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Extracellular alpha-synuclein induces calpain-dependent overactivation of cyclin-dependent kinase 5 in vitro

Grzegorz A. Czapski *, Magdalena Gąssowska, Anna Wilkaniec, Magdalena Cieślik, Agata Adamczyk

Department of Cellular Signalling, Mossakowski Medical Research Centre, Polish Academy of Sciences, Pawinskiego 5, 02-106 Warsaw, Poland

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ABSTRACT

Extracellular alpha-synuclein (ASN) could be involved in the pathomechanism of Parkinson's disease (PD) via disturbances of calcium homeostasis, activation of nitric oxide synthase and oxidative/nitrosative stress. In this study we analyzed the role of cyclin-dependent kinase 5 (Cdk5) in the molecular mechanism(s) of ASN toxicity.

We found that exposure of PC12 cells to ASN increases Cdk5 activity via calpain-dependent p25 formation and by enhancement of Cdk5 phosphorylation at Tyr15. Cdk5 and calpain inhibitors prevented ASN-evoked cell death. Our findings, indicating the participation of Cdk5 in ASN toxicity, provide new insight into how extracellular ASN may trigger dopaminergic cell dysfunction in PD.

1. Introduction

α-Synuclein (ASN) is a small 140-amino acid, intrinsically disordered protein that is highly abundant in the brain. Although the physiological role of ASN is not well understood, there is evidence suggesting that this protein is involved in synaptic vesicle recycling, neurotransmitter synthesis and release, and synaptic plasticity [9,24]. However, oligomerization/aggregation, deposition, and dysfunction of ASN are common events in neurodegenerative disorders known as synucleinopathies. ASN was shown to be the main component of characteristic intraneuronal protein aggregates called Lewy bodies (LB) and Lewy neurites (LN) in Parkinson's disease (PD), dementia with LBs (DLB) and in the LB variant of Alzheimer's disease (AD). Recent studies demonstrated that ASN aggregation may also occur in the sporadic form of AD with a frequency of ca. 60% [19,26]. A growing body of evidence has emphasized the importance of extracellular ASN in neurodegenerative disorders [13,37]. It was suggested that the spread of ASN pathology could contribute to the progression of neurodegeneration and clinical symptoms in PD [23,17]. Nevertheless, the precise mechanism(s) of secretion as well as the consequences of extracellular ASN action are still unclear. Our previous data showed that extracellular ASN evokes disturbances of calcium homeostasis, thus leading to activation of cytosolic nitric oxide synthase and to activation of NO-triggered caspase-dependent apoptosis [1,3,5]. An increase in the cytosolic calcium level activates many detrimental processes in the cell, including overactivation of cyclin-dependent kinase 5 (Cdk5). Unlike other cyclin-dependen kinases (CDKs), Cdk5 is not activated by cyclins and it is not directly involved in regulation of the cell cycle. Instead, Cdk5 has many other important functions, including regulation of the central nervous system's development, oxidative stress and apoptosis. The activity of Cdk5 is triggered by binding with the regulatory proteins p35 and p39. Additionally, the activity of Cdk5 might be regulated by phosphorylation at Tyrosine 15 and Serine 159. In conditions related to an increased calcium level, overactivated calpains catalyze the proteolytic cleavage of p35 and p39, thus leading to the formation of p25 and p29. Due to higher stability, these truncated forms are more potent activators of Cdk5 and may evoke prolonged activation and mislocalization of Cdk5. The consequence of Cdk5 overactivation is hyperphosphorylation of its targets, which may have detrimental consequences. This calcium-related, calpain-dependent mechanism of Cdk5 activation was demonstrated in several neurodegenerative disorders, including brain ischemia, AD and PD. Moreover, it was shown that inhibition of overactivated Cdk5 may be an effective method for neuroprotection in the experimental models of neurodegenerative diseases [8,25,33]. Previous studies demonstrated that overactivation of Cdk5 occurs in the brains of PD patients [6] and in the animal MPTP toxicity model [34,30].

* Corresponding author. Fax: +48 226086613.
E-mail address: gczapski@imdik.pan.pl (G.A. Czapski).

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Takahashi et al. [36] showed that ASN, ubiquitin and Cdk5 have a similar distribution pattern in LBs, and suggested that Cdk5 may participate in LB formation. It was also suggested that Cdk5 is a regulator of Parkin ubiquitin-ligase activity [7]. Our previous studies demonstrated that the cascade evoked by the non-Aβ component of AD amyloid (NAC) involves activation of Cdk5, however, the link between ASN toxicity and Cdk5 has never been confirmed [20]. The aim of the present study was to analyze the role of Cdk5 in an in vitro cellular model of ASN toxicity. Our results demonstrated, for the first time, that treatment with ASN evokes activation of calpains leading to p35 cleavage and overactivation of Cdk5. The protective effect of Cdk5 inhibition indicated the importance of Cdk5 in ASN toxicity.

