([A53T]aSyn) was obtained from Prof. V. Baekelandt and Dr. M. Gérard and the development of this line was described in [22]. Cells were grown at 37 °C and 5% CO_2 in a humidified atmosphere.

2.3. Induction of α-synuclein aggregation by rotenone and the effect of KYP-2047

Cells were cultured in 12-well plates for immunochemistry (8×10^4 cells/well), 96-well plates for cell viability and ROS experiments (6×10^4 cells/well) and in T25 flasks for Western blot (WB) (10^6 cells/flask). After 24 h, study groups were formed to test the effect of KYP-2047 on rotenone-induced aSyn aggregation during 3 days. Groups were as follows: (1) rotenone 50 nM + 0.01% DMSO (rotenone with vehicle), (2) rotenone 50 nM + KYP-2047 10 μM in 0.01% DMSO (rotenone with KYP), (3) KYP-2047 10 μM in 0.01% DMSO (KYP) and (4) 0.01% DMSO (vehicle). Cells in well plates had an additional group: (5) medium (negative control). WT cells were treated as above. However, no aSyn aggregates were seen, and therefore, this data is not shown.

2.4. aSyn immunocytochemistry

Cells cultured in 12-well plates were immunostained for aSyn after the 3-day treatments, using a protocol described in [18]. Briefly, after fixing and blocking, the cells were incubated overnight at +4 °C with a primary antibody against aSyn (rabbit anti-aSyn, #ab52168, AbCam, Cambridge, UK; 1:500 in 1% normal goat serum). On the next day, the cells were incubated for 2 h with a secondary antibody (anti-rabbit fluorescein, #31635, Thermo Fisher Scientific, Waltham, MA, USA; 1:500 in 1% normal goat serum).

Cells were photographed using Nikon Eclipse TE300 microscope with Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA). The cell calculations were done as previously reported [18].

2.5. aSyn fractionation and WB

For WB experiments, cells grown in T25 flasks were lysed and homogenized according to the protocol described earlier [18]. The procedure resulted in fractions containing soluble aSyn, its SDS-soluble monomers, and SDS-insoluble oligomers.

To detect the levels of different aSyn forms, and autophagy markers p62/SQSTM1 (p62; accumulation marker) and microtubule-associated protein 1 light chain 3 beta (LC3B; autophagosome marker), WB was used [23]. Protein levels were measured using the BCA Protein Assay Kit (#23227, Thermo Fisher Scientific) and the lysates were loaded onto a 12% SDS gel (20 μg of protein/well). Standard transfer and blocking techniques were used and the loading control was mouse anti-beta-actin (#ab8226, AbCam; 1:2500). Following primary antibodies were used: mouse anti-aSyn antibody (#ab1903, AbCam; 1:1000), anti-mouse p62 (#ab56416, AbCam; 1:5000) and anti-rabbit LC3B (#L7543; 1:1000). Anti-mouse HRP (#31430, Thermo Fisher Scientific; 1:2000) and anti-rabbit HRP (#31460, Thermo Fisher Scientific; 1:2000) were used as secondary antibodies. All antibodies were diluted in 5% skim milk in 0.05% Tween20 in Tris-buffered saline.

The images were captured using the C-Digit imaging system (Licor, Lincoln, NE, USA). Three independent WB experiments were performed. ImageJ (NIH, Bethesda, MD, USA) was used for analyzing bands, and the optical density (OD) value was calculated by comparing the OD value to the corresponding beta-actin OD value.

2.6. Detection of ROS

To detect the effects of rotenone and KYP-2047 on ROS in [A53T]aSyn and WT cells, DCFDA Cellular ROS Detection Assay Kit (#ab113851, AbCam) was used. The formation of 2,7-dichlorofluorescein (excitation and emission spectra of 495 nm and 529 nm, respectively) was measured using Wallac 1420 Victor2 fluorescence plate reader (Perkin Elmer, Waltham, MA, USA).

2.7. Cell viability assay

Cells were plated and treated with 50 nM and 100 nM rotenone in the presence of KYP-2047/vehicle (150 μL/well). To assess cell viability a standard LDH release assay was performed as previously described [10]. Absorbance was measured at 490 nm using Wallac 1420 Victor2 fluorescence plate reader (Perkin Elmer).

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