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Role of autophagy and proteasome degradation pathways in apoptosis of PC12 cells overexpressing human α-synuclein

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ABSTRACT

Parkinson's disease is a common neurodegenerative disease in the elderly. Its causes and mechanisms are not clearly understood. To explore the specific role of autophagy and the ubiquitin-proteasome pathway in apoptosis, a specific proteasome inhibitor and macroautophagy inhibitor and stimulator were selected to investigate pheochromocytoma (PC12) cell lines transfected with human mutant (A30P) and wild-type (WT) α-synuclein. The apoptosis ratio was assessed by flow cytometry. LC3, heat shock protein 70 (hsp70) and caspase-3 expression in cell culture were determined by Western blot. The hallmarks of apoptosis and autophagy were assessed with transmission electron microscopy. Compared to the control group or the rapamycin (autophagy stimulator) group, the apoptosis ratio in A30P and WT cells was significantly higher after treatment with inhibitors of the proteasome and macroautophagy. The results of Western blots for caspase-3 expression were similar to those of flow cytometry; hsp70 protein was significantly higher in the proteasome inhibitor group than in control, but in the autophagy inhibitor and stimulator groups, hsp70 was similar to control. These findings show that inhibition of the proteasome and autophagy promotes apoptosis, and the macroautophagy stimulator rapamycin reduces the apoptosis ratio. And inhibiting or stimulating autophagy has less impact on hsp70 than the proteasome pathway.

Parkinson's disease (PD) is a common degenerative disorder of the central nervous system, and its etiopathogenesis is not entirely clear. PD is caused by the degeneration of dopaminergic neurons in the substantial nigra, and its pathogenic hallmark is the accumulation and aggregation of α-synuclein in susceptible neurons [20,24,35,43]. The lysosomes and the ubiquitin-proteasome system are two major distinct proteolytic pathways in mammalian cells [6,16,40], so they play an important role in the degradation of α-synuclein. At the same time, many recent investigations have demonstrated that superabundant apoptosis is often associated with neurodegenerative diseases and apoptotic cell death is a common pathway for the loss and degeneration of dopaminergic neurons caused by different factors [4,17,25]. So, we set out determine whether autophagy and apoptosis have a mutual relationship with overexpression of human α-synuclein in pheochromocytoma (PC12) cells.

Programmed cell death (PCD), a critical mechanism for development and homeostasis in multicellular organisms, consists of two main forms: apoptosis and autophagy [39]. Apoptosis (type I PCD) is a cell-intrinsic suicide mechanism regulated by various signaling pathways [9]. Autophagy (type II PCD) has multiple physiological functions in multicellular organisms, including lysosome-dependent protein degradation and organelle turnover [11]. Some studies have pointed out that apoptosis and autophagy may be interconnected and even simultaneously regulated by the same trigger [42]. Other studies have further reported that autophagy can resist or suspend apoptosis [2]. Meanwhile, the cysteine proteases of the caspase family play essential roles in the induction and execution of apoptosis [5,16].

We selected a specific proteasome inhibitor and/or macroautophagy inhibitor and stimulator to determine their effects on PC12 cell lines transfected with human mutant (A30P) or wild-type (WT) α-synuclein, and found that autophagy and apoptosis were negatively correlated.

The recombinant vector pEGFP-C3-SNCA and the liposome transfer method were used to construct rat PC12 cell lines overexpressing human WT and A30P α-synuclein. Transfected PC12 cells were further screened with G418 Mediatech and were obtained by means of a limiting dilution assay. Stably transfected A30P and WT

