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Crosstalk between 6-OHDA–induced autophagy and apoptosis in PC12 cells is mediated by phosphorylation of Raf-1/ERK1/2

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Parkinson's disease (PD) is a degenerative brain disorder characterized by motor symptoms and loss of dopaminergic (DA) neurons in the substantia nigra. The mechanisms for DA cell death in PD have been extensively investigated using PC12 cells treated with a dopamine neurotoxin 6-hydroxydopamine (6-OHDA). 6-OHDA may induce both autophagy and apoptosis in PC12 cells. However, it remains unclear whether crosstalk occurs between autophagy and apoptosis in PC12 cells treated with 6-OHDA and whether Raf-1/ERK1/2 and their phosphorylation status play a role in autophagy. In this study, we used MDC staining assay and flow cytometry and found that 6-OHDA induced autophagy in PC12 cells. This induction was inhibited by the autophagy inhibitor 3-MA. Our electron microscopy observations also supported 6-OHDA induced autophagy in PC12 cells. Apoptosis of PC12 cells was increased with inhibition of autophagy by 3-MA. In addition, Inhibition of Raf-1 resulted in a decreased 6-OHDA–induced autophagy rate among PC12 cells. Phosphorylation levels of Raf-1 and ERK1/2 were increased in PC12 cells treated with 6-OHDA and inhibited by co-treatment with 6-OHDA and 3-MA. These data suggest that crosstalk between 6-OHDA-induced apoptosis and autophagy in PC12 cells may be regulated via the Raf-1/ERK1/2 signaling pathway. Our data suggest a mechanism for 6-OHDA toxicity in PC12 cells, contributing to our understanding of the pathogenesis of PD.

1. Introduction

Parkinson's disease (PD) is a degenerative brain disorder characterized by motor symptoms and loss of dopaminergic (DA) neurons in the substantia nigra (Michel et al. 2013; Brotchie et al. 2009). The mechanisms underlying DA cell death in PD remain poorly understood.

PC12 cells are rat pheochromocytoma cells that are located in the adrenal medulla in vivo. These cells have the same origin as the sympathetic nerve cells. Because the receptors and neurotransmitters in PC12 cells are very close to those of DA neurons in the midbrain, these cells have been widely used in studies of DA neurons in vitro after treatment with 6-hydroxydopamine (6-OHDA), especially in research related to the pathogenesis and pharmacology of neurodegenerative diseases such as PD (Ahn et al. 2015).

Autophagy is a process that is involved in protein and organelle degradation. It protects cells and also plays a role in cell death (Mizushima et al. 2008). Apoptosis is a process of programmed cell death. Both autophagy and apoptosis contribute to the maintenance of cellular and tissue homeostasis. Whereas autophagy controls the turnover of protein aggregates and damaged organelles within cells, apoptosis degrades and eliminates unwanted cells from organisms. These two processes are usually connected and mutually inhibited in determining the fate of cells, despite marked differences between them. However, autophagy may also induce apoptosis or necrosis by excessively degrading the cytoplasm and thus influences the normal clearance of dying cells (Maiuri et al. 2007; Marino et al. 2014). Several pathways mediate the crosstalk between autophagy and apoptosis, such as caspases (Wu et al. 2014; Mukhopadhyay et al. 2014), Bcl-2 family of proteins (Shimizu et al. 2004), and Ras/Raf/ERK signaling pathways (Cagnol and Chambard 2010; Ouyang et al. 2014; Liu et al. 2010; Zeng et al. 2012).

The Ras/Raf/MEK (mitogen-activated protein kinase/ERK kinase)/ERK (extracellular-signal-regulated kinase) pathway mediates the signals generated by exposure to growth factors and mitogens, transmitting signals from their receptors to regulate gene expression, which is essential for cell proliferation, differentiation and survival. The regulation of the Ras/Raf/MEK/ERK signaling cascade in cell growth is dependent on the stimuli and cell type (Kolch 2000; Zebisch et al. 2007; McCubrey et al. 2007; Chang et al. 2003).

6-OHDA, a dopamine neurotoxin, may cause cell death in vivo and in vitro. It has been used in generating animal models of Parkinson's disease (Tatenhorst et al. 2014; Oiwa et al. 2003; Morelli et al. 1991). Treatment of PC12 cells with 6-OHDA has been used as a model to study the pathogenesis and pharmacology of neurodegenerative diseases such as PD. Numerous studies have shown that 6-OHDA affects both apoptosis and autophagy in PC12 cells; for example, 6-OHDA increases the number of autophagosomes and activates autophagy, which may play a role in DA neuron death in a rat PD model (Xia et al. 2010). Further studies reveal that reactive intermediates of COX-2 mediate oxidation and cytotoxicity of 6-OHDA in PC12 cells (Tyurina et al. 2006). 6-OHDA induces cycle re-entry and apoptosis of PC12 cells by activating the ERK1/2 signaling pathway (Zhang et al. 2009; Li et al. 2012) and PKCδ (protein kinase C delta) which is required for subsequent ERK activation during cell death (Fan et al. 2014). Mitochondrial localization of ERK2 activity is sufficient to recapitulate the effects of 6-OHDA on mitophagy and autophagic cell death (Dagda et al. 2008). However, whether Raf-1/ERK1/2 and the corresponding phosphorylation status play a role in autophagy and whether crosstalk occurs between autophagy and apoptosis in PC12 cells treated with 6-OHDA remain unclear.

