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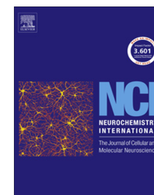

Alpha-synuclein overexpression increases phospho-protein phosphatase 2A levels via formation of calmodulin/Src complex

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ABSTRACT

Alpha-synuclein (α-Syn) is the principal protein component of Lewy bodies, a pathological hallmark of Parkinson's disease (PD). This protein may regulate protein phosphatase 2A (PP2A) activity, although the molecular mechanisms for α-Syn-mediated regulation of PP2A and the potential neuroprotective actions of PP2A against PD-associated pathology remain largely unexplored. We found that α-Syn gene overexpression in SK-N-SH cells and primary neurons led to PP2A/C phosphorylation at Y307, a known target of Src kinase, and consequent phosphatase inhibition. In addition, phospho-activated Src (p-Y416 Src, pSrc) was higher in SK-N-SH cells and primary neurons overexpressing α-Syn. Thus, α-Syn may promote Src activation and PP2A inactivation, leading to hyperphosphorylation of proteins. Immunoprecipitation revealed higher calmodulin/Src complex formation in α-Syn-overexpressing cells and α-Syn transgenic mice. A TUNEL apoptosis assay and an MTT cell viability assay demonstrated that the PP2A activator C_2-ceramide protected neurons against α-Syn-induced cell injury. Buffering the Ca^{2+} elevations induced by α-Syn overexpression ameliorated the cytotoxicity of α-Syn. Our findings define a potential molecular mechanism for α-Syn-mediated regulation of PP2A through formation of the calmodulin/Src complex, activation of Src, and Src-mediated phospho-inhibition of PP2A. Overexpression of α-Syn may lead to neurodegeneration in PD in part by suppressing the endogenous neuroprotective activity of PP2A.

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1. Introduction

Parkinson's disease (PD) is a movement disorder that results from selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). Many surviving SNc neurons contain cytoplasmic inclusions known as Lewy bodies. Parkinson's disease may progress into a wide-spectrum neurological disorder involving neurodegeneration in the olfactory, autonomic, limbic, and somatomotor systems (Braak et al., 2006). Many or all of these regions may also exhibit Lewy bodies, implicating formation of these protein inclusions in the pathogenesis of PD. Furthermore, Lewy bodies are found in several other neurodegenerative diseases, such as Lewy body dementia.

The primary component of Lewy bodies is α-synuclein (α-Syn), a highly conserved presynaptic protein of about 14 kDa. While α-Syn inclusions are ubiquitous in the PD brain, very little is known of the pathophysiological mechanisms leading from formation of Lewy bodies to neurodegeneration of dopaminergic and other neuronal types. Several lines of evidence suggest that the functional effects of α-Syn are closely related to protein phosphatase 2A (PP2A) expression and activity. Overexpression of wild type α-Syn led to increased PP2A activity in MN9D and PC12 cells (Peng et al., 2005), while α-Syn knockout mice exhibited decreased PP2A activity (Lou et al., 2010). Conversely, accumulation of α-Syn reduced PP2A activity in vitro and in vivo (Wu et al., 2012). Overexpression of α-Syn is toxic to neurons, at least in part by promoting oxidative stress (Chan et al., 2012; Siddiqui et al., 2012), protein aggregation (Lastres-Becker et al., 2012; Wan and Chung, 2012), and mitochondrial dysfunction (Devi and Anandatheerthavarada, 2010; Wu et al., 2009). Transgenic mice overexpressing α-Syn and reared on a diet supplemented with eicosanoyl-5-hydroxytryptamide, an agent that enhances PP2A activity, exhibited dramatically reduced α-Syn aggregation in the brain, as well as reduced neurodegeneration, larger dendritic arbors, reduced astroglial and microglial activation, and improved motor performance compared to α-Syn-overexpressing mice fed a normal diet (Lee et al., 2011).

Protein phosphatase 2A exists as a heterotrimer consisting of a 36-kDa catalytic subunit (PP2A/C), a 65-kDa scaffolding subunit (PP2A/A), and a variable regulatory subunit (PP2A/B). Post-translational modification of PP2A/C is the central mechanism for regulating phosphatase activity. For example, phosphorylation of PP2A/C at Y307 resulted in 90% inactivation of PP2A in vitro (Chen et al., 1992). As a major determinant of the phosphorylation status of many

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target proteins, including α-Syn (Lee et al., 2011), PP2A is an important regulator of numerous cellular processes, including growth and proliferation (Pitre et al., 2012), apoptosis (Kar et al., 2012), and transcription and translation (Mitra et al., 2012). Regulation of α-Syn phosphorylation state by PP2A is also a critical determinant of α-Syn cytotoxicity in neurodegenerative diseases like PD (Lee et al., 2011; Wu et al., 2012). Indeed, PP2A activity is regulated by α-Syn (Lou et al., 2010; Peng et al., 2005; Wu et al., 2012) but the signaling pathways remain poorly understood. Several lines of evidence indicate that inhibition of PP2A/C by phosphorylation at Y307 may be mediated by the Src tyrosine kinase (Chen et al., 1992, 1994) and that upstream Src activation depends on formation of a calmodulin–Src complex (Fedida-Metula et al., 2012).

In summary, the PD-associated protein α-Syn appears to regulate the functional activity of PP2A but it is not known if this suppression contributes to the pathogenic potential of α-Syn or if PP2A protects against α-Syn-induced neurotoxicity. The principal findings of the current study are that α-Syn overexpression can indeed enhanced Y307 PP2A/C phosphorylation and consequent inhibition as well as promote the formation of calmodulin–Src complexes. Furthermore, the PP2A activator C_2-ceramide alleviated α-Syn-induced cell injury. Our findings strongly suggest that PP2A inhibition may contribute to α-Syn-mediated neurodegeneration, thus defining an potential pathogenic mechanism for PD and other α-synucleinopathies.

