

Programmed cell death 4 blocks autophagy and promotes dopaminergic neuronal injury in Parkinson's disease

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Received August 31, 2023; Accepted November 17, 2023

DOI: 10.3892/etm.2024.12423

Abstract. Dysregulation of autophagy has previously been associated with the formation of toxic proteins, such as α -synuclein, in patients with Parkinson's disease (PD). In addition, it has been indicated that programmed cell death 4 (PDCD4) can inhibit autophagy in certain conditions, such as diabetic nephropathy, atherosclerosis and cardiac hypertrophy. Therefore, the hypothesis that PDCD4 can promote dopaminergic neuron damage through autophagy was proposed. To explore this hypothesis, the present study treated human neuroblastoma SK-N-SH cells with 1-methyl-4-phenylpyridinium (MPP⁺) to establish an *in vitro* model of PD. The potential effects of PDCD4 knockdown on lactate dehydrogenase (LDH) release, cell apoptosis, inflammatory response, oxidative stress and autophagy were then evaluated in this model of PD using an LDH assay kit, flow cytometry, western blotting, ELISA and immunofluorescence. The autophagy inhibitor 3-methyladenine (3-MA) was also applied to treat these cells, and its effects on these aforementioned parameters following PDCD4 knockdown were assessed. MPP⁺ was shown to increase the expression levels of PDCD4 in SK-N-SH cells. PDCD4 knockdown was revealed to suppress LDH release, cell apoptosis, secretion of inflammatory factors and oxidative stress. In addition, PDCD4 knockdown was demonstrated to enhance autophagy in cells treated with MPP⁺. By contrast, 3-MA treatment reversed the aforementioned effects of PDCD4 knockdown on cells, suggesting autophagy to be among the processes regulated by PDCD4 in SK-N-SH cells. The results of the present study suggested the existence of regulatory effects mediated by PDCD4 on autophagy in MPP⁺-induced SK-N-SH cells, offering potential future targets for PD therapy.

Introduction

High-incidence neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease (PD) and Huntington's disease, predominantly affect elderly individuals (1). The United Nations' aging report has predicted that the global population of individuals aged >60 years will reach 2.1 billion by 2050 (2), posing significant challenges for healthcare and social services in managing neurodegenerative diseases. Accumulating evidence has suggested that impaired protein turnover homeostasis and the subsequent accumulation of damaged or aberrantly-modified proteins are common mechanistic causes of these aforementioned neurological diseases (3). The autophagy-lysosome pathway is a cellular degradation process that serves a crucial role in maintaining protein homeostasis under metabolic stress or pathological conditions (4). Dysregulation of autophagy has been associated with the formation of toxic proteins, such as α -synuclein, in patients with PD (5,6), underscoring its potential importance in disease development. Therefore, understanding the signals that can regulate autophagy and intervening in this process may hold promise for disease control.

Programmed cell death 4 (PDCD4) is a tumor suppressor protein that was discovered in 2018, which has been reported to be involved in cell cycle progression and apoptosis (7). Initially studied in cancer, PDCD4 was found to modulate protein synthesis by inhibiting translation via reducing eukaryotic initiation factor 4A helicase activity in HeLa cells (8). In mitogen-stimulated cells, degradation of PDCD4 is required for efficient protein translation, which is a prerequisite for efficient cell proliferation (9). The potential role of PDCD4 in other physiologies, such as vascular remodeling and lipid metabolism, has only begun to be elucidated over recent years (10,11). In addition, in the context of protein synthesis, molecular pathways (such as mTOR, JAK/STAT and PI3k/Akt) involved in tumor cell development tend to overlap significantly with those associated with axonal growth and regeneration processes in the nervous system (12,13). Excluding nervous system malignancies, available research data on the role of PDCD4 in other nervous system diseases remain limited. Existing studies have revealed that PDCD4 knockout can reduce the chronic stress-induced depression-like behavior in mice (14), whereas increased PDCD4 levels *in vitro* can lead to significant reductions in axonal length (15). In addition, PDCD4 knockdown has been observed to reduce infarct damage and cortical neuronal apoptosis induced by brain ischemia/reperfusion injury (16).

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Key words: programmed cell death, Parkinson's disease, dopaminergic neuron, autophagy, neurodegeneration

Previous studies have suggested that PDCD4 can inhibit autophagy in certain conditions, such as diabetic nephropathy (17), atherosclerosis (18), and cardiac hypertrophy (19). However, the involvement of PDCD4 in neurodegenerative diseases and autophagy remains unclear. In the context of PD, which is primarily associated with damage and degeneration of the nigrostriatal dopaminergic pathway (20), it is conceivable that PDCD4 promotes dopaminergic neuron damage by inhibiting autophagy. To explore this hypothesis, the present study established an *in vitro* model of PD in SK-N-SH cells using 1-methyl-4-phenylpyridinium (MPP⁺) to investigate the regulatory mechanism of PDCD4 in this context. The results from the present study may offer novel insights and therapeutic targets for future PD treatment.