In this study, we investigated the crosstalk between autophagy and apoptosis in PC12 cells treated with 6-OHDA as well as the role of Raf-1/ERK1/2 and phosphorylation status in autophagy. We found that 6-OHDA induced both apoptosis and autophagy, which requires Raf-1/ERK1/2 activation and is associated with phosphorylation of Raf-1/ERK1/2. Apoptosis of PC12 cells was increased with inhibition of the autophagy by 3-MA. Our data suggest that crosstalk between 6-OHDA-induced autophagy and apoptosis in PC12 cells may be regulated by Raf-1/ERK1/2 signaling pathway. Our data provide a potential mechanism for 6-OHDA–induced autophagy and apoptosis of PC12 cells, supporting our understanding of the pathogenesis of PD.

2. Investigations and results

2.1. 6-OHDA induced autophagy in PC12 cells

To determine the effect of 6-OHDA on PC12 cells, we examined autophagy of PC12 cells treated with 6-OHDA using MDC staining. The results showed that more green fluorescence was present in autophagic vacuoles around nuclei of PC12 cells treated with 6-OHDA, compared with the control (Fig. 1A), indicating that treatment of PC12 cells with 6-OHDA resulted in accumulation of MDC in autophagosomes. Using flow cytometry, we found more MDC-positive PC12 cells after 6-OHDA treatment than among control cells (32.2%±4.44% vs. 0.97%±0.5%; Fig. 1B). The density of fluorescence was significantly decreased in PC12 cells treated with 10 mmol/L 3-MA and 6-OHDA compared with that in cells treated with 6-OHDA (Fig. 2A). The percentage of MDC-positive cells among PC12 cells treated with 10 mmol/L 3-MA and 6-OHDA was decreased to 4.51%±0.99% (Fig. 2B). 3-MA is a known autophagy inhibitor (Caro et al. 1988; Zhang et al. 2015). These results suggest that 6-OHDA (100 μmol) induced autophagy in PC12 cells.

We further examined the subcellular structure of PC12 cells treated with 6-OHDA under electron microscopy. The result showed that in PC12 cells treated with the vehicle control, the shape of cells was irregular, the karyoplasmic ratio was large, the cytoplasm contained a large number of ribosomes. In addition, there were vacuoles in some cells (Fig. 3A). In 6-OHDA–treated PC12 cells, we observed swelling of the Golgi bodies, proliferation of smooth endoplasmic reticulum, dilation of rough endoplasmic reticulum, degradation of Golgi bodies, degradation of polysomes, degradation of the endoplasmic reticulum, degeneration and lysis of mitochondria, and formation of autophagy lysosomes from fusion with primary lysosomes (Fig. 3B, C). Necrosis and apoptosis of the cells were observed. The spherical or leaf-like protrusions occurred on the surface of the cells (Fig. 3D). These observations confirmed that 6-OHDA induces autophagy in PC12 cells.

2.2. 6-OHDA–induced apoptosis in PC12 cells was increased as autophagy was inhibited

To determine the effect of autophagy on 6-OHDA–induced apoptosis in PC12 cells, we treated PC12 cells with 6-OHDA or both autophagy inhibitor 3-MA and 6-OHDA. The results showed that 6-OHDA increased apoptosis of PC12 cells as shown by shrunken, condensed, or fragmented nuclei, compared with the control (Fig. 4). Interestingly, 6-OHDA–increased apoptosis of PC12 cells was increased after 3-MA treatment. These results suggest that 6-OHDA–induced apoptosis of PC12 cells was increased as autophagy was inhibited.

