Mutant A53T α-Synuclein Induces Neuronal Apoptosis by regulating Autophagy

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Abstract: Previous studies have shown that mutant A53T α-synuclein leads to a variety of cytotoxicity and the mechanism remain to further investigate. In our study, SH-SY5Y cells were transfected with EGFP-N1, EGFP-α-synuclein (WT) and EGFP-α-synuclein (A53T) and incubated for 24 hours. MTT assays showed that the mutant A53T α-synuclein decreased the viability of SH-SY5Y cells. Flow cytometry analysis showed that the mutant A53T α-synuclein increased apoptosis. The mutant A53T α-synuclein up-regulated autophagy was evidenced by punctuate monodansylcadaverine (MDC) staining and microtubule-associated protein light chain 3 (LC3) immunoreactivty. The mutant A53T α-synuclein also increased protein levels of beclin I and membrane form LC3 (LC3-II). 3-methylademine (3-MA), the inhibitor of autophagy, can decreased the apoptotic cell death of SH-SY5Y cells indicated by mutant A53T α-synuclein. Together, these findings indicate that mutant A53T α-synuclein induces apoptosis through up-regulating autophagy in SH-SY5Y cells, furthermore, autophagy inhibitors can decrease the cytotoxicity of mutant A53T α-synuclein.

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1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease of undiscovered origin mainly characterized by the progressive loss of dopaminergic neurons caused by programmed cell death from the substantia nigra pars compacta and the presence of intracytoplasmic inclusions called Lewy bodies consisted of ubiquitinated α-synuclein containing in related brain region (Martinez-Vicente and Vila, 2013; Chau et al., 2009). The presence of Lewy bodies in PD suggests that defective protein handling, in particular of α-synuclein, may participate in the pathogenesis of PD.

α-synuclein is a protein of 140 amino acids which is predominantly distributed in presynaptic terminals throughout the central nervous system. α-synuclein oligomerization and aggregation are considered a role in neurodegenerative diseases pathogenesis. Under pathologic conditions, α-synuclein aggregates to form neuronal inclusions in several neurodegenerative diseases (Unal-Cevik et al., 2011; Li et al., 2009). For example, α-synuclein is the major component of Lewy bodies found in PD, dementia with Lewy bodies, as well as the Lewy body variant of Alzheimer's disease. The clearance of α-synuclein involves a variety of cellular intrinsic mechanisms. While α-synuclein was originally thought to be exclusively degraded by the ubiquitin-proteasome system (UPS), we now know that it can also be degraded inside the lysosomes, where it can be delivered by different pathways, like macroautophagy, chaperone-mediated autophagy and endocytosis (Martinez-Vicente and Vila, 2013). In nature, autophagy is a recycling process by which a cell can handle damaged organelles and long-lived proteins by segregating them in a bilayer autophagic vacuole called autophagosome and transporting them to lysosome for enzymatic degradation (Xiong et al., 2013; Venderova and Park, 2012). The cell can then reuse the resultant energy and components to survive under stress conditions (Venderova and Park, 2012).

It is known that both missense and multiplication mutations of α-synuclein cause early-onset autosomal dominant familial form of PD (Chau et al., 2009; Warner and Schapira, 2003). Previous genetic and pathological studies clearly point out that there is an important role of α-synuclein in the pathogenesis of PD. Extensive studies have been performed to investigate the underlying pathogenic mechanisms of α-synuclein-induced cell loss. It has been indicated that overexpression of both wild type and mutant α-synuclein such as mutant A53T α-synuclein leads to a variety of cytotoxicity, including the injury of proteasome and lysosome activities, the

disruption of ER-Golgi transport, the perturbation of the mitochondrial function, and the inhibition of synaptic transmission (Lin et al., 2012). Here, we provide evidence that shows that the mutant A53T α-synuclein induced apoptosis by regulating autophagy in SH-SY5Y cells.