Materials and methods

Cell culture and treatment. SK-N-SH cells (American Type Culture Collection) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; Cytiva) and treated with 1 mM MPP⁺ (Sigma-Aldrich; Merck KGaA) for 12 h at 37°C to mimic PD *in vitro* (21). Cells were transfected with either of two types of pLKO.1-Neo short-hairpin RNA (shRNA; 100 nM) against PDCD4 or with scrambled shRNA as a negative control (sh-NC; Changsha Aibiwei Biotechnology Co., Ltd.) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h. The target sequences for shRNAs (5'-3') were as follows: sh-PDCD4-1, GCGGTTTGTAGAAGAATGTTT; sh-PDCD4-2, CCTCCATTAACGAAGCTAGAA; and sh-NC, TTCTCCGAACGTGTCACGT. The expression levels of PDCD4 were measured 48 h post-transfection. For mechanistic studies, transfected cells were pre-treated with 5 mM autophagy inhibitor 3-methyladenine (3-MA; Sigma-Aldrich; Merck KGaA) for 30 min at 37°C (22) and then treated with MPP⁺.

Reverse transcription-quantitative PCR (RT-qPCR). SK-N-SH cells were homogenized with TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) on ice. Total RNA was quantified using NanoDrop[®] equipment (NanoDrop; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a SuperScript[™] III RT kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, before 2X SYBR Green PCR Mastermix (Beijing Solarbio Science & Technology Co., Ltd.) was applied for qPCR. The thermocycling conditions used for qPCR were as follows: 94°C for 2 min, followed by 40 cycles of 94°C for 15 sec and 60°C for 30 sec. The relative mRNA expression levels were normalized to β -actin using the $2^{-\Delta\Delta C_q}$ method (23). The primer sequences (5'-3') were as follows: PDCD4, forward AACCTGCAGAA AATGCTGG, reverse CGCCTTTTTCCTTGGCATT; and β -actin, forward CTTGCGGGGCGACGAT and reverse CCA CATAGGAATCCTTCTGACC.

Western blotting. SK-N-SH cells were lysed using RIPA buffer (Beyotime Institute of Biotechnology) on ice, before total protein was quantified using the BCA method. Proteins (25 μ g/lane) were separated by SDS-PAGE using 10 or 12% gels according to molecular weight and electro-transferred onto PDVF membranes. The membranes were blocked in

5% fat-free milk for 2 h at room temperature before being incubated with primary antibodies against PDCD4 (cat. no. 9535S; 1:1,000; Cell Signaling Technology, Inc.), Bcl-2 (cat. no. 3498S; 1:1,000; Cell Signaling Technology, Inc.), Bax (cat. no. 50599-2-Ig; 1:10,000; ProteinTech Group, Inc.), cleaved-caspase3 (cat. no. 25128-1-AP; 1:1,000; ProteinTech Group, Inc.), LC3 (cat. no. 14600-1-AP; 1:2,000; ProteinTech Group, Inc.), p62 (cat. no. 18420-1-AP; 1:20,000; ProteinTech Group, Inc.), Beclin1 (cat. no. 11306-1-AP; 1:5,000; ProteinTech Group, Inc.) and GAPDH (cat. no. 10494-1-AP; 1:20,000; ProteinTech Group, Inc.) at 4°C overnight. The next day, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibodies (cat. no. ab6721; 1:2,000; Abcam) for 2 h at room temperature. The membranes were then developed with a BeyoECL Plus reagent (Beyotime Institute of Biotechnology) before the results were analyzed using ImageJ software (version 1.52; National Institutes of Health).

Lactate dehydrogenase (LDH) activity. SK-N-SH cells were seeded into 96-well plates and grown to 80% confluence. Cells received MPP⁺ stimulation, before the LDH release reagent (cat. no. C0016; Beyotime Institute of Biotechnology) diluted 10-fold was added to the wells and incubated at 37°C for an additional 1 h. Cells without MPP⁺ stimulation were also supplemented with LDH release reagent and served as a maximum enzyme release control. After centrifugation at 400 x g for 5 min at room temperature, the supernatant of each well was collected and the absorbance was measured at 490 nm using a microplate reader (Molecular Devices, LLC). LDH release (%) = absorbance of the experimental group/absorbance of the maximum release control group x100, and the result was normalized to the control group.

Flow cytometry. SK-N-SH cells were subjected to MPP⁺ stimulation, washed twice with PBS and suspended in 1X binding buffer at a density of 1x10⁶/ml. Cells were incubated with 5 μ l Annexin V-FITC working solution for 5 min and 5 μ l propidium iodide working solution for 5 min both at room temperature in the dark. These solutions were contained within the Annexin V-FITC/PI apoptosis detection kit (cat. no. CA1020; Beijing Solarbio Science & Technology Co., Ltd.). Cell apoptosis was then immediately analyzed using a flow cytometer (BD FACSCanto[™]; BD Biosciences) and FlowJo software (version 10.0.7; FlowJo LLC).

ELISA. The levels of inflammatory factors TNF- α (cat. no. PT518), IL-1 β (cat. no. PI305) and IL-6 (cat. no. PI330) were assessed using ELISA kits (Beyotime Institute of Biotechnology) according to the manufacturer's protocols. Cell supernatant was gathered after centrifugation at 300 x g for 5 min at 4°C, before the absorbance was measured using a microplate reader (Molecular Devices, LLC).

Cellular reactive oxygen species (ROS) levels. SK-N-SH cells were incubated with diluted DCFH-DA probe (Nanjing KeyGen Biotech Co., Ltd.) at a final concentration of 10 μ mol/l in the dark at 37°C for 30 min. The cells were then washed twice with PBS and imaged from five fields of view using a fluorescence microscope (Olympus Corporation). The fluorescence intensity was analyzed using ImageJ software (version 1.52; National Institutes of Health).

