Research Report

α-Synuclein knockdown attenuates MPP⁺ induced mitochondrial dysfunction of SH-SY5Y cells

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ABSTRACT

α-Synuclein is one of the main constituents of Lewy bodies and plays an important role in the pathology of Parkinson’s disease. Mutation or overexpression of α-synuclein causes Parkinson’s disease, and downregulation of α-synuclein resists MPP⁺-induced cell death, but the mechanism remains elusive. In this study, we attempted to explore the effect of α-synuclein knockdown on mitochondrial function in MPP⁺-treated SH-SY5Y cells. We reconstructed the short hairpin RNA expression vector, pGenesil-2, specially targeting α-synuclein mRNA, and it was stably transfected into SH-SY5Y cells. Cell viability, nuclear morphology, and mitochondrial membrane potential were then detected, and the expression of α-synuclein, cytochrome c, Bcl-2 and Bax were analyzed by Western blotting. The results showed that after exposure to 500 μM MPP⁺ for 24 h, about 41.0±1.5% control cells showed low mitochondrial membrane potential. However, the percentage was 13.6±1.2% in MPP⁺ treated α-synuclein knockdown cells. MPP⁺ induced cytochrome c release significantly, which was about 3.1-fold compared with that of control. However, in α-synuclein knockdown cells, the release of cytochrome c was blocked, which was about 1.4-fold compared with that of control. The Bcl-2/Bax ratio of SH-SY5Y cells reduced to 35.5±3.8% after MPP⁺ treatment, and this ratio was 85.2±3.0% in MPP⁺ treated α-synuclein knockdown cells. These data suggest that knockdown of α-synuclein might be an effective means in rescuing MPP⁺-induced mitochondrial dysfunction of SH-SY5Y cells.

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1. Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder. It is characterized pathologically by the selective loss of dopaminergic neurons in substantia nigra and the presence of intracytoplasmic protein inclusions called Lewy bodies (LB). Histological research showed that α-synuclein is one of the main components of LB (Spillantini et al., 1997).

Although the precise function of α-synuclein remains unknown, growing evidences have investigated that mutant or high level of α-synuclein is neurotoxic. It’s reported that missense and gene multiplication mutations of α-synuclein gene caused rare familial PD (Chartier-Harlin et al., 2004;
It has also been shown that sporadic PD patients have increased level of α-synuclein mRNA in the midbrain (Chiba-Falek et al., 2006). The expression of α-synuclein is also upregulated in the in vivo/vitro PD models (Purisai et al., 2005; Kalivendi et al., 2004; Gómez-Santos et al., 2002). The involvement of an α-synuclein in PD points to the possibility that strategies aimed at suppressing α-synuclein may potentially halt or slow down the progression of dopaminergic cell death in PD.

SH-SYSY cell, a human neuroblastoma cell line, which possesses intracellular substrates for dopamine synthesis, metabolism and transportation, is one of the most widely used neuronal cell lines for PD researches. Treatment of SH-SYSY cells with 1-methyl-4-phenylpyridinium (MPP⁺) represents an in vitro experimental model for the study of PD. MPP⁺ is taken up via dopamine transporter (DAT) and accumulates in dopaminergic neurons. MPP⁺ could induce cell death of dopaminergic neurons. MPP⁺ could induce cell death of dopaminergic neurons by inhibition of the mitochondrial complex I (Shimohama et al., 2003).

Mitochondrion is an energy producer and is vital to cellular survival and normal function. MPP⁺ can induce mitochondrial dysfunction in SH-SYSY cells while α-synuclein knockdown protected SH-SYSY cells against MPP⁺ (Fountaine and Wade-Martins, 2007). However, whether the mitochondrial function in MPP⁺-treated α-synuclein knockdown cells remains normal is unknown.

In the present study, we downregulated α-synuclein expression by short hairpin RNA (shRNA) in SH-SYSY cells. To evaluate the effect of α-synuclein knockdown on cell survival and mitochondrial function, we detected cell viability, nuclear morphology, mitochondrial membrane potential (MMP), cytochrome c release and Bcl-2/Bax expression in MPP⁺-treated SH-SYSY cells and α-synuclein knockdown cells. The results suggested that shRNA-mediated α-synuclein knockdown may inhibit MPP⁺-induced mitochondrial dysfunction of SH-SYSY cells.