2.Materials and Methods

2.1.Cell culture and transfections

SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) containing 10% newborn calf serum (GIBCO). SH-SY5Y cells were seeded in six-well plates (9.6 cm^2/well; Falcon Labware, Oxnard, CA, USA) at a density of 400 000 cells/well, incubated for 24 hours at 37 ℃ in humidified 5% CO_2. Later, SH-SY5Y cells were divided into three groups and trasnfected with the recombinant plasmid EGFP-N1, EGFP-α-synuclein (WT) and EGFP-α-synuclein (A53T), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, UAS) according to the manufacturer's instructions.

2.2.Cell viability assay

Cell viability was assessed by MTT assay. Cells were plated in 96-well plates at the density of 5000 cells in 100 μL medium per well one day before the experiment. MTT solution was added to the culture medium (5mg/ml final concentration) and 4 hours later the reaction was stopped by addition of 10% acidified SDS (100 μl) to the cell culture. The absorbance value at 570 nm was read using an automatic multiwell spectrophotometer (Bio-Rad, Richmond CA, USA). The percentage of cell death (growth inhibition) was calculated as follows: cell death (%) = (1-A of experiment well/A of positive control well) ×100%.

2.3.Apoptosis detection by flow cytometry

Apoptosis of SH-SY5Y cells overexpressing EGFP-N1, EGFP-α-synuclein (WT) and EGFP-α-synuclein (A53T) was detected using an annexin V-FITC kit (Biovision, Inc., California, USA), according to the manufacturer's instructions. Briefly, cells were plated in 96-well plates at the density of 5000 cells in 100 μL medium per well and incubated for 24 hours in the presence or absence of 3-MA (10mmol/L), and then cells were harvested, washed twice in PBS, and then stained with Annexin V-FITC and PI according to the manufacturer's instructions. The resulting fluorescence was detected by flow cytometry with Cell Quest analysis software. Each experiment was repeated three times.

2.4.Visualization of MDC-labelled vacuoles

Autophagic vacuoles were labelled with MDC by incubating SH-SY5Y cells plated in 96-well plates with 0.05 mM MDC in PBS at 37℃ for 10 min. After incubation, cells were washed four times with PBS and immediately analysed by fluorescence microscopy using an inverted microscope (Olympus BX50).

2.5.Statistical analyses

Statistical analysis was performed using standard statistical software (SPSS for Windows, version 13.0; SPSS Inc., Chicago, IL, USA). Comparisons between two groups were performed using the Student's t-test, while comparisons involving more than two groups were performed by one-way analysis of variance. A P value < 0.05 was considered to be statistically significant.

3.Results

3.1.Overexpression of mutant A53T α-synuclein induces apoptosis of SH-SY5Ycells

The SH-SY5Y cells were divided into three groups respectively and overexpressing EGFP-N1, EGFP-α-synuclein (WT) and EGFP-α-synuclein (A53T). The cell viability of SH-SY5Y cells was detected using the MTT assay, and values were given as mean ± SD. The results of three groups were 89.12±7.82, 84.33±6.98 and 54.34±4.56. Overexpression of WT α-synuclein reduced SH-SY5Y cell viability ($p>0.05$, compared to the control group), although to a lesser extent than mutant A53T α-synuclein (*, $p<0.05$) (Figure. 1).

Apoptotic morphological characteristics such as chromatin condensation and nuclear fragmentation were viewed in different groups of SH-SY5Y cells. Flow cytometry of annexin V-FITC-labeled cells showed that the overexpressing of both WT and

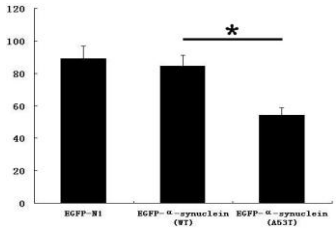

Figure 1. Effects of mutant A53T α-synuclein on viability of SH-SY5Ycells.

Mutant A53T α-synuclein induced apoptosis, however the extent was significantly higher for cells overexpressing mutant A53T α-synuclein (**,$P<0.01$). The overexpressing of EGFP-N1, EGFP-α-synuclein (WT) and EGFP-α-synuclein (A53T) resulted in 3.23±0.67%, 5.98±0.79% and 22.45±6.12% apoptotic

cell death, respectively (Figure. 2).