Oxidative stress parameters. Malondialdehyde (MDA; cat. no. D799761; Sangon Biotech Co., Ltd.) and 4-hydroxynonenal (4-HNE; cat. no. D751041; Sangon Biotech Co., Ltd.) levels, and catalase (CAT; cat. no. BC0200; Beijing Solarbio Science & Technology Co., Ltd.) and superoxidase dismutase (SOD; cat. no. D799593; Sangon Biotech Co., Ltd.) activities were quantified using the corresponding commercial assay kits according to the manufacturers' instructions. Optical density was measured using a microplate reader.

Immunofluorescence (IF). SK-N-SH cells were subjected to MPP⁺ stimulation followed by 4% paraformaldehyde fixation at room temperature for 15 min. These cells were blocked with 5% goat serum (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 1 h, and then incubated with a primary antibody against LC3B (cat. no. ab51520; 1:2,000; Abcam) at 4°C overnight, followed by incubation with FITC-labelled goat anti-rabbit secondary antibody (cat. no. SA00003-2; 1:500; ProteinTech Group, Inc.) for 1 h at room temperature. The nuclei were counterstained with 0.5 µg/ml DAPI for 5 min at room temperature. Cells were finally imaged from five fields of view under a fluorescence microscope (Olympus Corporation). The fluorescence intensity was analyzed using ImageJ software (version 1.52; National Institutes of Health).

Statistical analysis. All experiments were performed at least three times independently. GraphPad Prism 8.0 software (Dotmatics) was used for statistical analysis and data are presented as the mean ± standard deviation. Statistical differences between two groups were assessed using the unpaired Student's t-test, whereas one-way ANOVA followed by Tukey's post hoc test was applied for comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Role of PDCD4 in cytotoxic injury. After SK-N-SH cells were treated with MPP⁺, the mRNA and protein expression levels of PDCD4 were significantly increased (Fig. 1A and B). To explore the role of PDCD4, cells were subjected to PDCD4 knockdown by shRNA transfection. Transfection efficiency was then verified by RT-qPCR and western blotting (Fig. 1C and D). PDCD4 mRNA and protein levels were significantly reduced in the sh-PDCD4-1 and sh-PDCD4-2 groups compared with the sh-NC group. Since transfection efficiency in the sh-PDCD4-2 group was superior, this transfection group was selected for subsequent experiments. This group of transfected cells and cells in the sh-NC group were then treated with MPP⁺, and PDCD4 expression was significantly decreased in the MPP⁺ + sh-PDCD4 group compared with that in the MPP⁺ + sh-NC group (Fig. 1E and F). Subsequently, the effects of MPP⁺ and PDCD4 on cytotoxic injury in each group were evaluated. MPP⁺ caused a significant increase in LDH activity, which was significantly reversed by PDCD4 knockdown (Fig. 2A). The results of flow cytometry (Fig. 2B) showed that MPP⁺ promoted cell apoptosis, which was supported by the western blotting results (Fig. 2C). This was indicated by the significant increase in Bax and cleaved-caspase3 protein expression levels, and the significant decrease in Bcl-2 expression levels (Fig. 2C). After

cells with PDCD4 knockdown were treated with MPP⁺, the level of apoptosis was significantly lower compared with that in the MPP⁺ + sh-NC group, according to both flow cytometry and western blotting results (Fig. 2C and D).

Role of PDCD4 in the inflammation and oxidative stress. The effects of MPP⁺ and PDCD4 on the inflammatory response and oxidative stress of cells in each treatment group were next evaluated. After measuring the levels of inflammatory factors in the cell supernatant, it was found that MPP⁺ significantly increased the levels of TNF-α, IL-1β and IL-6, which was significantly reversed by PDCD4 knockdown compared with those in the MPP⁺ + sh-NC group (Fig. 3A). The results of DCFH-DA probe imaging revealed that MPP⁺ induced a significant increase in ROS levels in the cells, which was also significantly reversed by PDCD4 knockdown (Fig. 3B). In addition, MPP⁺ significantly increased MDA and 4-HNE levels, an effect that was significantly reversed by PDCD4 knockdown. The activities of CAT and SOD were significantly weakened by MPP⁺, but were significantly restored after PDCD4 knockdown (Fig. 3C).

Role of autophagy in PDCD4 regulation. Immunofluorescence revealed that MPP⁺ slightly but significantly promoted the autophagic behavior of cells, which was significantly potentiated by PDCD4 knockdown (Fig. 4A). Similar findings were also reflected in the results of western blotting (Fig. 4B). Specifically, LC3II/LC3I and Beclin1 expression levels were significantly increased, whereas p62 expression was decreased, after MPP⁺ treatment. In addition, these aforementioned alterations were significantly potentiated in the MPP⁺ + sh-PDCD4 group (Fig. 4B).

