Phosphorylation of α-synuclein upregulates tyrosine hydroxylase activity in MN9D cells

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Introduction

α-Synuclein is the major protein component of Lewy Bodies and dystrophic neurites, the hallmark lesions of Parkinson’s disease (PD). It is widely expressed in the brain but selectively neurotoxic to dopaminergic neurons (Rochet et al., 2004; Yasuda et al., 2007). Accumulating evidence has demonstrated that α-synuclein is involved in dopamine (DA) homeostasis (for example, in DA synthesis). The regulatory effect of α-synuclein likely contributes to the decrease in cytoplasmic DA. It was reported that overexpression of α-synuclein in MN9D cells significantly reduced the activity of tyrosine hydroxylase (TH), a rate-limiting enzyme in DA biosynthesis (Perez et al., 2002). We also observed that silenced α-synuclein enhanced the activity of TH and the biosynthesis of DA significantly (Liu et al., 2008). In PD, the main modification of α-synuclein appears to be a single phosphorylation at Ser129 (Anderson et al., 2006). Normally, only 4% of Ser129 is phosphorylated, while approximately 90% is phosphorylated in synucleinopathic lesions (Fujikawa et al., 2002). Thus α-synuclein phosphorylation plays an important role in the pathogenesis of PD, but its function remains elusive. In the present study the effect of α-synuclein phosphorylation on TH expression and activity was examined.

Materials and methods

Construction of mutant α-synuclein cDNA, retroviral packaging

The overlapping extension PCR (OE-PCR) method (An et al., 2005) was used to produce one site-directed mutagenesis of α-synuclein S129D. pLNCX2 wild-type α-synuclein was used as the template. Two pairs of primers were used, including outer primers pLNCX Seq/PCR Primers #K1060-F and mutagenic primers (sense 5’-CTTATGAAATGCCTGATGAGGAAGGGTATC-3’, antisense 5’-GATACCCCTCCTCATCAGGCAATTCTCATAAAG-3’). The resulting construct with the S129 site alteration was confirmed by sequencing (Shanghai Invitrogen Biotechnology Co., China.). Retrovirus vectors pLNCX carrying WT/S129D α-synuclein were transfected into PT67 cell to package using Lipofectamine 2000 (Gibco BRL, Carlsbad, CA, USA) transfection according to the manufacturer’s instruction. Following transfection, the retrovirial supernatant was harvested.

Cell culture and infection

The dopaminergic cell line (Choi et al., 1991) (MN9D, gift from Dr. Bastian Hengerer, Novartis AG) was cultured in DMEM/F12 media supplemented with 10% fetal bovine serum (Gibco BRL, Carlsbad, CA; pH 7.2) and incubated at 37 °C in an atmosphere of 5% CO2. The cells were infected with retrovirus to express...
wild-type or S129D α-synuclein. The expression of α-synuclein in infected cells was determined by Western blot 72 h after infection.

**Western blot**

Western blots were performed as described previously (Liu et al., 2008). Briefly, MN9D cells (10⁷) were lysed in the presence of a protease-inhibitor cocktail. Whole cell protein extracts (50 μg) were resolved by 10% SDS-PAGE and processed by Western blot analysis using the following specified antibodies: α-Synuclein monoclonal antibody (1:1000, gift from Dr. Shun Yu (Yu et al., 2007)), TH monoclonal antibody (1:5000), pser40TH monoclonal antibody (1:1000), β-actin polyclonal antibody (1:1000), PKA and pPKA monoclonal antibody (1:1000) (all the above antibodies were from Sigma-Aldrich, St. Louis, MO, USA), pERK and total ERK polyclonal antibody (1:1000 Cell Signaling Technology, Beverly, MA, USA).

**HPLC**

The contents of DA were measured using reverse-phase high-performance liquid chromatography (HPLC) as described previously (Liu et al., 2008). MN9D cells (10⁶) were collected and lysed in HPLC buffer. Then 50 μl supernatant samples were injected onto a Phase II Column. Compounds were detected and quantified with an LC-4C detector (Bioanalytical Systems, Inc., West Lafayette, IN, USA). Levels of DA were measured and peaks were identified by retention times set to known standards.