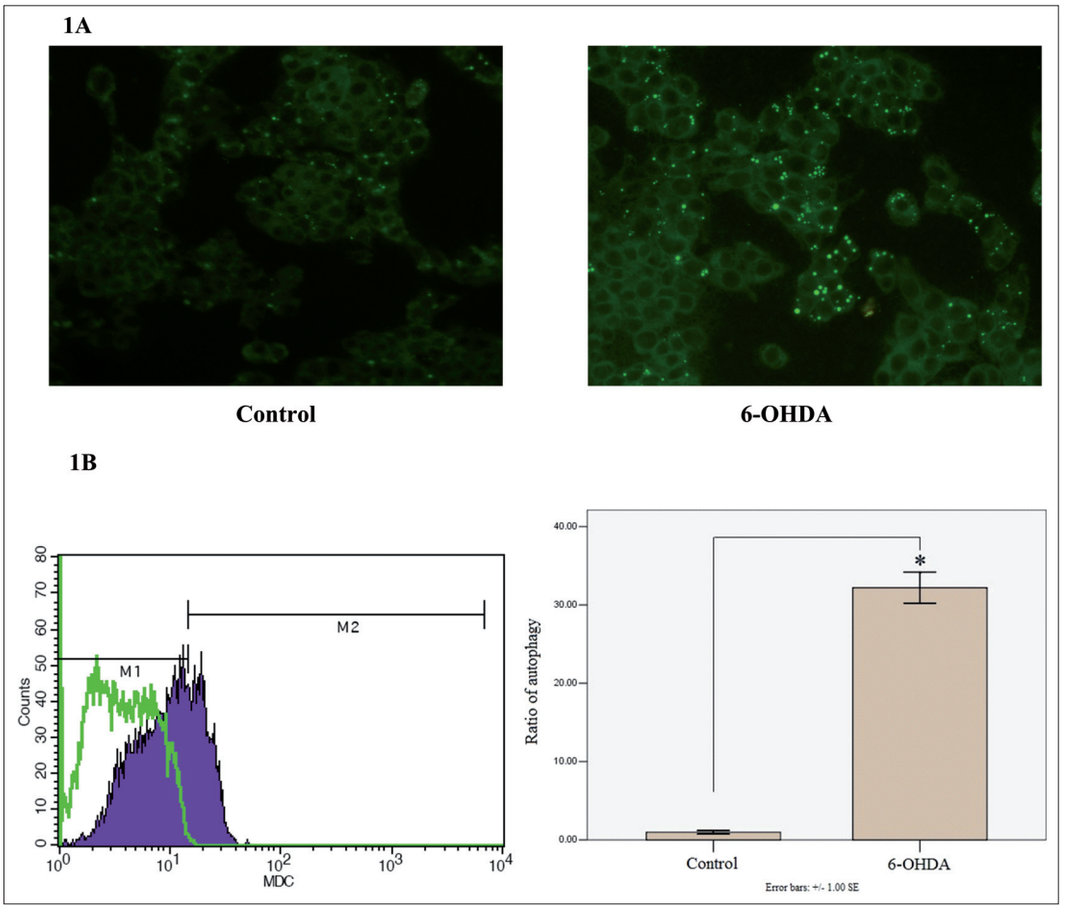

Fig. 1: 6-OHDA induced autophagy in PC12 cells. (A) PC12 cells were treated with 6-OHDA (100 μmol) for 24 h. PC12 cells were stained with MDC and examined under fluorescence microscopy. (B) MDC-positive PC12 cells were counted using flow cytometry. * p<0.01 compared to the control.

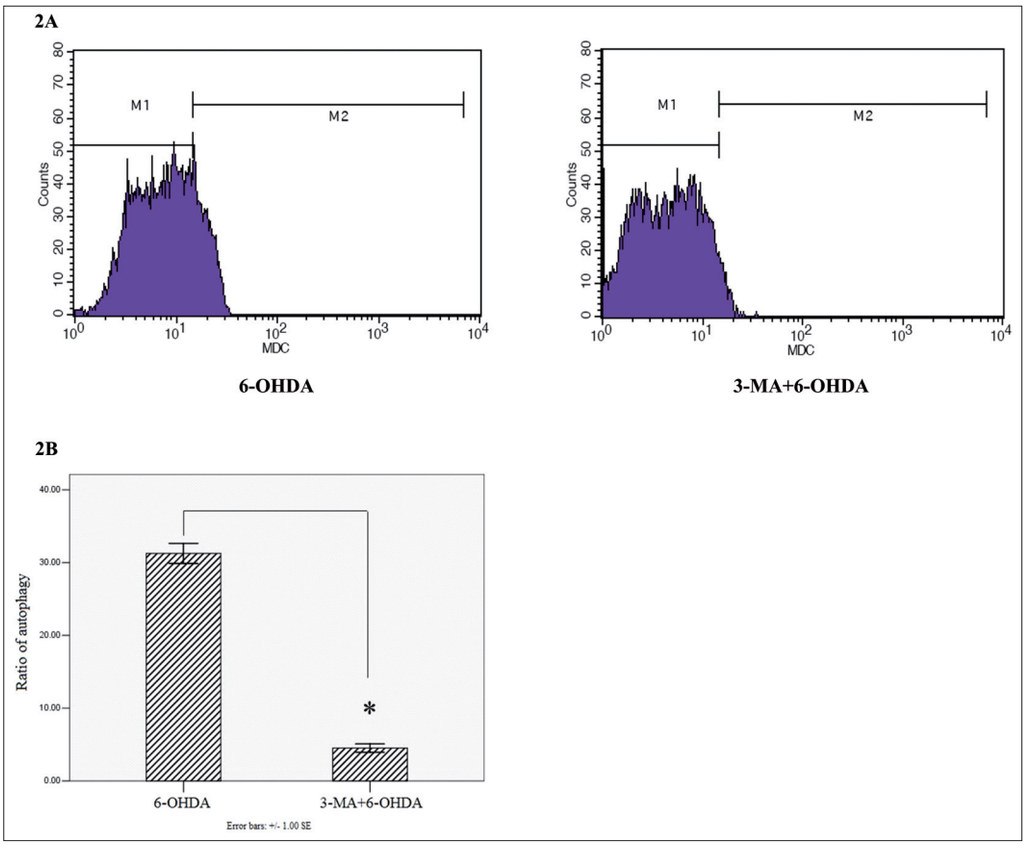

Fig. 2: 3-MA inhibited 6-OHDA–induced autophagy in PC12 cells. (A) MDC-positive PC12 cells were counted using flow cytometry. PC12 cells were treated with 10 mmol/L 3-MA and/or 6-OHDA (100 µmol). (B) Quantitation of the MDC-positive cells among PC12 cells.