To explore whether autophagy mediated the regulatory mechanism of PDCD4, cells were treated with 3-MA. Under conditions of MPP⁺ stimulation and PDCD4 knockdown, 3-MA significantly increased the activity of cellular LDH (Fig. 5A) and significantly promoted cell apoptosis compared with those in the MPP⁺ + sh-PDCD4 group, which was reflected in the significant increase in the proportion of apoptotic cells (Fig. 5B). Additionally, 3-MA increased the expression levels of Bax and cleaved-caspase 3 and reduced Bcl-2 levels, corroborating the increase in apoptosis (Fig. 5C). 3-MA treatment was also found to significantly increase the levels of inflammatory factors TNF-α, IL-1β and IL-6 in cells (Fig. 6A). Furthermore, the levels of cellular ROS, as reflected by fluorescence imaging (Fig. 6B), and the levels of oxidative stress indicators MDA and 4-HNE, as detected using respective kits, were all significantly increased in response to 3-MA compared with those in the MPP⁺ + sh-PDCD4 group. By contrast, CAT and SOD activities were significantly decreased after 3-MA treatment compared with those in the MPP⁺ + sh-PDCD4 group (Fig. 6C). These results suggested that 3-MA largely reversed the effects of PDCD4 knockdown.

Discussion

PD is the second most common neurodegenerative disease, which pathologically manifests as the damage and progressive loss of dopaminergic neurons (24). Although current treatment methods, such as medication (Levodopa, monoamine

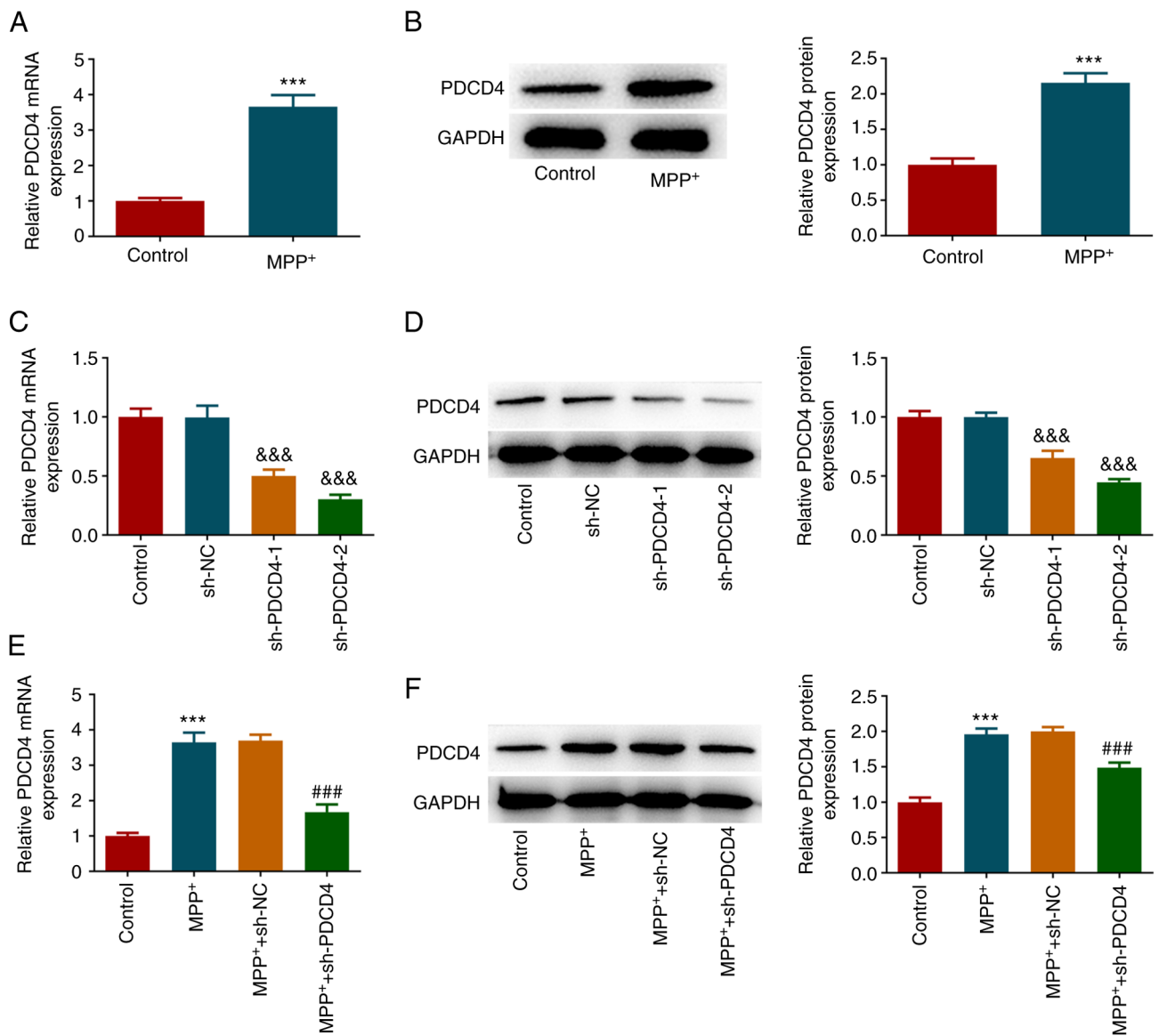


Figure 1. PDCD4 expression after MPP⁺ treatment. SK-N-SH cells were treated with MPP⁺ before PDCD4 (A) mRNA and (B) protein expression levels were determined using reverse transcription-quantitative PCR and western blotting, respectively. PDCD4 (C) mRNA and (D) protein expression levels were determined after transfection with sh-NC or sh-PDCD4. PDCD4 (E) mRNA and (F) protein expression levels were determined following transfection and MPP⁺ treatment. ***P<0.001 vs. control; &&&P<0.001 vs. sh-NC; ###P<0.001 vs. MPP⁺ + sh-NC. MPP⁺, 1-methyl-4-phenylpyridinium; PDCD4, programmed cell death 4; sh, short hairpin; NC, negative control.