**Measurement of cytotoxicity**

Cytotoxicity was assessed using the Cell Counting Kit-8 (CCK8, Dojindo Laboratories, Tokyo, Japan) to count living cells by combining WST-8 and 1-Methoxy PMS (Morita et al., 2000; Wang et al., 2005). Briefly, MN9D cells (1.0 × 10⁴ cells/well) were seeded into 96-well plates and transiently infected with retroviral supernatant. After 72 h, 10 μl of CCK-8 solution was added to each well of the plates and then incubated for 2 h in the incubator (37 °C and 5% CO₂). The absorbance was measured with a microplate reader at 450 nm.

**Immunofluorescence staining and confocal fluorescence microscopy**

Cells were fixed with 4% formaldehyde diluted in phosphate-buffered saline containing calcium and magnesium. Fixed cells were incubated overnight with the Alexa Fluor 594/488 conjugated secondary antibody (1:400, Invitrogen, Carlsbad, CA) and a He/Ne laser at 561 nm was used for Alexa 488 and a He/Ne laser at 546 nm was used for Alexa 594. Photography of cells was obtained using a 40 × oil immersion objective. Images were collected in the 512 × 512 pixel format, stored on a magnetic mass memory and processed by Leica LAS AF software.

**Statistical analysis**

Experiments were repeated independently, three to five times; the mean value was used for the statistical analysis. ANOVA followed by Bonferroni post hoc multiple comparisons were used to examine the significance between differences among the experimental groups. A value of p < 0.05 was considered to be statistically significant. All data are presented as means ± SE.

**Results**

**Overexpression of α-synuclein in MN9D cells**

The protein expression of α-synuclein was determined in uninfected (MN9D), vector (VEC), wild-type α-synuclein (MN9D/WT) and S129D α-synuclein (MN9D/S129D) infected MN9D cells with Western blot. There was a significant increase in α-synuclein protein levels in MN9D/WT and MN9D/S129D cells compared with that in uninfected and vector-alone cells (Fig. 1A). Moreover, α-synuclein aggregation in cytoplasm and nuclei appeared only in MN9D/S129D cells but not in MN9D/WT cells (Fig. 1B).

![Image](image_url)

**Fig. 1. Expression of α-synuclein in infected MN9D cells.** (A) Western blot analysis was performed for detection of protein levels of α-synuclein in uninfected (MN9D), vector (VEC), wild-type (WT) and S129D α-synuclein (S129D) infected MN9D cells. There was a significant increase of α-synuclein expression levels in the WT and S129D groups (**p < 0.01, *p < 0.05 compared with vector control cells**). (B) Distribution of TH and α-synuclein in MN9D cells. TH (red) and α-synuclein (green) were labeled with monoclonal anti-TH and anti-α-synuclein antibody, and visualized with goat anti-mouse secondary antibodies conjugated to Alexa Fluor 594 and Alexa Fluor 488, respectively. Nuclei were stained with DAPI (blue). Arrows showed the aggregation of α-synuclein in cytoplasm and nuclei of MN9D/S129D cells. Scale bar, 10 μm. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.
Increase in TH activity and DA content by phosphorylation of \( \alpha \)-synuclein at Ser129

There was no significant difference in the TH protein levels among the various groups (Fig. 2A). However, the level of TH phosphorylation at Ser40 was significantly decreased in MN9D/WT cells but significantly increased in MN9D/S129D cells (Fig. 2B). Cytosolic DA was measured with HPLC. DA level was significantly lower in MN9D/WT but significantly higher in MN9D/S129D cells, compared with uninfected and vector-alone MN9D cells (Fig. 3A). These results suggest that wild-type \( \alpha \)-synuclein plays a role in controlling DA balance by inhibiting TH activity in MN9D cells while phosphorylation of \( \alpha \)-synuclein at Ser129 may relieve this inhibition and result in an increase of DA content in cells.

Involvement of ERK, but not PKA, in regulation of TH activity by \( \alpha \)-synuclein in MN9D cells

It has been shown that TH Ser-40 is mainly phosphorylated by cAMP-dependent protein kinase (Fujisawa and Okuno, 2005), but there was no significant difference in PKA and PKA phosphorylation levels among the various groups (Fig. 4A, B). ERK1/2, which is also regulated by \( \alpha \)-synuclein (Iwata et al., 2001), has been reported to be involved in the regulation of TH phosphorylation at Ser40 (Sutherland et al., 1993; Lindgren et al., 2002). Western blot results showed that wild-type \( \alpha \)-synuclein overexpression suppressed phosphorylation of ERK1, but not ERK2, in MN9D cells (Fig. 4D). In contrast, the phospho-ERK1/2 level in MN9D/S129D cells was increased compared to that in control cells (Fig. 4D). No significant difference was found in total ERK1/2 expression among the cells (Fig. 4C).