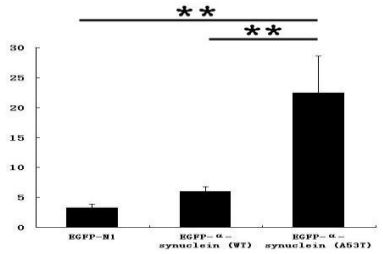

Figure 2. Apoptotic cell death was analysed by flow cytometry.

3.2.Overexpression of mutant A53T α-synuclein increases autophagic activity in SH-SY5Y cells

Autophagic vacuoles could be detected by MDC staining. Under a fluorescence microscope, MDC-stained AVs appear as distinct dot-like structures distributing within the cytoplasm or localizing in the perinuclear regions. There was an increase in the number of MDC-labeled vesicles for both WT and mutant A53T α-synuclein, however in cells overexpressing mutant A53T α-synuclein the increasing extent was higher (Figure. 3). The result indicates that mutant A53T α-synuclein may induce AV formation.

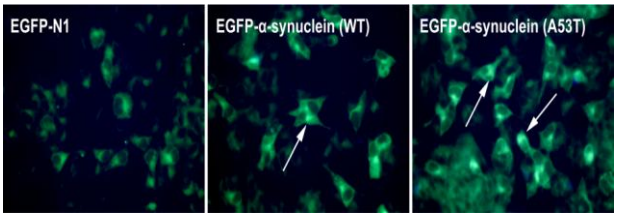

Figure 3. Mutant A53T α-synuclein induced MDC-labeled vesicles in SH-SY5Ycells. Fluorescence particles in the cytoplasm stand for autophagic vacuoles. Magnification ×620.

LC3 is viewed localizing in autophagosome membranes during amino acid starvation-induced autophagy. LC3 is regarded as a molecular marker of autophagosomes. Previous studies find that there are two forms of the LC3 proteins in various cells: LC3-I and LC3-II. LC3-I is the cytoplasmic form and translate into LC3-II, which is autophagosome membrane form. Therefore, the amount of LC3-II is related with the autophagosome formation extent. We examined the expressions of LC3-I (18 kDa) and LC3-II (16 kDa) in cells overexpressing WT or mutant A53T α-synuclein by western blot analysis. An apparent increase in the levels of LC3-II protein was detected in cells overexpressing WT or mutant A53T α-synuclein, the increasing extent was higher in mutant A53T α-synuclein group (Figure. 4). Beclin I is a part of type III PI3 kinase complex that play an important role in the formation of the autophagic vesicle and we can prevent induction of autophagy by interference with beclin I. Therefore, beclin I is required in the processes of autophagy. The western blot analysis shown that the levels of beclin I protein were markedly increased for both WT and mutant A53T α-synuclein, however the extent of increasing was significantly higher in cells overexpressing mutant A53T α-synuclein.

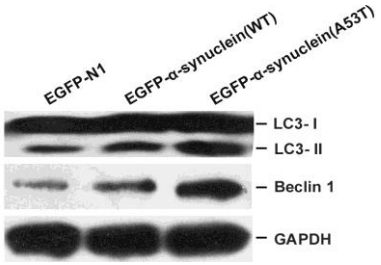

Figure 4. LC3 and beclin I protein expression in different groups of SH-SY5Ycells.

3.3.Autophagy increases mutant A53T α-synuclein -induced death of SH-SY5Y cells

We can prevent autophagy by adding 3-MA, the specific class III PI3 kinase inhibitor. To investigate the effects of autophagy on mutant A53T α-synuclein -induced apoptosis, 3-MA was added 12h after the cells had been transfected with EGFP-N1, EGFP-α-synuclein (WT) and EGFP-α-synuclein (A53T). Results showed treatment of SH-SY5Y cells with 3-MA decreased the apoptotic cell death to 6.72±2.32%, 8.23±3.54% and 17.32±4.34% (*, $p < 0.05$), respectively (Figure. 5).

