

## Original Article

# Pharmacological manipulations of autophagy modulate paraquat-induced cytotoxicity in PC12 cells

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Received March 3, 2017; Accepted April 9, 2017; Epub June 15, 2017; Published June 30, 2017

**Abstract:** Environmental exposure to paraquat has been reported to be associated with Parkinson's disease (PD). In experimental animal models paraquat reproduces features of PD, however, the exact mechanism of PD-induced neurotoxicity has not been fully established. This study was designed to investigate paraquat-mediated interference with mitochondrial function and autophagy, and determine the impact of the modulation of autophagy flux on paraquat-induced cell toxicity. Rat adrenal pheochromocytoma PC12 cells were treated with paraquat for 24 h to establish a cellular mode of PD induced neurotoxicity. Pre-incubation of PC12 cells with an antioxidant N-acetyl-L-cysteine (NAC) or autophagy modulators rapamycin and chloroquine was conducted to determine the effect of modulation of oxidative status and autophagy flux on paraquat-elicited cytotoxicity. Mitochondrial functions and dynamics were analyzed by measuring oxygen consumption in a high-resolution oxygraph and imaging with a fluorescent mitochondrial dye (MitoTracker). Reactive oxygen species was determined by flow cytometry using fluorescent probe DCFH-DA. Autophagic flux was determined by Western blot analysis of autophagy marker LC3-II as well as p62 expression. It was found that treatment of cells with paraquat caused a concentration-dependent loss of cell viability that was accompanied by a decrease in cell respiration and reduction of polarized mitochondria, which was prevented by pretreatment of cells with NAC. Analysis of autophagy showed that NAC inhibited basic autophagy flux of PC12 cells, as evidenced by a decrease in LC3-II level and an increase in p62 expression. However, this modulation of autophagy by NAC may not be implicated into its cellular protective mechanism over paraquat cytotoxicity as inhibition of autophagy by chloroquine significantly enhanced paraquat induced cytotoxicity. Furthermore, the autophagy inducer rapamycin dramatically decreased paraquat induced cellular toxicity in PC12 cells. The present study demonstrates that basal autophagy plays a protective role in paraquat-induced cell toxicity. Antioxidant NAC confers protective role in paraquat toxicity mainly through maintaining mitochondrial dynamics and function, other than a modulation of autophagy flux.

**Keywords:** Paraquat, mitochondrial respiration, autophagy, LC3-I/II, p62

## Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta. Most PD cases are sporadic, and the etiology of PD remains complex. There is growing agreement among scientists that environmental exposure to neurotoxic agents play a key role in the development and progression of PD [1]. Exposure to pesticides, paraquat (PQ) for instance, has been identified as one of the important environmental factors linking to higher risk of PD incidence. Epidemiologic studies have revealed that both environmental and occupational exposure to PQ is

significantly associated with an increased risk of developing PD [2-5]. In spite of these investigations, a causal relationship between PQ exposure and PD has yet to be established. On the contrary, it remains controversial in relating increased incidence of PD to PQ exposure [6]. While more rigorous epidemiological studies are required to gain further understanding of the relationship between the herbicide PQ exposure and PD, dissecting molecular mechanism of PQ neurotoxicity may unravel more insights with regard to the role of PQ in the development and progression of PD.

It has been well documented that PQ causes selective killing of nigrostriatal dopaminergic

neurons [7, 8], characteristic pathological changes in human PD. Although the mechanism underlying PQ-induced neuronal cell loss remains fully elucidated, numerous studies suggest that mitochondrial impairment is a critical subcellular alteration leading to the cell death caused by PQ. However, it is difficult to precisely define how mitochondrial alterations contribute to the mechanisms of PQ induced cell death as mitochondrial dysfunction lies in the hub of a set of cytotoxic events including increased oxidative stress, decreased bioenergetics and activation of apoptosis, all of which have been reported to be associated with PQ treatment in cells as well as in rodent models [9-12]. PQ-induced mitochondrial impairment may result in increased oxidative stress which in turn causes damage to mitochondria. This reciprocal interplay between mitochondrial dysfunction and oxidative stress makes it complicated to dissect mitochondrial signaling in PQ mediated cells death.

Studies in recent years have demonstrated the importance of autophagy in neurodegenerative disorders including PD. In particular, mitophagy, an autophagic process, which selectively removes damaged or old mitochondria, has been suggested to play an important role in neuroprotection mechanisms, while excessive activation of autophagy is implicated into the mechanism of pathogenic process of PD [13, 14]. It has been well documented that PQ causes impairment of mitochondrial function, however, whether autophagy participates in the removal of dysfunctional mitochondria and therefore play a protective role in the PQ neurotoxicity remains to be investigated.

In the present study, we demonstrate that autophagy plays a role in the PQ-induced mitochondrial impairment in PC12 cells. We found that activation of autophagy enhances cellular resistance to PQ induced cytotoxicity, whereas inhibition of autophagy increased cell toxicity of PD. Antioxidant NAC mediated cell defense against PQ toxicity is acted mainly through maintaining mitochondrial function other than the modulation of autophagy.