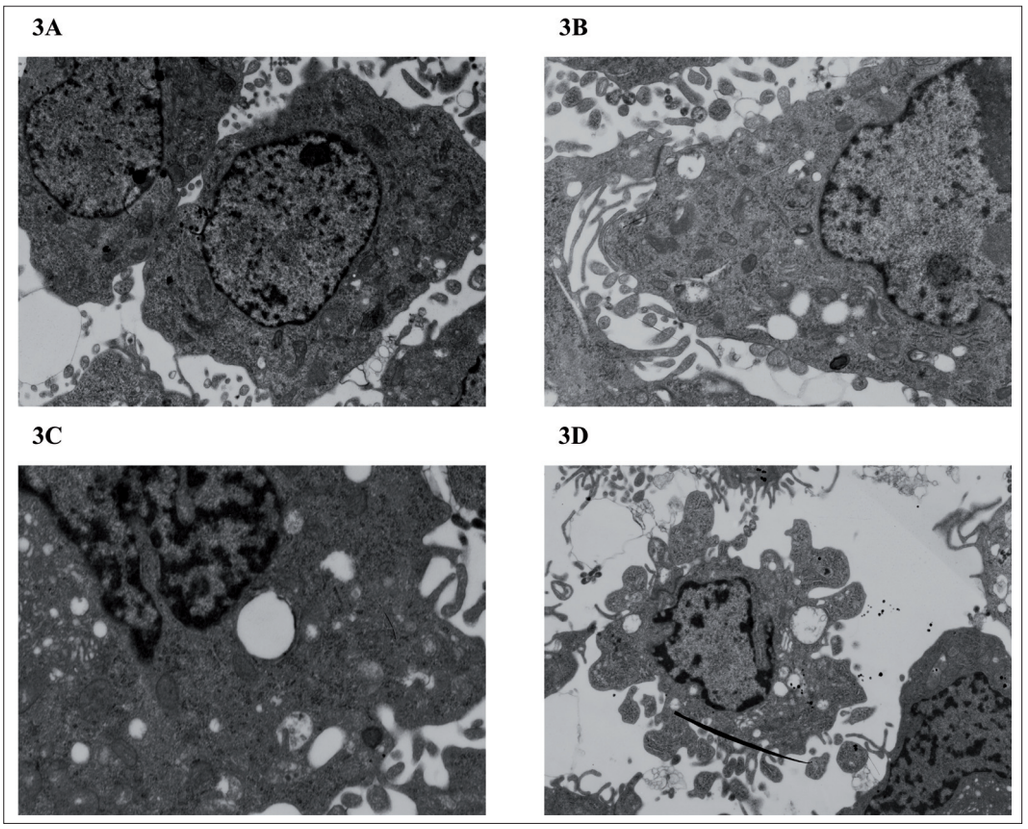

Fig. 3: 6-OHDA induced both autophagy and apoptosis in PC12 cells. PC12 cells were treated with 6-OHDA (100 µmol) for 24 h and examined under electron microscopy. (A) The control, normal PC12 cells. (B, C, D) PC12 cells treated with 6-OHDA (100 µmol). Representive pictures are shown.