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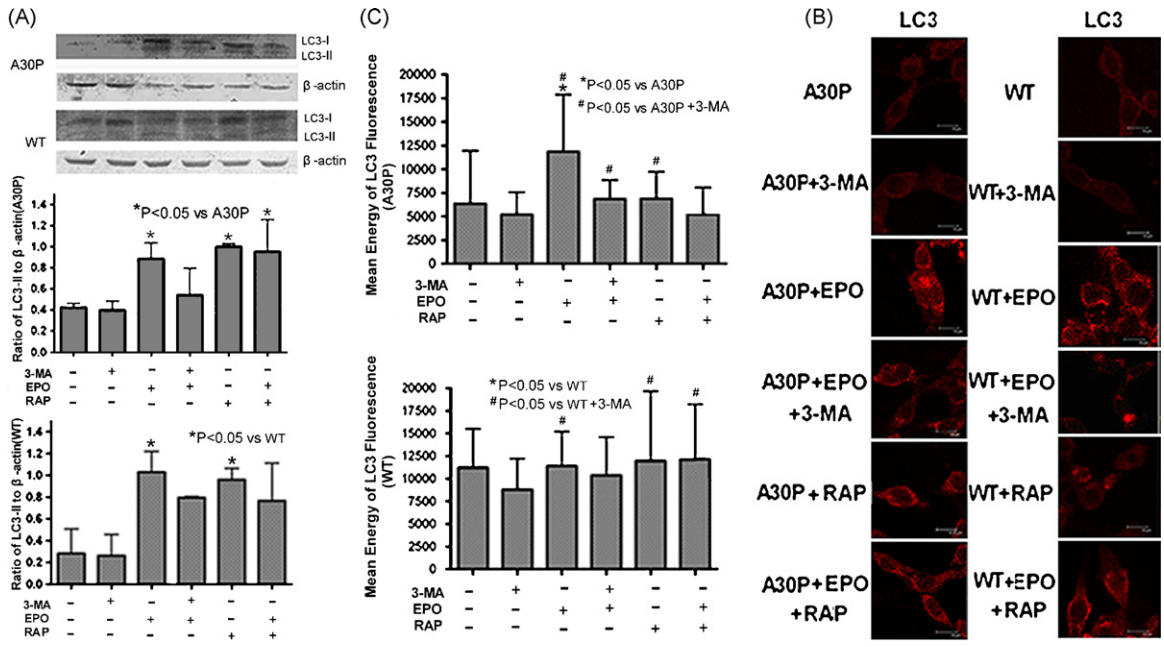

Fig. 1. Effect on autophagy of the inhibition of proteasome activity and/or macroautophagy in different cells. (A) Western blot analysis of LC3 following drug treatment (24 h) in A30P and WT PC12 cell lines. PC12 cells stably transfected with WT and A30P α-synuclein were treated with the proteasome inhibitor epoxomicin and/or the macroautophagy inhibitor 3-MA and macroautophagy stimulator rapamycin for 24 h before Western blot analysis. EPO: epoxomicin; RAP: rapamycin; WT: wild-type. (B) Cells were imaged by confocal microscopy. A30P and WT cells treated with indicated drugs were permeabilized and immunostained with anti-LC3 antibody, followed by Cy3-labeled secondary antibodies for imaging. (C) Mean immunofluorescence mean intensity of cells.

α-synuclein PC12 cell lines were cultured in RPMI1640 (Gibco, USA) containing 10% calf serum, at 37 °C in 5% CO_2 (details in Ref. [36]).

After adherence, cells were incubated in control medium (Gibco), or media containing either 10 mM 3-methyladenine (3-MA; Sigma), 0.2 μg/ml rapamycin (RAP; Sigma), or 100 nM epoxomicin (EPO; Sigma). 3-MA was made up in water and rapamycin and epoxomicin were dissolved in DMSO.

For flow cytometry analysis, WT and A30P cells treated with epoxomicin and/or 3-MA and rapamycin were harvested, washed with PBS and stained with propidium iodide (PI; Sigma). Percentages of apoptotic cells were determined by flow cytometry (FACSCalibur; Becton Dickinson). The results were from four independent tests.