### Materials and methods

#### *Chemicals and reagents*

Rapamycin and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St Louis, MO,

USA). Chloroquine diphosphate was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). Paraquat was purchased from Nantong Feiyu Fine Chemical Co., Ltd (Nantong, China).

#### *Cell culture and drug treatment*

The differentiated rat adrenal pheochromocytoma PC12 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml). PC12 cells were seeded in culture plates at a density of  $5.0 \times 10^4$  cells/cm<sup>2</sup> and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Cells were grown for 24 h before exposure to the tested chemicals. Following 2 h pretreatment with NAC, autophagy inhibitor chloroquine or inducer rapamycin, cells were exposed to paraquat in the incubation medium for 24 h. The cells were then subject to the subsequent experiments and assays.

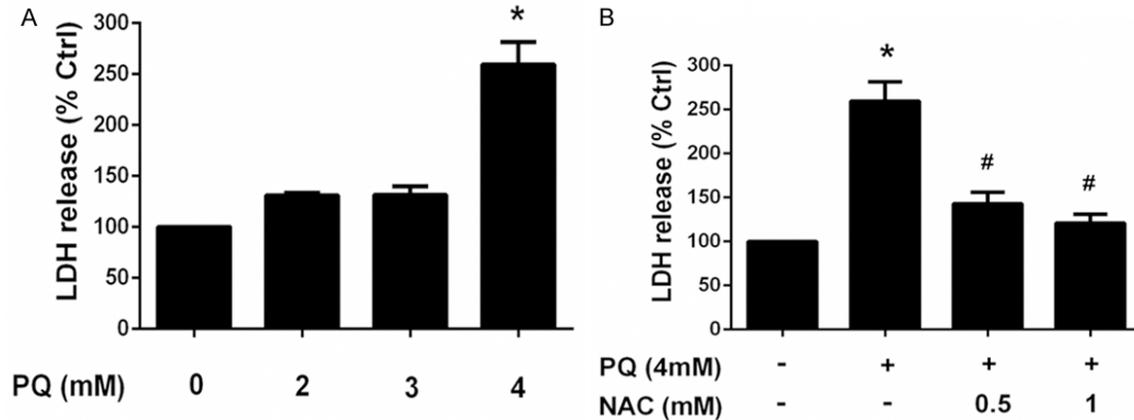
#### *Determination of cell viability of PC12 cells*

Cell viability was determined by measuring lactate dehydrogenase (LDH) release from cells using a LDH assay kit (Beyotime Biotechnology, China) per manufacture's instruction. Briefly, at the end of drug exposure, 10 µl of culture supernatant was mixed with 90 µl of the LDH substrate mixture contained in the assay kit. After incubation for 30 min at room temperature, absorbance was measured at 490 nm. The viability of the cells was expressed as a percentage of the absorbance measured in control cells.

#### *Determination of reactive oxygen species (ROS)*

Intracellular ROS levels were measured using 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA, Beijing Solarbio Science & Technology). Briefly, cells ( $10^5$  cells/well) were seeded onto 6-well culture plate and incubated with the indicated chemicals and drugs. Upon completion of treatment of drugs, the cells were collected, and followed by incubation with serum-free medium containing 10 µM DCF-DA for 20 min at 37°C in the dark. The cells were then washed twice with phosphate buffered saline (PBS), trypsinized, and resuspended. The DCF-DA fluorescence was analyzed using Gallios™ Flow Cytometer (Beckman). The each determination of fluorescence was the mean fluorescence intensity of 10,000 cells.

## Paraquat cytotoxicity



**Figure 1.** Paraquat (PQ)-induced cytotoxicity in PC12 cells. A: Concentration-dependent LDH release caused by paraquat. B: Effect of NAC on paraquat-induced LDH release. PC12 cells were treated with paraquat (0-4 mM) for 24 h. The cells were pre-incubated with NAC where indicated for 2 h before exposure to paraquat. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with paraquat alone.

### Determination of oxygen consumption

Determination of mitochondrial respiration of PC12 cells was performed at 37°C in a high-resolution oxygraph (Oxygraph-2 k Oroboros Instruments, Austria). Measurements of oxygen consumption were performed in a closed chamber at a final concentration of  $2 \times 10^6$  cells/ml with a magnetic stirring. Oxygen flux (pmol  $O_2$ /s/ $10^5$  cells), being directly proportional to oxygen consumption, was recorded continuously using DatLab software.

### Staining of mitochondrial morphology and nuclear staining

Mitochondrial morphology was detected with a mitochondrial selective probe MitoTracker Red CMXRos (Invitrogen). The probe was diluted with serum-free medium (1:1000). After 24 h of paraquat treatment, the living cells were incubated with the staining solution containing MitoTracker probe at 37°C for 30 min. After the staining was complete, the cells were washed with PBS three times, 5 min each. The nuclear staining was then performed with DAPI. The cells were fixed with 4% paraformaldehyde 10 min, then treated with 0.3% Triton for 10 min. DAPI stock solution was diluted with PBS (1:1000). The cells were stained with DAPI working solution for 5 min. The cells were observed under an inverted fluorescence microscope (Olympus IX73).