2. Results

2.1. α-syn-shRNA downregulated α-synuclein expression

To get long-term suppression of gene expression, we used pGenesil vector containing sequence targeting α-synuclein gene to reduce α-synuclein expression in SH-SYSY cells. Stable transfectants were selected by G418. α-Synuclein expression in control cells, cells transfected with pGenesil-scrambled shRNA (control vector) or pGenesil-α-synuclein-shRNA(α-syn-shRNA) was assayed by Western blotting. The result showed that, compared with control, α-syn-shRNA inhibited α-synuclein protein expression significantly, while pGenesil-scrambled shRNA had no effect on α-synuclein protein level.

2.2. α-syn-shRNA enhanced cell survival against MPP⁺

Active mitochondria of living cells can cleave MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium) to produce formazan, the amount of which is directly correlated to the living cell number. Cells were incubated in drug-free medium or medium containing 500 μM MPP⁺ for 24 h, and then cell viability was measured by MTT assay. After treatment with MPP⁺ for 24 h, the cell viability of control cells, control vector transfected cells and α-syn-shRNA transfected cells was decreased to 67.7±2.9%, 66.5±3.3% and 89.6±2.7%, respectively (Fig. 2). The result suggested that SH-SYSY cells with downregulated α-synuclein expression can resist the toxicity of MPP⁺.

2.3. α-syn-shRNA prevented MPP⁺-induced morphological changes in nuclei

To investigate the nuclear morphological changes, the cells were stained by Hoechst 33258. Hoechst freely passes cell membranes and stains nuclear blue. Cells with intact blue
nuclei were considered as living cells, and cells exhibiting reduced nuclear size and intense blue fluorescence were considered as apoptotic cells. As shown in Fig. 3, very few control cells or cells transfected with α-syn-shRNA showed reduced nuclear size and intense blue fluorescence (0.89±0.4% and 1.03±0.6%, respectively). After 500 μM MPP⁺ treatment for 24 h, 30.2±1.6% of cells exhibited reduced nuclear size, chromatin condensation, nuclear fragmentation, and intense fluorescence. This change in nuclear morphology was rescued in cells transfected with α-syn-shRNA, and only 14.2±1.3% of cells had nuclear condensation or fragmentation.

2.4. α-syn-shRNA attenuated MPP⁺-induced MMP reduction

In order to explore changes in mitochondrial membrane function, we detected mitochondrial membrane potential with fluorescent probe DiOC6 (3). As shown in Fig. 4, in normal cells, about 11.2±0.8% cells showed low mitochondrial membrane potential, whereas this proportion dramatically increased to approximately 41.0±1.5 % after MPP⁺ treatment. In cells transfected with α-syn-shRNA, the percentage of cells showing low mitochondrial membrane potential after MPP⁺ treatment was about 13.6±1.2%. The result showed that knockdown of α-synuclein expression prevented disruption of MMP.

![Graph showing effect of α-synuclein knockdown on mitochondrial membrane potential](image)

Fig. 4 – Effect of α-synuclein knockdown on mitochondrial membrane potential in control and α-syn-shRNA cells. Cells were incubated in drug-free medium or medium containing 500 μM MPP⁺ for 24 h, and then the mitochondrial membrane potential was estimated by flow cytometry. Data were expressed as the percentage of cells with low MMP. * p<0.05 vs. control+MPP⁺.

![Photomicrographs of SH-SY5Y cell nuclei by Hoechst 33258 staining](image)

Fig. 3 – Photomicrographs of SH-SY5Y cell nuclei by Hoechst 33258 staining. Cells were incubated in drug-free medium or medium containing 500 μM MPP⁺ for 24 h, and then the nuclear morphology was captured after incubated with 10 μg/ml Hoechst 33258. A: control cells; B: control cells+MPP⁺; C: cells transfected with pGenesil-α-syn-shRNA; D: cells transfected with pGenesil-α-syn-shRNA+MPP⁺. Scale bar=50 μm.
2.5. α-syn-shRNA prevented cytochrome c efflux from mitochondrial

Release of cytochrome c into cytosol is a marker of mitochondrial dysfunction. To evaluate the level of cytochrome c in cytosol, cells were fractionated and cytosolic proteins were detected by Western blotting with anti-cytochrome c antibody. The result showed that MPP+ could induce cytochrome c release markedly, which was about 3.1-fold compared with that of control. However, in cells transfected with α-syn-shRNA, the induction was blocked significantly, and the release of cytochrome c was 1.4-fold compared with that of control (Fig. 5).