Cells were seeded on coverslips placed in 24-well dishes. Then, cells were incubated in media containing autophagy/proteasome drugs for 24 h. Cells were fixed with 4% paraformaldehyde (Sigma) for 15 min, washed with PBS, and permeabilized with 0.1% Triton-X-100 (Sigma) for 15 min. Anti-LC3 antibody (Santa Cruz) was used at 1:250 for 2 h, the cells were washed, and 1:500 Cy3-conjugated anti-goat antibodies (Beyotime Institute of Biotechnology) were added for 1 h. Finally, slides were mounted in Citifluor (Citifluor Ltd.) with 3 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Cells were visualized under a Leica TCS ST2 confocal microscope.

Cells were seeded in culture flasks at 1.5×10^8 cells/ml treated with the drugs as above, and further cultured for 24 h. Cell pellets were collected and lysed in lysis buffer before electrophoresis on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with the antibodies anti-β-actin (Santa Cruz), anti-LC3 (Santa Cruz), anti-hsp70 (Abcam) and anti-caspase-3 (cell signaling) followed by detection using an enhanced chemiluminescence system (Pierce) or TMB stabilized substrate for HRP (Promega). Densitometric quantification of the films of the immunoblotted membranes and stained gels was done with an image software analysis system.

The procedures for cell culture and drug treatment were the same as above. At 24 h after treatment, the cells were collected, fixed in 2.5% glutaraldehyde for 2 h, and washed with 0.1 M phosphate buffer. The pellet was then postfixed in 1% osmium tetraoxide for 2 h, dehydrated through a graded series of ethanol and acetone, and embedded in epoxy resin. Ultrathin sections were cut on an ultramicrotome and stained with uranyl acetate and then lead citrate, and viewed on a transmission electron microscope (JEM-1011, Japan).

Normally distributed data are shown as mean ± S.D. and were analyzed by one-way ANOVA using SPSS 12.0 software. A value of $P < 0.05$ was regarded as significant.

Since the ratio of LC3-II to β-actin is considered to be an accurate indicator of autophagy, immunoblotting was carried out to detect the protein expression of LC3 [19,29]. We did not quantify LC3-II versus LC3-I, as some LC3-II can be converted back to LC3-I [41]. We consistently saw activation of macroautophagy by treatment with the proteasome inhibitor epoxomicin, in both cycling and differentiated cells in all cell lines [1,43] by directly analyzing macroautophagy markers (Fig. 1A). 3-MA is a specific inhibitor of autophagy [1,43] at the sequestration stage, where a double-membrane structure forms around a portion of the cytosol. In the presence of the proteasome inhibitor 3-MA, levels of LC3-II in cells were higher (Fig. 1A). Immunofluorescence was carried out to verify that changes in the intracellular distribution of LC3 also supported constitutive activation of macroautophagy in cells treated with epoxomicin (Fig. 1B and C). At the same time, the combination of proteasome and autophagy inhibitor had a neutralizing effect on LC3 expression, and the proteasome inhibitor and macroautophagy stimulator did not act synergistically on LC3 (Fig. 1).

The ubiquitin-proteasome system participates in numerous biological processes including the cell cycle, apoptosis, signal transduction, the immune response, and turn-over of misfolded proteins [14,24,40]. Our results on the apoptosis ratio indicated that the proteasome inhibitor epoxomicin promoted apoptosis, and 3-MA, a macroautophagy inhibitor, also increased the apoptosis ratio (Fig. 2A). But rapamycin, which inhibits the mammalian target of rapamycin (mTOR), a negative regulator of autophagy, to upreg-