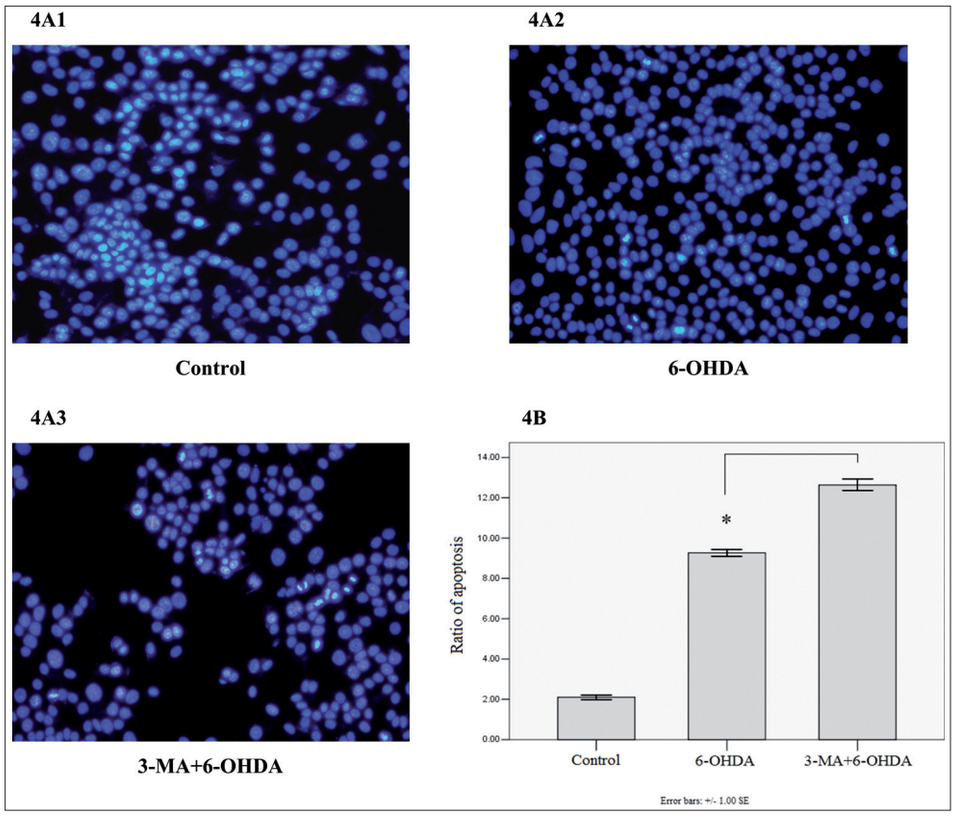

Fig. 4: 3-MA increased cell death and apoptosis in PC12 cells treated with 6-OHDA. (A) PC12 cells were treated with 6-OHDA (100 μmol) for 24 h. Cell death and apoptosis were determined using Hoechst 33258 staining and fluorescent microscopy. (B) Quantitation of the rate of apoptosis after treatment.

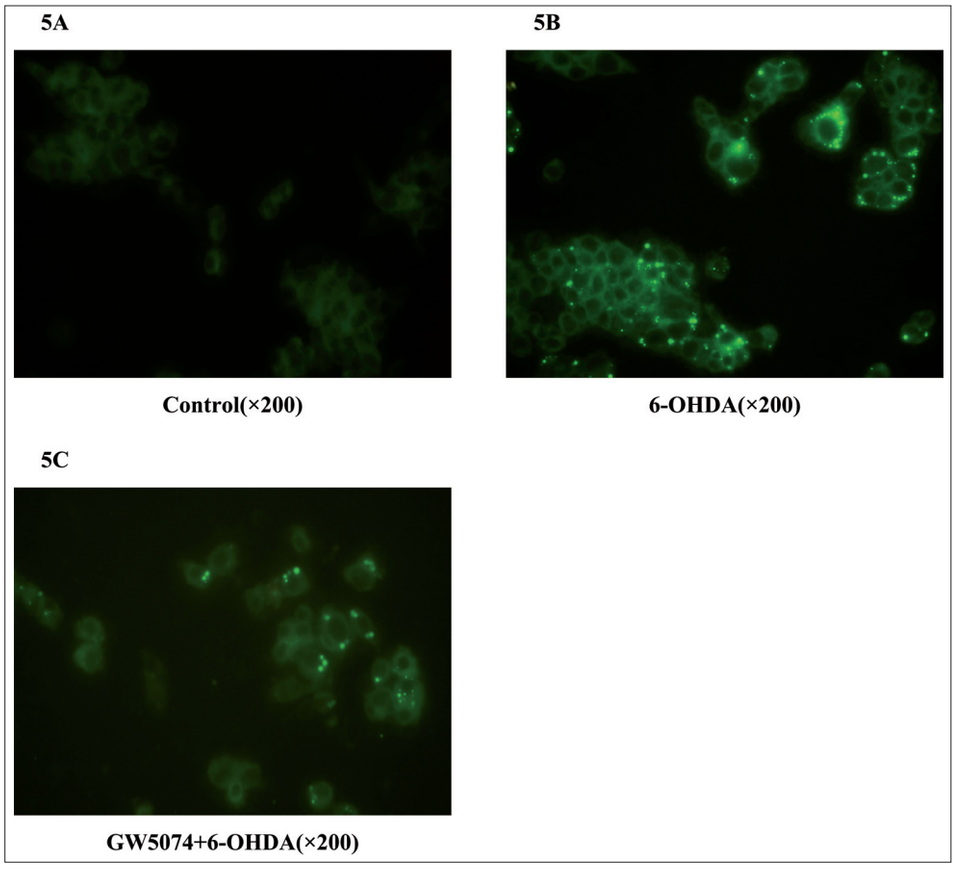

Fig. 5: Raf-1 inhibitor GW5074 inhibited 6-OHDA–induced autophagy in PC12 cells. PC12 cells were treated with 6-OHDA (100 μmol) and/or GW5074 for 24 h. PC12 cells were stained with MDC and examined under fluorescence microscopy.