oxidase type B inhibitors, dopamine receptor agonists), deep brain stimulation surgery and rehabilitation, can alleviate symptoms in some patients, they cannot halt disease progression (3). Therefore, elucidating the etiology of this disease is critical for the development of effective diagnostics and therapies. Neurodegenerative diseases are marked by the presence of deleterious protein aggregates in the cytoplasm and nucleus, leading to cytotoxicity and neuronal cell death (25). Autophagy is a process that can engulf long-lived proteins and protein aggregates, which are then targeted for lysosomal degradation (26,27). Therefore, autophagy serves a key role in maintaining cytoplasmic homeostasis by clearing damaged proteins and/or organelles (28). Although advances have been made in elucidating the role of the autophagy machinery and how its components are regulated, the mechanism underlying its dysfunction and how it influences PD pathology remains

unclear. The present study found that MPP⁺ increased PDCD4 expression and enhanced the extent of autophagy in cells, suggesting that PDCD4 may be an upstream regulator of the autophagy process in this cell type. Knockdown of PDCD4 expression was found to promote autophagy further, and to reduce cell apoptosis and mitigate oxidative stress. These findings suggested that PDCD4 could be a potential target for autophagy regulation in PD.

In the context of PD, the relationship between PDCD4 and autophagy was assessed in the present study. Similar associations have been previously documented. During atherosclerosis, macrophages exposed to low-density fatty acids have been reported to trigger autophagy at the initial stages of foam cell formation (18). PDCD4 deficiency was also found to enhance lipophagy in macrophages, but to suppress apoptosis and mitochondrial dysfunction. Another previous study demonstrated

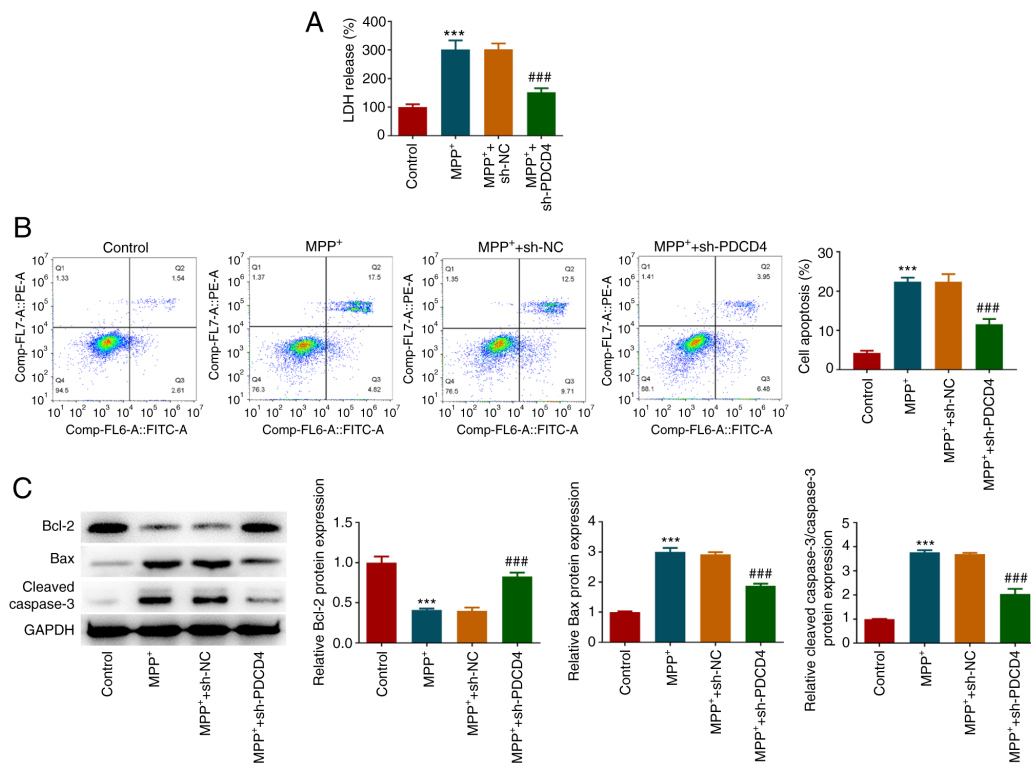


Figure 2. Role of PDCD4 in cytotoxic injury. (A) LDH activity in SK-N-SH cells after transfection and MPP⁺ treatment was measured. (B) Cell apoptosis was assessed using flow cytometry. (C) Expression levels of apoptosis marker proteins were assessed using western blotting. ***P<0.001 vs. control; ###P<0.001 vs. MPP⁺ + sh-NC. LDH, lactate dehydrogenase; MPP⁺, 1-methyl-4-phenylpyridinium; PDCD4, programmed cell death 4; sh, short hairpin; NC, negative control.