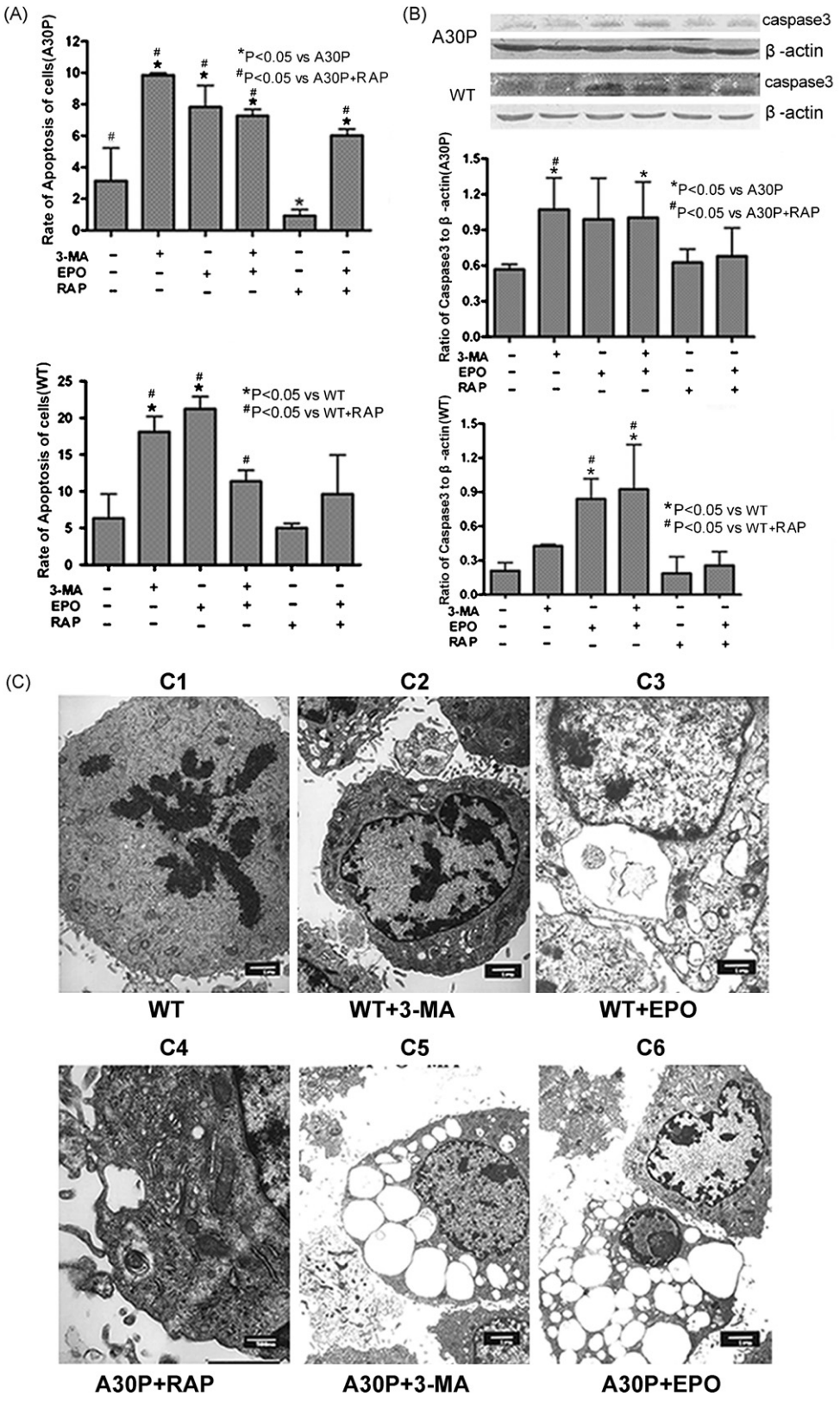

Fig. 2. Inhibition of proteasome or autophagy promotes apoptosis and stimulating autophagy inhibits apoptosis. (A) Apoptosis ratios were determined by flow cytometry after drug treatment. (B) Caspase-3 was determined by immunoblotting with the same cells used in (A). (C) Transmission electron microscope images. Representative images from different groups of cells. C1: WT α-synuclein PC12 cells showed normal structure. C2: WT cells treated with 3-MA showed chromatin condensation, bulges, and early apoptosis. C3: mitochondrial bulges, blebbing, and chromatin condensation in WT cells treated with EPO. C4: autophagosome in cells treated with rapamycin. C5: A30P cells treated with 3-MA showed blebbing, mitochondrial bulges, nuclear fragmentation, chromatin condensation, and cell debris. C6: A30P cells treated with epoxomicin led to a characteristic apoptosis morphology and death, including blebbing, mitochondrial bulges, nuclear fragmentation, and chromatin condensation.