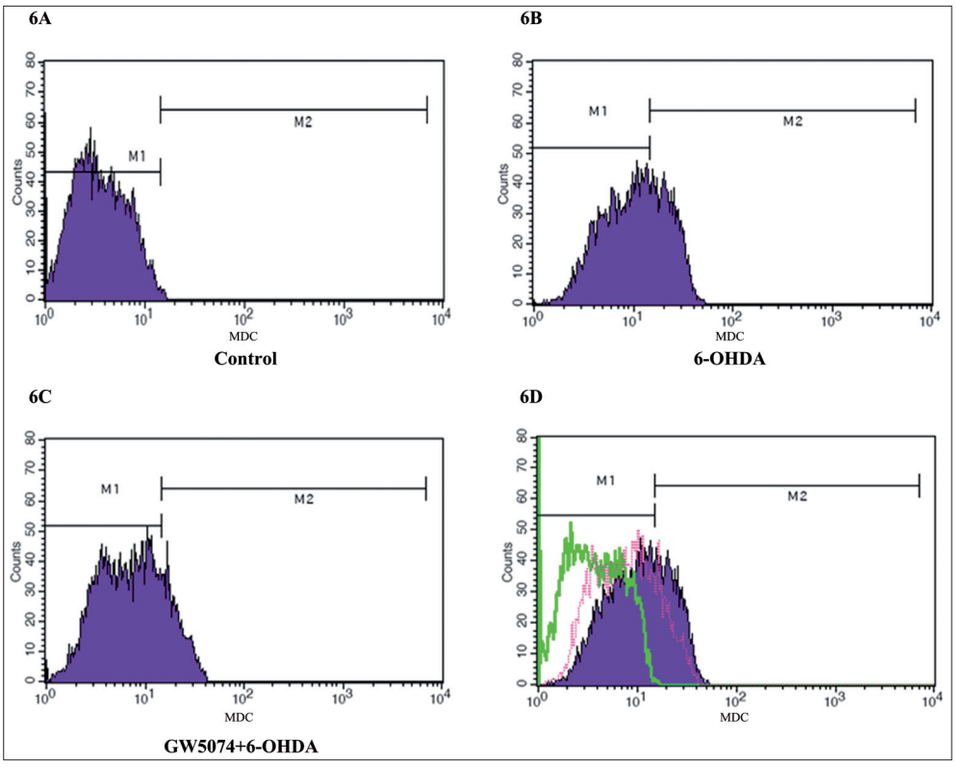

Fig. 6: Quantitation of the inhibiting effect of GW5074 on 6-OHDA–induced autophagy in PC12 cells. PC12 cells were treated with 6-OHDA (100 μmol) and/or GW5074 for 24 h. PC12 cells were stained with MDC and examined by flow cytometry. (A) Control. (B) 6-OHDA, (C) GW5074+6-OHDA, and (D) merged profiles of (A), (B), and (C). Representative data from three replicates were shown.

2.3. Raf-1 activity was required for 6-OHDA–induced autophagy in PC12 cells

To determine the role of Raf-1/ERK in the 6-OHDA–induced autophagy of PC12 cells, we treated PC12 cells with GW5074 which is a Raf-l inhibitor (Li et al. 2012; Jensen et al. 2015; Wang et al. 2004; Su et al. 2014). The results showed that treatment of PC12 cells with GW5074 and 6-OHDA resulted in decreases in autophagy as indicated by reduction in the number of autophagic vacuoles, compared with that resulting from 6-OHDA treatment (Fig. 5). Consistently, treatment of PC12 cells with GW5074 and 6-OHDA resulted in decreases in the density of fluorescence and the number MDC-positive cells, compared with the 6-OHDA treatment (Fig. 6). The percentage of cells undergoing autophagy was decreased from 36.05% to 19.97% (Fig. 6). These results suggest that activation of Raf-1 is required to promote 6-OHDA–induced autophagy in PC12 cells.

2.4. Changes in the phosphorylation of Raf-1 and ERK1/2 in PC12 cells treated with 6-OHDA and 3-MA

To examine the effect of 6-OHDA and 3-MA on phosphorylation of Raf-1 and ERK1/2 in PC12 cells, we determined the levels of Raf-1 and ERK1/2 and their phosphorylated forms in PC12 cells treated with 6-OHDA or a combination of 3-MA and 6-OHDA. The results showed that the levels of total ERK1/2 were not changed in PC12 cells treated with 6-OHDA or the combination of 3-MA and 6-OHDA. The phosphorylated ERK1/2 levels were increased by 6-OHDA, and this increase was compromised by co-treatment with 3-MA (Fig. 7). The levels of total and phosphorylated Raf-1 were also increased by 6-OHDA, but these increases were compromised by co-treatment with 3-MA (Fig. 7). These results suggest that phosphorylation levels of Raf-1 and ERK1/2 were increased in PC12 cells treated with 6-OHDA during autophagy and associated with the autophagy process.