2. Materials and methods

2.1. Preparation of soluble alpha-synuclein

Alpha-synuclein (ASN, rPeptide, Bogart, GA, USA) or beta-synuclein (BSN, rPeptide, Bogart, GA, USA) was dissolved in phosphate-buffered saline pH 7.4 at a concentration of 100 μM and immediately used for experiments [2,5]. Aliquots containing 2 μg of ASN or BSN protein were analyzed by SDS–PAGE, which was followed by silver staining.

2.2. Cell culture

Rat pheochromocytoma PC12 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5% heat-inactivated horse serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin in a 5% CO_2 atmosphere at 37 °C. Cell treatment was performed in low-serum (2% FBS) DMEM to stop proliferation. Calpeptin, an inhibitor of calpains, Roscovitine and BML-259, inhibitors of Cdk5, were dissolved in DMSO. The final concentration of DMSO in the cell culture medium did not exceed 0.05%.

2.3. Cytotoxicity assays

For an analysis of the effect of ASN and BSN on cell survival and death, assays discriminating live and dead cells were performed. Equal numbers of cells were cultured in a 96-well PEI-coated plate, and after 24 h the medium was changed to (2% FBS) DMEM and the tested compounds were added. After 48 h incubation in the presence of the tested compounds, MTT assay and trypan blue staining were performed.

MTT (0.25 mg/ml) was added to the culture medium and the cells were incubated for 2 h. The medium was removed, the cells were dissolved in DMSO and absorbance at 595 nm was measured.

Trypan blue solution (0.4% in 0.81% sodium chloride and 0.06% potassium phosphate dibasic) was added to the culture medium. The cells were examined immediately under an optical microscope. The number of blue stained cells and the total number of cells were counted. If cells took up trypan blue, they were considered non-viable.

2.4. Cytosolic Ca^{2+} measurements

Measurement of the intracellular Ca^{2+} concentration was carried out using fluorescent indicator Fluo-4-acetoxymethyl (AM)

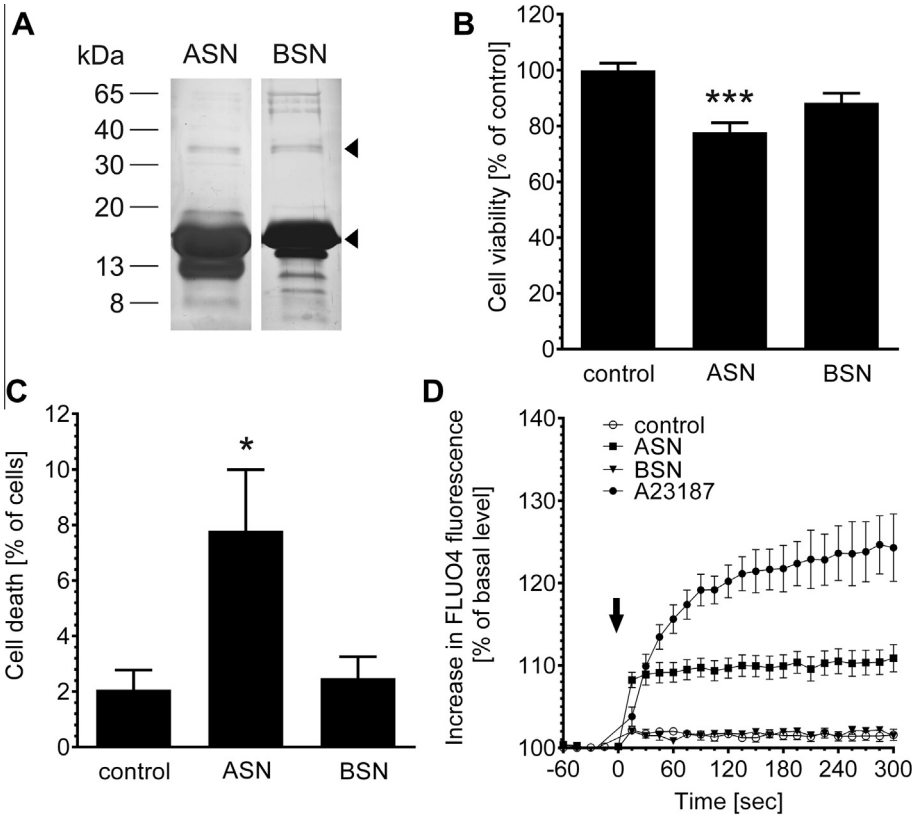

Fig. 1. The effect of ASN and BSN on PC12 cells. PC12 cells were incubated with 10 μM ASN or BSN for 48 h. (A) Electrophoretic analysis of the ASN and BSN aggregation form. Proteins were subjected to denaturing SDS–PAGE followed by silver staining. Note the presence of monomers and dimers (indicated by the arrows). (B) The effect of ASN and BSN on cell viability determined by MTT assay, as described in the Methods section. (C) The effect of ASN and BSN on cell death determined by trypan blue staining, as described in the Methods section. (D) The effect of ASN and BSN on the cytoplasmic calcium level determined by the Fluo-4 probe, as described in the Methods section. A23187 used as a positive control. Data represent the mean value ± S.E.M. for 3–14 independent experiments. *, *** $P < 0.05$ and 0.001, respectively, compared to the control cells using a one-way ANOVA followed by the Newman–Keuls test.