Prevention of phosphomimic mutant on cytotoxicity of wild-type \( \alpha \)-synuclein in MN9D cells

As it has been suggested that \( \alpha \)-synuclein is involved in cell death, we studied the effect of wild-type (WT) and phosphomimic mutant \( \alpha \)-synuclein on the viability of MN9D cells. Thus there is a significant reduction of cell count in MN9D/WT cells but not in MN9D/S129D cells (Fig. 3B). These data indicated that overexpression of wild-type \( \alpha \)-synuclein was toxic, while the S129D mutation could prevent \( \alpha \)-synuclein toxicity.

Discussion

In the present study, Ser129 at the C terminus of \( \alpha \)-synuclein was altered to an Asp residue to mimic constitutive phosphorylation modification. It has been shown that substitution of serine residues with negatively charged amino acid mimics structural and functional consequences of phosphorylation at these sites (Leger et al., 1997). In fact, many earlier studies have reported that Ser129 phosphomimetic mutation of \( \alpha \)-synuclein plays the same role as ser129 phosphorylation in enhancing eosinophilic inclusion formation (Smith et al., 2005), disrupting axonal transport (Saha et al., 2004), etc.
We demonstrated that wild-type α-synuclein inhibited TH activity, which was consistent with the earlier studies (Perez et al., 2002). Moreover, we found that phosphorymic S129D α-synuclein significantly enhanced TH activity. So these findings suggest that α-synuclein plays a role in the regulation of TH activity and that phosphorylation at Ser129 enhances TH activity. No differences in TH protein levels were seen among the various groups, consistent with earlier studies that neither knock down with RNAi (Liu et al., 2008) nor overexpression (Perez et al., 2002) of α-synuclein had any effect on TH expression in MN9D cells. However, in MES23.5 dopaminergic cells, TH mRNA and protein levels are significantly reduced when α-synuclein was over-expressed (Yu et al., 2004). A similar effect has also been reported in M17 dopaminergic cells transfected with wild-type α-synuclein (Baptista et al., 2003). We propose that the differences in TH expression were mainly due to the different cell types.

The possible mechanisms involved in regulation of TH activity by phosphorylation of α-synuclein were examined. TH is known to be phosphorylated at Ser40, not only by PKA but also by other protein kinases such as ERK. The present results indicated that ERK, but not PKA, is involved in α-synuclein-overexpressing-induced TH activity in MN9D cells. It has been shown that overexpression of wild-type α-synuclein induces apoptosis (Saha et al., 2000) and phosphorylation level of ERK1/2 is closely related to cell apoptosis (Franklin and McCubrey, 2000). In the present study, phosphorylated α-synuclein prevented cell death, possibly by increasing the amount of available active ERK1/2. In fact, we also found that apoptosis-inhibiting factor was enhanced in MN9D/WT but not in MN9D/S129D cells (unpublished data).

It has been shown that phosphorylated α-synuclein often forms aggregations (Smith et al., 2005). In our study, α-synuclein aggregation was found in the cytoplasm and nuclei of MN9D/S129D cells. The inclusions may play a protective role in cell death α-synuclein as inclusions are mostly present in surviving cells and less so in apoptotic cells (Tanaka et al., 2004). Thus, phosphorylation of Ser129 might relieve neurotoxicity of wild-type α-synuclein. Such toxicity relief also appears in rat PD models that expressed α-synuclein as inclusions are mostly present in surviving cells and less so in apoptotic cells (Tanaka et al., 2004). Thus, phosphorylation of Ser129 might relieve neurotoxicity of wild-type α-synuclein. Such toxicity relief also appears in rat PD models that expressed α-synuclein as inclusions are mostly present in surviving cells and less so in apoptotic cells (Tanaka et al., 2004). Thus, phosphorylation of Ser129 might relieve neurotoxicity of wild-type α-synuclein. Thus, phosphorylation of α-synuclein is involved in the upregulation of TH activity and inhibits cytotoxicity of WT α-synuclein.

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**References**