ulate autophagy in mammals [1,21,43], decreased the apoptosis ratio (Fig. 2A). Therefore, inhibiting macroautophagy or interdiction of the proteasome pathway promoted apoptosis in both WT and A30P cells. However, inhibiting the macroautophagy pathway had less impact on apoptosis than the proteasome pathway in WT cells; this was different in A30P cells (Fig. 2A). To further understand the relationship between the proteasome pathway or autophagy and apoptosis, we combined proteasome inhibitor and macroautophagy inhibitor/autophagy stimulator treatment. Our results showed that inhibiting the proteasome together with macroautophagy in WT and A30P cells did not have an additive effect on promoting apoptosis (Fig. 2A). The apoptosis ratio when using the proteasome inhibitor and macroautophagy stimulator together was lower than that of using the proteasome inhibitor alone. In other words, rapamycin down-regulated the increased apoptosis induced by epoxomicin. The results of caspase-3 active segment analysis by Western blot were similar to those for the apoptosis ratio (Fig. 2B). The effect of autophagy manipulation on susceptibility to apoptosis differed between the cell types. But, although rapamycin decreased apoptosis in A30P cells, the caspase-3 activity did not decrease.

We further identified the hallmarks of apoptosis and autophagy in our cell lines using electron microscopy after 24 h drug treatment (Fig. 2C). The results showed that the proteasome inhibitor epoxomicin increased apoptosis and autophagy. Rapamycin, an autophagy inducer, reduced the occurrence of apoptosis. 3-MA, an inhibitor of autophagy, impaired autophagosomes but increase apoptosis. These data on ultrastructural changes provide morphological evidence supported by the data on LC3 expression with Western blot and apoptosis ratio. The results for A53T mutant α-synuclein PC12 cells were similar to those for A30P cells (data not shown).

To further confirm the mechanisms of communication between autophagy, the proteasome system and apoptosis, we checked the amount of hsp70 in each group of cells. We found that hsp70 levels were higher in the EPO intervention groups than in the non-EPO groups (Fig. 3).

Parkinson's disease is characterized by the loss of dopaminergic neurons in the substantia nigra and the formation of aggregates (Lewy bodies) in neurons [20]. The conformational change and abnormal aggregation of α-synuclein and other aggregate-prone proteins are generally considered to lead to neuronal death [31]. Many studies indicate that abnormalities in the autophagy-lysosome pathway and the ubiquitin-proteasome system are involved in the development of PD [12,28,37,43]. Recent research suggests that the ubiquitin-proteasome (UPS) pathway is correlated with apoptosis, and several regulator molecules related to apoptosis are degraded via the UPS pathway. Nevertheless, the specific function of the UPS pathway in apoptosis is still controversial [32]. Other investigations also demonstrated that depending on the cellular context and stimulus, the occurrence of apoptosis is preceded by and even depends on the occurrence of autophagy [8,44]. Moreover, some studies have reported that autophagy resists or suspends apoptosis [2]. The co-regulation of both apoptosis and autophagy may participate in mammalian cell death [34]. Accordingly, under some situations, there are multiple connections between apoptotic and autophagic processes that can together seal the fate of cells [30]. Previous results indicated that activation of autophagy in two cell lines (Huh7 and OUMS29) rescue them from apoptosis, but inhibition of autophagy by 3-MA has a significant effect on Huh7cells but not OUMS29 cells [15]. At the same time, that the inhibition of autophagy increases the apoptotic ratio in oridonin-induced L929 cells was also found in the current study, indicating that autophagy antagonizes apoptosis [5]. Our results are consistent with the recent report that proteasome inhibitors and macroautophagy inhibitors facilitate apoptosis. This suggests that