2.6. α-syn-shRNA preserved Bcl-2/Bax ratio

Since Bcl-2 family proteins play critical roles in programmed cell death induced by a wide array of death signals, the levels of Bcl-2 family proteins in three groups were studied by Western blotting. After MPP+ treatment, the Bcl-2 level in SH-SY5Y cells didn’t change significantly, while Bax increased remarkably. The Bcl-2/Bax ratio was reduced to 35.5±3.8%. In α-synuclein knockdown cells, the Bcl-2/Bax ratio was 99.5±3.1%. After α-synuclein knockdown cells were exposed to MPP+, Bcl-2 maintained the same level as control, but the upregulated Bax level could be attenuated. The Bcl-2/Bax ratio of α-synuclein knockdown cells changed to 85.2±3.0%. The data indicated that α-syn-shRNA inhibited the reduction of the Bcl-2/Bax ratio observed after MPP+ treatment (Fig. 6).

3. Discussion

MPP+ decreased cell viability, induced morphological changes and impaired mitochondrial function in SH-SY5Y cells (Fall and Bennett 1999). In this study, we showed that α-synuclein knockdown could inhibit mitochondrial dysfunction and cell death caused by MPP+.

One of the models usually used for the study of PD is the administration of MPP+. Its toxicity is mainly induced by inhibiting mitochondrial function in dopaminergic neurons. In this study, we used the neuroblastoma cell line, SH-SY5Y cells, mainly because they have many properties similar to dopaminergic neurons and they are simpler and easier to culture than primary neuronal cultures, which are complicated, as the cell population is changing in cell type, cell number, and phenotypic expression over time in culture (Valverde et al., 2008; Murayama et al., 2001). Furthermore, primary cultures may exhibit inconsistency between different donors. It has been suggested that MPP+-induced apoptosis in SH-SY5Y cells could be an in vitro model for PD study (Shimohama et al., 2003; Cheung et al., 2009).

α-Synuclein, a naturally unfolded protein of 140 amino acids, is the main constituent of Lewy bodies, which is a morphological hallmark of PD. Though its precise function remains elusive, growing evidences show that missense and
gene multiplication mutations of the gene encoding α-synuclein cause early onset of family PD (Chartier-Harlin et al., 2004; Polymeropoulos et al. 1997; Zarranz et al., 2004). So its downregulation may be helpful for PD. Our results showed that α-syn-shRNA reduced α-synuclein expression successfully, suggesting that it was an effective, long-term gene silencing means. Our results also showed that MPP+ treatment decreased SH-SYSY cell viability and increased apoptotic nuclei dramatically, which was consistent with previous reports (Dauer et al., 2002; Klivenyi et al., 2006). However, compared with the common SH-SYSY cell, the survival of α-synuclein knockdown SH-SYSY cells were significantly increased after the same treatment. The data suggested that α-synuclein knockdown protected SH-SYSY cells against MPP+ toxicity.

In this study we found that after MPP+ treatment, mitochondrial membrane potential depression and cytochrome c release occurred, consistent with previous reports (Wang and Xu 2005; Lee et al., 2008). In α-synuclein knockdown cells, however, these phenomena of mitochondrial membrane potential depression and cytochrome c release occurred, consistent with previous reports (Wang and Xu 2005; Lee et al., 2008). In α-synuclein knockdown cells, however, these phenomena of mitochondrial membrane potential depression and cytochrome c release occurred, consistent with previous reports (Wang and Xu 2005; Lee et al., 2008).

4. Experimental procedures

4.1. Cell culture and MPP+ treatment

SH-SYSY cells were grown in DMEM (Gibco, CA, USA) with 10% fetal bovine serum (FBS, Hyclone, South America). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2. The culture medium was changed every 48 h. Cells were exposed to 500 μM MPP+ (Sigma, St Louis, USA), and then cultured for 24 h respectively.

4.2. Plasmid reconstruction and transfection

Small interfering RNA (siRNA) oligonucleotides specifically targeting α-synuclein mRNA were confirmed to be valid by a previous report (Sapru et al., 2006). The forward hairpin oligonucleotides (S-‘TTTGGACCAAGTTGGCGAACATTCAAGGCGCATGC-3) and reverse oligonucleotides (S-’AGCTCAAAAAAGGACCAGTGGCGCAAAGATCGTTCCTGAAAATCTGCGTCCGAACTGCTGTC-3) were ligated into pGenesil-2 vector (Genesil, Wuhan, China). The basic pGenesil-2 vector map, the forward and reverse oligonucleotides, and the transcript-shRNA were shown in Fig. 7.