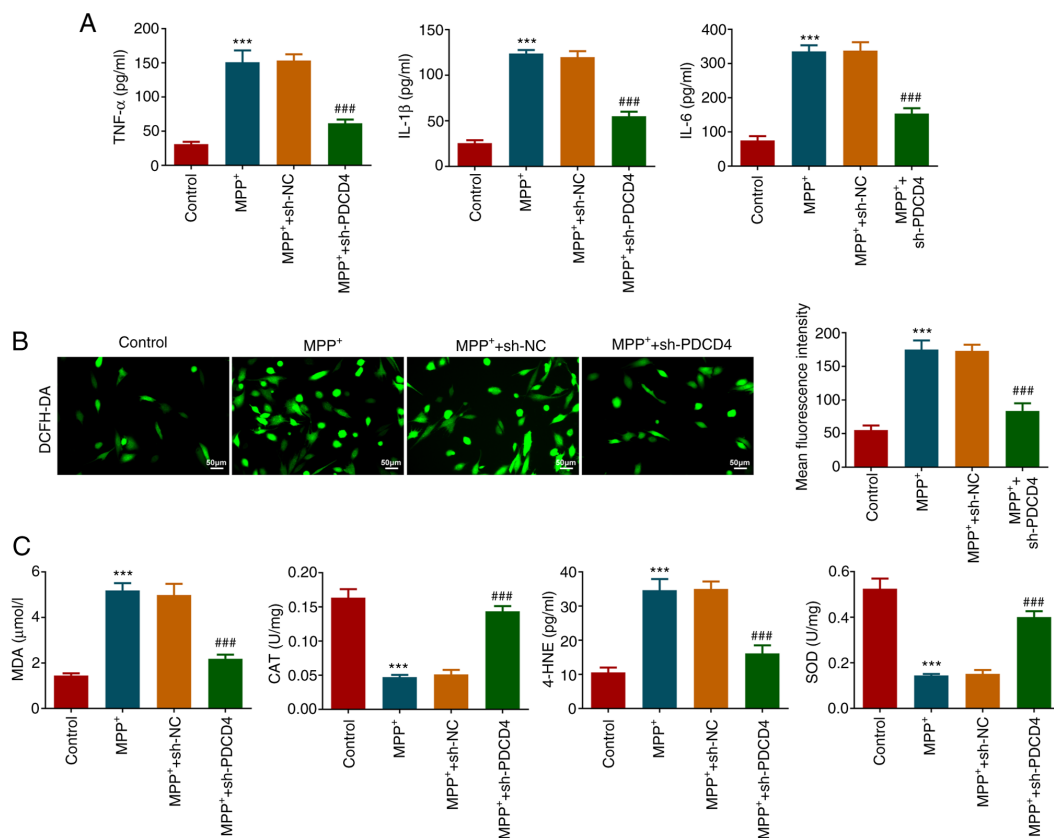


Figure 3. Role of PDCD4 in inflammation and oxidative stress. (A) Levels of inflammatory factors in the cell supernatant were measured by ELISA. (B) DCFH-DA probe was used to measure reactive oxygen species content in cells. Magnification, x200. (C) Levels of MDA and 4-HNE, and activities of CAT and SOD, were used to measure the degree of oxidative stress in cells. ***P<0.001 vs. control; ###P<0.001 vs. MPP⁺ + sh-NC. MPP⁺, 1-methyl-4-phenylpyridinium; PDCD4, programmed cell death 4; sh, short hairpin; NC, negative control; MDA, malondialdehyde; CAT, catalase; 4-HNE, 4-hydroxynonenal; SOD, superoxide dismutase.