Fig. 3. Epoxomicin increased hsp70, and autophagy had less impact on hsp70 than the proteasome pathway. (A) Detection of hsp70 expression following drug treatment (24 h) in A30P cells. (B) Hsp70 protein detected in PC12 cells expressing WT α-synuclein.

accelerating apoptosis is one mechanism of inducing and promoting PD provoked by proteasome or macroautophagy inhibitors. But macroautophagy inhibitors were more effective on A30P than WT cell lines, and this may be because the degradation of α-synuclein is likely to vary between different cell types. Some studies suggest that WT α-synuclein is mainly degraded by chaperone-mediated autophagy (CMA) and degradation of mutant α-synuclein by CMA is impaired [7].

Gonzalez-Polo et al. found that when HeLa or HCT116 cancer cells are cultured in the absence of nutrients, they rapidly induce autophagy in order to recycle essential metabolites, such as lipids and amino acids, for fuelling the bioenergetic machinery [13]. In these circumstances, inhibition of autophagy results in accelerated cell death that manifests the hallmarks of apoptosis including chromatin condensation, MOMP and activation of caspases [13]. The same phenomena were shown in our TEM

results; when macroautophagy was inhibited, we observed morphological changes including chromatin condensation, chromatin margination, karyopyknosis and nuclear fragmentation in cells, and cytoplasmic vacuolization.

We checked the amount of hsp70 and cysteine protein hydrolyze (caspase-3) in each group of cells to further understand the mechanisms underlying autophagy, the proteasome pathway and apoptosis. Hsp70 is an anti-apoptosis partner molecule, and plays an important role in maintaining proteasome function [18]. Hsp70 also helps misfolded proteins refold into the natural structure, and promotes them to be decomposed by the proteasome. In this way, it takes part in the protein management process and controls apoptosis by various mechanisms [18]. The cysteine protein hydrolysis family has very important effects on mediating apoptosis, and caspase-3 is a key executive molecule. Recent research showed caspase-3 is the final effector of dopamine neuron apoptosis in PD patients and animal models [16]. Our Western blot study showed that caspase-3 expression was consistent with the apoptosis ratio data detected by flow cytometry. When caspase-3 was up-regulated, there was a compensatory increase of hsp70 to protect the cell. However, aggregated protein combines with hsp70 and prevents such protection [3]. Therefore, the EPO group did not protect cells from apoptosis even when there was a compensatory increase of hsp70. The macroautophagy stimulator rapamycin and the macroautophagy inhibitor 3-MA had effects on hsp70 expression in A30P and WT cells similar to control. In conclusion, either stimulation or inhibition of macroautophagy, has less impact on hsp70 than on the proteasome pathway. We found that rapamycin decreased apoptotic cells in A30P cells independent of caspase-3 activity. Although several lines of evidence recently demonstrated crosstalk between autophagy and caspase-independent apoptosis [10], we could not confirm that autophagy activation protects cells from caspase-independent cell death. Undoubtedly, there are multiple connections between the apoptotic and autophagic processes.

The mechanisms by which the inhibition of autophagy may favor cell death are not entirely clear. It is possible that the inhibition of autophagy results in a bioenergetic shortage that triggers apoptosis [27,23,26]. Inhibition of autophagy may subvert the capacity of cells to remove damaged organelles or to remove misfolded proteins, which would favor apoptosis [27,38]. But we noticed that proteasome inhibition activated macroautophagy and accelerated apoptosis. This is probably because inhibition of the proteasome favors oxidative reactions that trigger apoptosis, presumably through a direct effect on mitochondria, and the absence of NADPH2 and ATP may de-inhibit the activation of caspase-2 or MOMP [22,33]. Another possibility is that aggregated proteins induced by proteasome inhibition increase apoptosis. In short, the relationships between autophagy, the proteasome system and apoptosis are very complicated, and many factors participate.

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