2. Materials and methods

2.1. Plasmid constructs and lentivirus

The human WT-α-Syn (hα-Syn) cDNA was obtained by RT-PCR from human brain RNA. Primers 5′-CTGGAAGATATGCCTGTGGATC-3′ and 5′-AGCACTTGTACAGGATGGAAC-3′ were designed according to the α-Syn sequence in GenBank (NM_000345.3). The cDNA was directionally cloned into the pCMV-myc plasmid (which were kindly provided by Dr. Zhiqin Xu, Capital Medical University, China) and the orientation verified by DNA sequencing. The human PP2A/B′γ cDNA was obtained by RT-PCR from human RNA. Primers 5′-ATCTAGCCAAAGCCAATCCC-3′ and 5′-CTCGTCCTTCACTGTC TTTCTC-3′ were designed according to the PP2A B′γ sequence in GenBank (NM_001161725.1). The cDNA was directionally cloned into the PLNCX2 plasmid (which were also kindly provided by Dr. Zhiqin Xu, Capital Medical University, China) and the orientation verified by DNA sequencing. The human PP2A/Bα cDNA was obtained by RT-PCR from human RNA. Primers 5′-TAGCAACAGG AGATAAAGGTGG-3′ and 5′-AACTCTGGTTCATGGCTCTG-3′ were designed according to the PP2A Bα sequence in GenBank (NM_002717.3). The cDNA was directionally cloned into the PLNCX2 plasmid and the orientation verified by DNA sequencing.

Lentivirus (LV) gene transfer vectors encoding GFP-shRNAs (LV-GFP-sh-1#, 5′-GCA CAC TGT TCC TCG TTA TGA-3′; LV-GFP-sh-2#, 5′-GGA GCA GTT ACT TAC AGA AGA-3′) targeting specific regions of rat α-Syn mRNA (NM_019169.2), a scrambled negative control (LV-GFP-sh-con, 5′-GGA TTG ATT CAA CAC GGA AGA-3′), or a GFP-hα-Syn fusion protein (LV-GFP-α-Syn) were constructed by Genechem (Shanghai, China).

2.2. Cell cultures

2.2.1. Human SK-N-SH cells

Human SK-N-SH cells obtained from the American Type Culture Collection (ATCC) were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, cells were plated at 1×10^7 cells per culture flask one day before transfection. Samples (9 μg) of pcDNA3.1-myc (myc plasmid) or pcDNA3.1-myc-α-Syn (α-Syn/myc plasmid) DNA and 20 μl of Lipofectamine 2000 reagent were gently mixed in Opti-MEM (Invitrogen). Cells were incubated at 37 °C in a CO_2 incubator for the indicated times. Protein expression was verified by Western blotting. For another experiment, SK-N-SH cells were infected with lentivirus gene transfer vectors encoding GFP-α-Syn. After 3 days of infection, these cells were transfected with PLNCX2 plasmid, PLNCX2-PP2A/Bα, or PLNCX2-PP2A/B′γ for 24 h for Western blot and PP2A activity assay.

2.2.2. Primary rat cortical neurons

All experiments were approved by the Institutional Animal Care and Use Committee of Capital Medical University of Science and Technology (approval No. SCXK-2011-004)) and were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Primary cortical neurons were prepared from Sprague Dawley rat E14.5–E15.5 embryos. Briefly, cortical neurons were cultured in 3.5-cm dishes (1.4×10^6 cells/dish) on cover slips coated with 100 μg/ml poly-L-lysine. The culture medium was Neurobasal medium (Invitrogen) supplemented with basic fibroblast growth factor (10 ng/ml), nerve growth factor (10 ng/ml), L-glutamine (0.5 mM), and B27 supplement minus AO 50× (1×). After 7 days, primary neurons were infected with different Lentivirus (LV) gene transfer vectors.

2.3. Transgenic mice

The animal protocol was approved by the Animal Care and Use Committee of Capital Medical University and was consistent with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23). Male transgenic mice (18–22 g) over-expressing hα-Syn (Tg-α-Syn) under the control of the PDGF promoter were kindly provided by Dr. Changan Jiang (Sichuan University, China) and maintained on a C57BL/C3H background. Transgenic mice and wild type littermates (WT) were housed under a 12-h light/12-h dark cycle at 20–23 °C with free access to food and water.

2.4. Measurement of cell viability

To evaluate the effects of the various transfection protocols on cell viability, 1×10^4 SK-N-SH cells were transfected with myc or α-Syn/myc plasmid for 0, 6, 12, 24, 48, or 72 h and then plated in 96-well plates. Some cultures were then treated with the PP2A activator C_2-ceramide (5 μM) or the membrane permeant calcium chelator BAPTA/AM (50 μM). Following treatment, cell viability was estimated by the MTT assay, which determines relative live cell count by the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to colored formazan crystals (Williams et al., 2002). All assays were performed according to the manufacturer's instructions.

2.5. Sample preparation and Western blot analyses

Primary cortical neurons from Sprague Dawley rats were infected with LV-GFP, LV-GFP-α-Syn, LV-GFP-sh-1#, LV-GFP-sh-2#, or LV-GFP-sh-con for 3 days, harvested, and lysed in TNE buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA; all from Sigma–Aldrich) containing 1% Nonidet P-40 (Calbiochem) and both protease and phosphatase inhibitor cocktails (Roche). Alternatively, cultured SK-N-SH cells were transfected with myc or α-Syn/myc plasmid for 24 h. Total cell extract was centrifuged at 12,000×g for 30 min, and the supernatant protein concentration

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