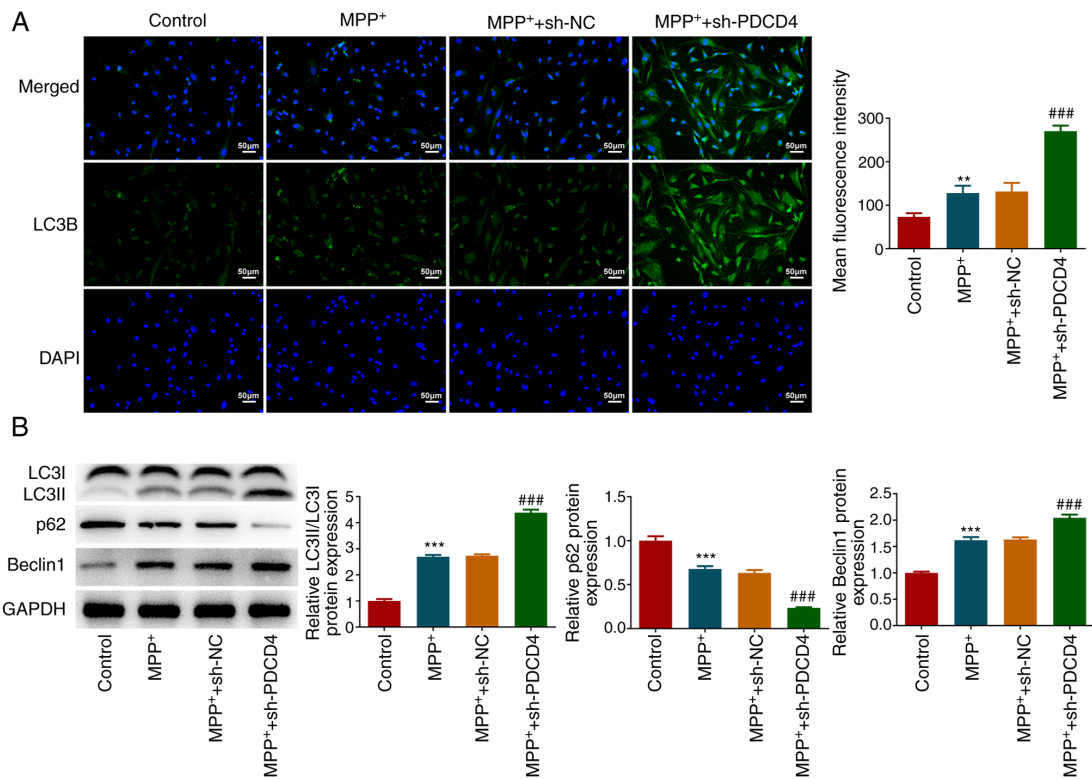


Figure 4. Role of PDCD4 in autophagy. (A) Immunofluorescence of LC3B was used to reflect the extent of autophagy in cells. Magnification, x200. (B) Expression levels of autophagy marker proteins were assessed using western blotting. ** $P < 0.01$, *** $P < 0.001$ vs. control; ### $P < 0.001$ vs. MPP⁺ + sh-NC. MPP⁺, 1-methyl-4-phenylpyridinium; PDCD4, programmed cell death 4; sh, short hairpin; NC, negative control.

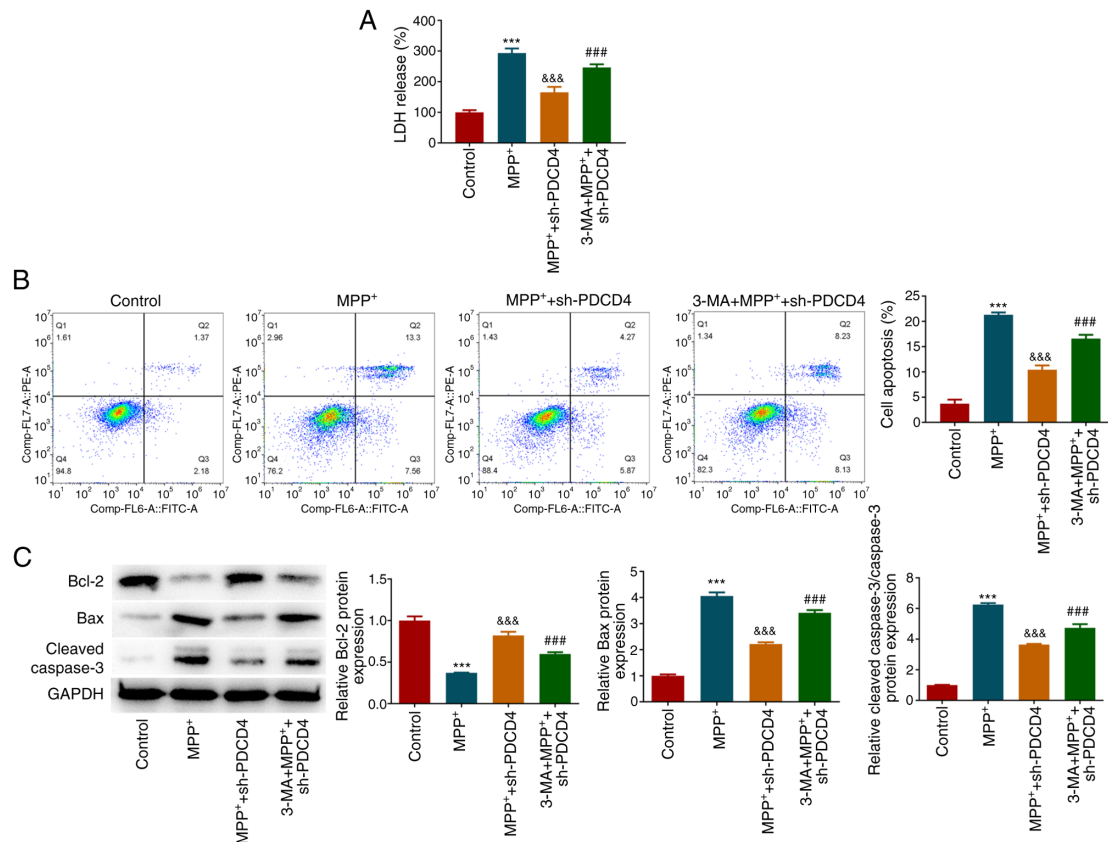


Figure 5. Role of autophagy in cytotoxic injury. To explore whether autophagy mediated the regulatory mechanism of PDCD4, cells were treated with 3-MA. (A) Effect of 3-MA on LDH activity in SK-N-SH cells was measured. The effect of 3-MA on cell apoptosis was measured using (B) flow cytometry and (C) western blotting. *** $P < 0.001$ vs. control; &&& $P < 0.001$ vs. MPP⁺; ### $P < 0.001$ vs. MPP⁺ + sh-PDCD4. 3-MA, 3-methyladenine; MPP⁺, 1-methyl-4-phenylpyridinium; PDCD4, programmed cell death 4; sh, short hairpin.