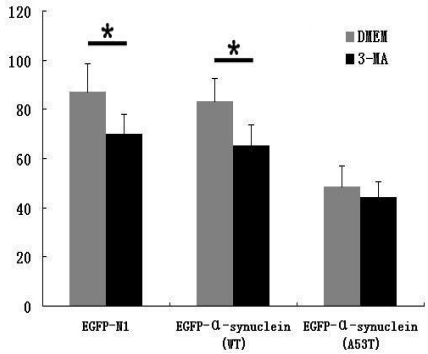

Figure 5. Effects of 3-MA on mutant A53T α-synuclein -induced apoptotic death of SH-SY5Y cells.

4.Discussion

In this study, we have shown that, after cells were transfected with EGFP-N1, EGFP-α-synuclein (WT) and EGFP-α-synuclein (A53T) and incubated for 24 hours, MDC-labeled vesicles and the protein levels of beclin I and membrane form LC3 (LC3-II) increased and the apoptotic cell death increased as well in the EGFP-α-synuclein (A53T) group. These data demonstrate that overexpression of mutant A53T α-synuclein increases autophagic activity and induces apoptosis in SH-SY5Y cells. These data consistent with previous findings that A53T mutant α-synuclein aggregates more rapidly in neurons, is degraded more slowly and has greater toxicity than WT α-synuclein. Furthermore, the inhibitor of autophagy, can decreased the apoptotic cell death of SH-SY5Y cells indicated by mutant A53T α-synuclein. So we come to an conclusion that mutant A53T α-synuclein induces neuronal apoptosis by regulating autophagy.

It is believed that increasing autophagy, the intracellular degradation machinery, is beneficial for neurodegenerative diseases related with protein aggregation. For example, the deletion of the endogenous inhibitor of intracellular cathepsins, Cystatin B, significantly reduced the brain amyloid plaque load in a mouse model of Alzheimer disease (Huang et al., 2011). It has been also confirmed that enhancers of autophagy enhance the clearance of mutant huntingtin and decrease toxicity in cellular and Drosophila models of Huntington disease (Huang et al., 2011; Sarkar et al., 2007). Autophagy induced by rapamycin also decreases mutant Ataxin-3 levels and toxicity in a mouse model of spinocerebellar ataxia type (Menzies et al., 2010). However, there are also some evidences that suggest that overactivation of the degradative system may have harmful effects. Here are some examples, even the partial loss of Cystatin B bring about neuronal loss, ataxia, and seizures in mice (Kaasik et al., 2007). Xilouri's study demonstrated that the inhibition of autophagy by down-regulating ATG5, one of autophagy related genes, attenuates mutant A53T α-synuclein ininduced toxicity. Consistent with our study, Xilouri *et al.* also confirmed that mutant A53T α-synuclein overactivates the macroautophagic degradation of long-lived proteins (Xilouri et al., 2009).

The two sets of studies have indicated that at some point autophagy is neuroprotective, on the other is nerve lesion (Pan et al., 2008; Li et al., 2011; Xue et al., 1999; Canu et al., 2005). Previous studies have suggested that the difference may be attributed to different models, different mechanisms involved in these models and different treatment phases. Increasing evidence supports that activating autophagy in early-stage is protective and over activating autophagy in late-stage eventually leads to cell death (Takacs-Vellai et al., 2006). The activation of autophagy has both yin and yang sides. The positive side is that it increase the clearance of aggregated proteins (Kanki et al., 2009; Atwal et al., 2007), while the negative side could be that the clearance is so much that some "innocent" intracellular components are involved in.

Taken together, our data suggest that mutant A53T α-synuclein induces neuronal apoptosis by regulating autophagy. While the relationship between apoptosis and autophagy remains unclear and requires further investigation. Therefore, it should be a therapeutic direction of PD by regulating autophagy precisely rather than enhancing or inhibiting autophagy simply.

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