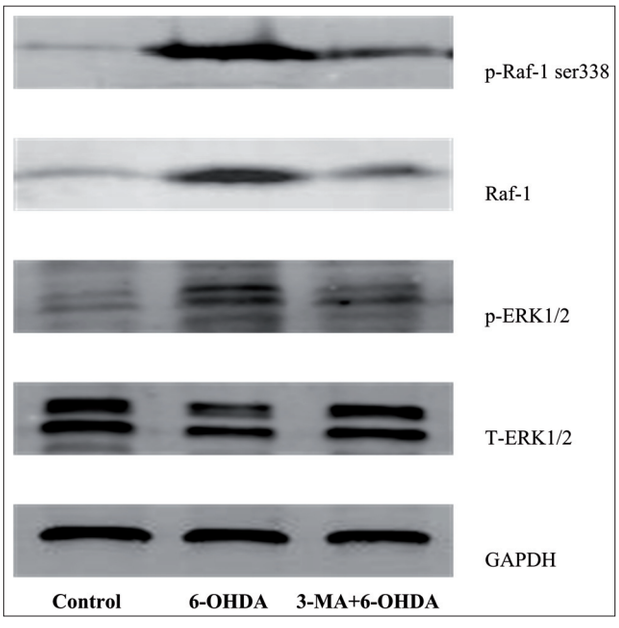

Fig. 7: Changes in the phosphorylation of Raf-1 and ERK1/2 in PC12 cells treated with 3-MA and 6-OHDA. PC12 cells were treated with 10 mmol/L 3-MA and/or 6-OHDA (100 μmol). The Raf-1 and ERK1/2 protein levels and their phosphorylation status were determined using Western blotting. GAPDH was used as a loading control.

3. Discussion

In the current study, we used MDC staining assay, flow cytometry, and electronic microscopy and found that 6-OHDA induces autophagy in PC12 cells. This induction was inhibited by an autophagy inhibitor

3-MA. We also found that apoptosis of PC12 cells is increased as autophagy is inhibited. Inhibition of Raf-1 results in a decrease in the 6-OHDA–induced autophagy rate of PC12 cells. Phosphorylation levels of Raf-1 and ERK1/2 are increased in PC12 cells treated with 6-OHDA during autophagy and associated with the autophagy process. These findings support that 6-OHDA induces both autophagy and apoptosis, and crosstalk between them may be mediated through modulating the phosphorylation of Raf-1 and ERK1/2.

In the current study, we found that the phosphorylation levels of Raf-1 and ERK1/2 were increased in PC12 cells after 6-OHDA treatment and decreased upon co-treatment with 3-MA and 6-OHDA, revealing that phosphorylation of Raf-1 and ERK1/2 is associated with the autophagy process in PC12 cells. This was confirmed by the observation that the Raf-1 inhibitor GW5074 downregulated the autophagy rate. Consistently, during autophagy in the human colon cancer cell line HT-29 cells, galpha-interacting protein (GAIP) accelerates hydrolysis of GTP and facilitates autophagy. GAIP is regulated by Raf-l/ERK1/2 (Petiot et al. 2000; Pattingre and Codogno 2003). 6-OHDA activates mitochondrial ERK2 activity in SH-SY5Y cells. The activated ERK2 activity plays a key role in mitophagy and autophagic cell death (Dagda et al. 2008). Therefore, phosphorylation of Raf-1 and ERK1/2 is likely associated with autophagy and apoptosis induced by 6-OHDA in PC12 cells.

Here, we found that 6-OHDA induced both autophagy and apoptosis in PC12 cells. Furthermore, inhibition of autophagy resulted in increases in the apoptosis of PC12 cells, as seen by the increases in PC12 cell apoptosis after 3-MA treatment, because 3-MA is a known autophagy inhibitor (Caro et al. 1988; Zhang et al. 2015). This reveals the presence of crosstalk between autophagy and apoptosis in PC12 cells treated with 6-OHDA.

Previous studies have shown that the Ras/Raf/ERK signaling pathway may mediate crosstalk between autophagy and apoptosis (Cagnol and Chambard 2010; Ouyang et al. 2014; Liu et al. 2010; Zeng et al. 2012). The Raf-1 inhibitor GW5074 and the ERK1/2 pathway inhibitor U0126 ameliorated apoptosis in PC12 cells induced by 6-OHDA (Li et al. 2012). In the current study, we also found that the phosphorylation of Raf-1 and ERK1/2 is increased in PC12 cells after 6-OHDA treatment and decreased upon co-treatment with 3-MA and 6-OHDA. The Raf-1 inhibitor GW5074 decreased the autophagy rate among PC12 cells. Therefore, we speculate that Raf-1 may be a mediator for crosstalk between autophagy and apoptosis in PC12 cells treated with 6-OHDA.

In summary, we found that that 6-OHDA induces both autophagy and apoptosis probably through modulating the phosphorylation of Raf-1 and ERK1/2, which may mediate crosstalk between autophagy and apoptosis in PC12 cells. Our findings may provide a clue for deciphering the mechanisms of PD pathogenesis.