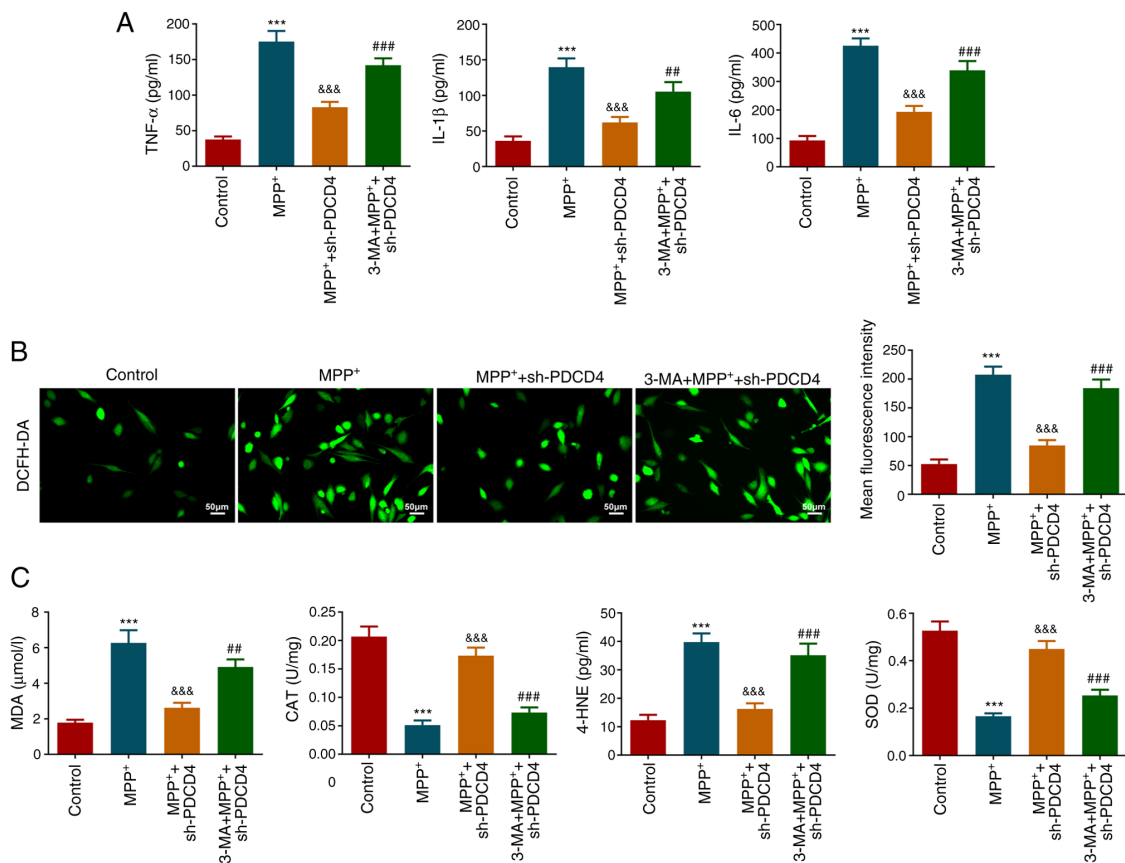


Figure 6. Role of autophagy in inflammation and oxidative stress. (A) Effect of 3-MA on the levels of inflammatory factors was measured. (B) Effect of 3-MA on the level of reactive oxygen species production was assessed using DCFH-DA probe. Magnification, x200. (C) Levels of MDA and 4-HNE, and activities of CAT and SOD, were measured following 3-MA treatment. *** $P < 0.001$ vs. control; &&& $P < 0.001$ vs. MPP⁺; ## $P < 0.01$ and ### $P < 0.001$ vs. MPP⁺ + sh-PDCD4. 3-MA, 3-methyladenine; MPP⁺, 1-methyl-4-phenylpyridinium; PDCD4, programmed cell death 4; sh, short hairpin; MDA, malondialdehyde; CAT, catalase; 4-HNE, 4-Hydroxynonenal; SOD, superoxide dismutase.

that PDCD4 can reduce the expression of autophagy-related (ATG) 5 and hence formation of the ATG12/ATG5 complex in human ovarian cancer Skov3 cells, thereby exerting a negative regulatory function on autophagy (29). In addition, in mouse neuro-inflammation models, PDCD4 has been found to potentially serve as a hub regulatory molecule, simultaneously boosting microglial inflammatory activation and neuronal apoptosis in the central nervous system (30).

To assess the effects of PDCD4 regulatory signaling on autophagy, the present study used 3-MA to inhibit autophagy in cells. Previous studies have used 3-MA to investigate the regulation of autophagy in neurological disease models. Autophagy has been observed to be impaired in the hippocampus of rats with doxorubicin-induced neuronal injury, but 3-MA treatment resulted in persistent oxidative stress and neuronal apoptosis (31). In a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced PD mouse model, 3-MA was found to reverse the therapeutic effects of kaempferol on tyrosine hydroxylase-positive neuron injury and neuroinflammation (32). Furthermore, dopaminergic neurons are particularly metabolically active with high mitochondrial energy demands (33). Therefore, they are particularly vulnerable to the insufficient clearance of damaged mitochondria (33). A recent study found that PDCD4 knockdown was able to maintain mitochondrial membrane potential in mouse dopaminergic neuronal MN9D cells (34).

In summary, to the best of our knowledge, the present study was the first to reveal the regulatory effects of PDCD4 on autophagy in MPP⁺-induced SK-N-SH cells, offering potential targets for PD therapy. The results of the present study may be used to potentially advance the development of targeted interventions to mitigate the pathophysiology of PD.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GC and PL contributed to the project design and experiments. TK and NL contributed to experiments and formal analysis. All authors read and approved the final manuscript, and confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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