The sequence of antisense strand used in pGenesil-scrambled shRNA(control vector, Genesil, Wuhan, China) is S-’GACTTCATAAGGCGCATGC-3, which bears no homology to any sequences in the human genome database. Therefore, the transcript-shRNA was expected to have no interference on human genes.

RNA interference expression vector (α-syn-shRNA) or control vector were transfected into SH-SYSY cells using Lipofectamine™ 2000 (Invitrogen, CA, USA) according to the manufacturers’ instructions. Briefly, the complexes of diluted RNA interference expression vector or control vector and Lipofectamine™ 2000 were added to wells containing 90% confluence cells and Opti-MEM medium (Invitrogen, CA, USA). 6 hours later, medium was changed to DMEM with 10%FBS. Stably transfected cells were selected by G418 (400 μg/ml, Alexis, Switzerland) 24 h after transfection.

4.3. Measurement of cell viability

Cell viability was measured by MTT assay, which is based on the conversion of yellow MTT to a purple formazan compound by the dehydrogenase activity of intact mitochondria. Briefly, SH-SYSY cells were seeded on 96-well plate at a density of 5 x 10⁴ cells/200 μl. After the cells were incubated in drug-free medium or medium containing 500 μM MPP+ for 20 h, 20 μl of 5 mg/ml MTT (Sigma, Milwaukee, USA) solution was added to each well for 4 h. Then the supernatant was removed and 150 μl/well DMSO was added to dissolve the formazan. The absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as percentage of the control group (Feng et al., 2008).
4.4. Morphological changes

The changes in nuclear morphology of apoptotic cells were investigated by staining the cells with Hoechst 33258. Hoechst freely passes cell membranes and stains nuclear DNA blue. After incubating in drug-free medium or medium containing 500 μM MPP+ for 24 h, cells were washed twice with PBS and incubated with 10 μg/ml Hoechst 33258 (Sigma, St. Louis, USA) dye for 20 min at 37 °C in the dark. Then cells were washed with PBS and observed under an Olympus IX70 inverted fluorescence microscope (Japan).

4.5. Measurement of mitochondrial membrane potential (MMP)

In order to evaluate the mitochondrial respiratory function, the mitochondrial membrane potential was assessed by DiOC6 (3) (Aldrich, Milwaukee, USA). After incubating in drug-free medium or medium containing 500 μM MPP+ for 24 h, the cells were incubated with 40 nM DiOC6 (3) for 15 min at 37 °C away from light. Then the cells were harvested by trypsin and washed with cold PBS. After resuspending in cold PBS, the cells were analyzed with flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm (Liu et al., 2007).

4.6. Preparation of proteins of the whole cell and the cytosolic fractions

The whole cell proteins were extracted in lysis buffer with protease inhibitor cocktails on ice for 30 min. The lysates were then centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was the whole cell proteins.

To get proteins of the cytosolic fractions, the cells were harvested, washed twice in ice-cold PBS, and resuspended in ice-cold extract buffer (20 mM HEPES-KOH, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF, pH 7.5). The resuspended cells were homogenized with ten strokes of a Teflon homogenizer. The homogenates were centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was centrifuged at 100,000 g for 1 h at 4 °C and the resulting supernatant was used as proteins in the cytosolic fractions (Liu et al., 2007).

4.7. Western blotting

For Western blot analysis, the concentration of the whole cell proteins or cytosolic proteins was calculated with BIO-RAD DC protein assay kit (BIO-RAD, USA). Each extract was denatured in loading buffer, separated in SDS-PAGE and transferred onto nitrocellulose membrane. After blockage, the membrane was incubated with the first antibodies followed by horseradish peroxidase-conjugated second antibodies. The first antibodies used were as follows: rabbit anti-α-syn polyclonal antibody, goat anti-β-actin polyclonal antibody, mouse anti-cytochrome c monoclonal antibody, mouse anti-Bcl-2 antibody, and rabbit anti-Bax antibody (Santa Cruz biotechnology, CA, USA). Signals were detected with ECL Western Blotting Detection Reagents (GE healthcare, UK).

4.8. Statistical analysis

Data were expressed as means±S.E.M. Statistical analysis was performed using one-way ANOVA. p<0.05 denoted the presence of a statistically significant difference.
REFERENCES