4. Experimental

4.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and horse serum were obtained from Invitrogen (Carlsbad, CA, USA). 6-OHDA, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazoliumbromide (MTT), U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene] (ERK1/2 pathway inhibitor), and GW5074 (Raf-1 inhibitor) were all obtained from Sigma-Aldrich Co. (Poole, Dorset, UK). PhosSTOP Phosphatase Inhibitor Cocktail Tablets and Complete Protease Inhibitor Cocktail Tablets were from Roche, Germany. The antibodies to ERK1/2 (L34F12, mouse monoclonal), phospho-ERK1/2 (Thr-202/Tyr-204, rabbit polyclonal), and phospho-Raf-1 (Ser-338, rabbit polyclonal) were purchased from Cell Signaling Tech, Inc. The antibody used to detect phospho-Raf-1 (Ser-259, rabbit polyclonal) was obtained from Santa Cruz Biotechnology, Inc. The apoptosis detection kit with Annexin V-FITC and propidium iodide was purchased from Beyotime Institute of Biotechnology (China). The IRDyeTM800-conjugated anti-rabbit, IRDyeTM700-conjugated anti-mouse IgG second antibodies, and anti-GAPDH mouse monoclonal antibodies were the kind gifts of Dr. Xiangning Meng. Other chemicals were of analytical grade and from standard commercial sources.

4.2. Cell culture and treatment

PC12 cells were cultured in DMEM supplemented with 10% FBS in a humidified atmosphere with 5% CO_2 at 37 °C. Cells in logarithmic growth phase were treated with vehicle, 6-OHDA (100 µmol/L), GW5074 (10 nmol/L)+6-OHDA, or 3-MA (3-methyladenine, 10 mmol/L) +6-OHDA (100 µmol/L). GW5074 and 3-MA were added 1 h before 6-OHDA treatment.

4.3. Detection of autophagy by MDC staining

Autophagic vacuoles (Av) were stained using monodansyleadaverin (MDC, Sigma) using a published approach (Biederbick et al. 1995). In brief, PC12 cells were seeded at a density of $3x10^5$ cells/ml in 6-well plates and cultured overnight. GW5074 was added to the media l h prior to addition of 6-OHDA. After incubation for 24 h, MDC (dissolved in water) was added and incubated at 37 °C for 60 min. Afterwards, 4% paraformaldehyde was added, and the cells incubated for 15 min. After washing with PBS twice, the cells were visualized at 356 nm (emission filter) and 545 nm (monitor filter) under a fluorescence microscope (IX73-U, Olympus, Japan). The density of fluorescence in each separate PC12 cell stained with MDC was determined using flow cytometry at 488 nm. Overall, 10000 cells were analyzed for each treatment.

4.4. Transmission electron microscopy

Cell were collected by centrifugation twice at 1000 rpm for 3 min and then fixed in 2.5% glutaraldehyde. The cells were subjected to acetone dehydration and resin embedding, and then 50-nm sections were cut on a LKB machine (EMUC7, Leica, Germany). The sections were stained with uranium acetate and lead citrate and visualized and documented using a transmission electron microscope (HT7700, Hitachi, Japan).

4.5. Western blotting

PC12 cells were lysed in buffer containing 20mM Tris HCl (PH: 7.5), 1 mM EDTA, 1% NP40, 140 mM NaCl, 50 mM NaF, PhosSTOP Phosphatase Inhibitor, and Complete Protease Inhibitor Cocktail. The concentration of the total protein was determined using a BCA protein assay kit (Beyotime). Each sample containing 70 g protein was loaded and resolved on 10% and 12% SDS-PAGE and then transferred to a PVDF membrane (Millipore). The membranes were blocked in 5% BSA and Tris-buffered saline (TBS) for 1 h and washed with TBS-T (containing 0.1% Tween-20) three times for 5 min each. TBS-T buffer containing appropriate primary antibodies (the ERK1/2 antibody 1:1000, phospho-ERK1/2 antibody 1:1000, phospho-Raf-1 antibody 1:500, GAPDH antibody 1:5000) was added, and the sections were incubated overnight at 4 °C. Membranes were washed with TBS-T three times for 10 min each and incubated with the corresponding IRDye 800– or IRDye 700–labeled IgG secondary antibody in the dark for 40 min at room temperature. After another three washes with TBS-T for 5 min each, the membranes were scanned using an Odyssey infrared imaging system (LI-COR). Quantification of immunoreactive bands was performed using Scion Image software.

4.6. Apoptosis assay using Hoechst 33258 staining

PC12 cells were seeded on sterile coverslips in 6-well plates. After treatment with chemicals such as 6-OHDA, cells were fixed, rinsed three times with PBS, and then stained with 0.5 mg/ml Hoechst 33258 (Beyotime, China) for 5 min in the dark at room temperature. After three rinses with PBS, cells were examined under a fluorescent microscope (IX71, Olympus, Japan). Uniformly stained nuclei were scored as healthy and viable cells. Shrunken, condensed, or fragmented nuclei were scored as apoptotic. The apoptotic rate was calculated by the relative the number of condensed nuclei to the total cell number.

4.7. Statistical analysis

Data were analyzed using SPSS13.0 (SPSS, USA). The Student's t test was used to compare differences between two groups. $P<0.05$ was considered statistical significant.

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