### Western blotting

After 24 h treatment of the cells with paraquat, cells were washed with PBS buffer and lysed in

RIPA lysis buffer. The whole-cell lysates were centrifuged at 17,000×g for 30 min at 4°C. The protein concentrations of the supernatants were determined using Pierce BCA Protein Assay Kit (Thermo Scientific). The proteins were separated on SDS-polyacrylamide gel, and immunoblotted with primary antibodies (anti-β-actin from Beyotime Biotechnology; anti-Caspase 3, cleaved-caspase 3, and SQS-TM1/P62 from Cell Signaling Technology; and anti-LC3-I/II from Abcam) and with horseradish peroxidase-conjugated secondary antibody for 2 h. The membrane-bound secondary antibody was detected with ECL Western blot detection kit. The band intensities were quantified using Quantity One 1-D analysis software v4.52 (BioRad).

### Statistical analysis

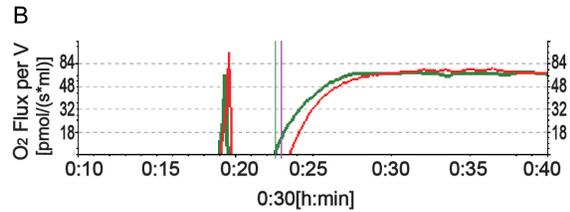
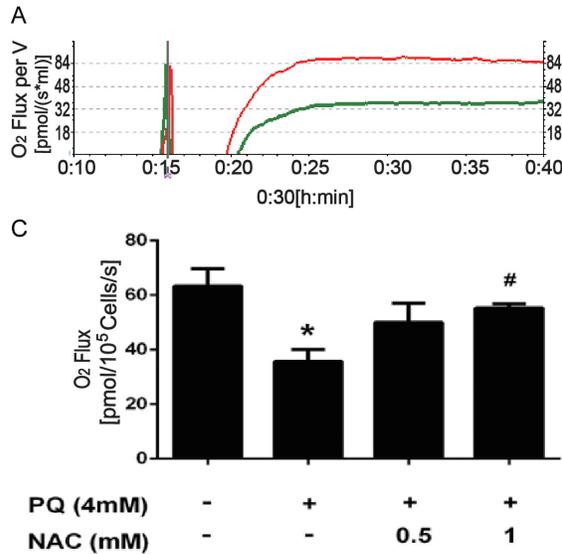
The statistical significance of the difference between three or more groups of individual data was analyzed by one-way analysis of variance and *post hoc* multiple comparisons using Turkey's test. A  $p$  value  $< 0.05$  was considered as statistical significance. Data were expressed as mean  $\pm$  SD of three or more individual experiments.

## Results

### Paraquat-induced cytotoxicity in PC12 cells

PC12 cells were exposed to different concentrations of paraquat for 24 h to establish a cellular model of paraquat toxicity. Results showed a concentration-dependent cytotoxicity with 4

## Paraquat cytotoxicity



**Figure 2.** Effect of paraquat on cellular oxygen consumption. Oxygen consumption of PC12 cells was measured by Oxygraph-2 k high-resolution respirometry. A: A typical graph of high-resolution respirometry of the control (red line) and PQ-treated (green line) cells showing oxygen flux. B: A representative graph of high-resolution respirometry of cells treated with PQ + 0.5 mM NAC (red line) and PQ + 1.0 mM NAC. C: Quantification of oxygen flux. Oxygen flux was expressed as pmol/s/10<sup>5</sup> cells. \**P* < 0.05 compared with control; #*P* < 0.05 compared with paraquat alone.

mM of paraquat causing significant toxicity compared with control, as determined by measuring LDH release, while pretreatment of cells with antioxidant NAC dramatically decreased paraquat-elicited cytotoxicity (**Figure 1**).

### *Paraquat suppresses oxygen consumption and reduces mitochondrial dynamics*

To determine the effect of paraquat on mitochondrial function, oxygen consumption was measured by Oxygraph-2 k high-resolution respirometry. As shown in **Figure 2**, treatment of PC12 cells with paraquat significantly suppressed cellular oxygen flux, which was reversed by pretreatment of cells with NAC, as compared with the control. Further, mitochondrial dynamics was analyzed with fluorescence microscopy using MitoTracker Red CMXRos probe which irreversibly binds to the polarized mitochondrial membrane [15]. Compared with the control, treatment with paraquat profoundly decreased fluorescence intensity, indicating a loss of polarized mitochondria in cells, while pretreatment of cells with NAC markedly reversed fluorescence intensity (**Figure 3**).

### *Increased ROS generation induced by paraquat*

Interference with mitochondrial respiration may result in increased oxidative stress. Therefore, flow cytometry was employed to assess cellular ROS generation using fluorescence probe DCF-DA. PC12 cells treated with paraquat exhibited significantly higher level of ROS

as compared with control cells, while preincubation with NAC decreased paraquat-induced ROS generation (**Figure 4**).

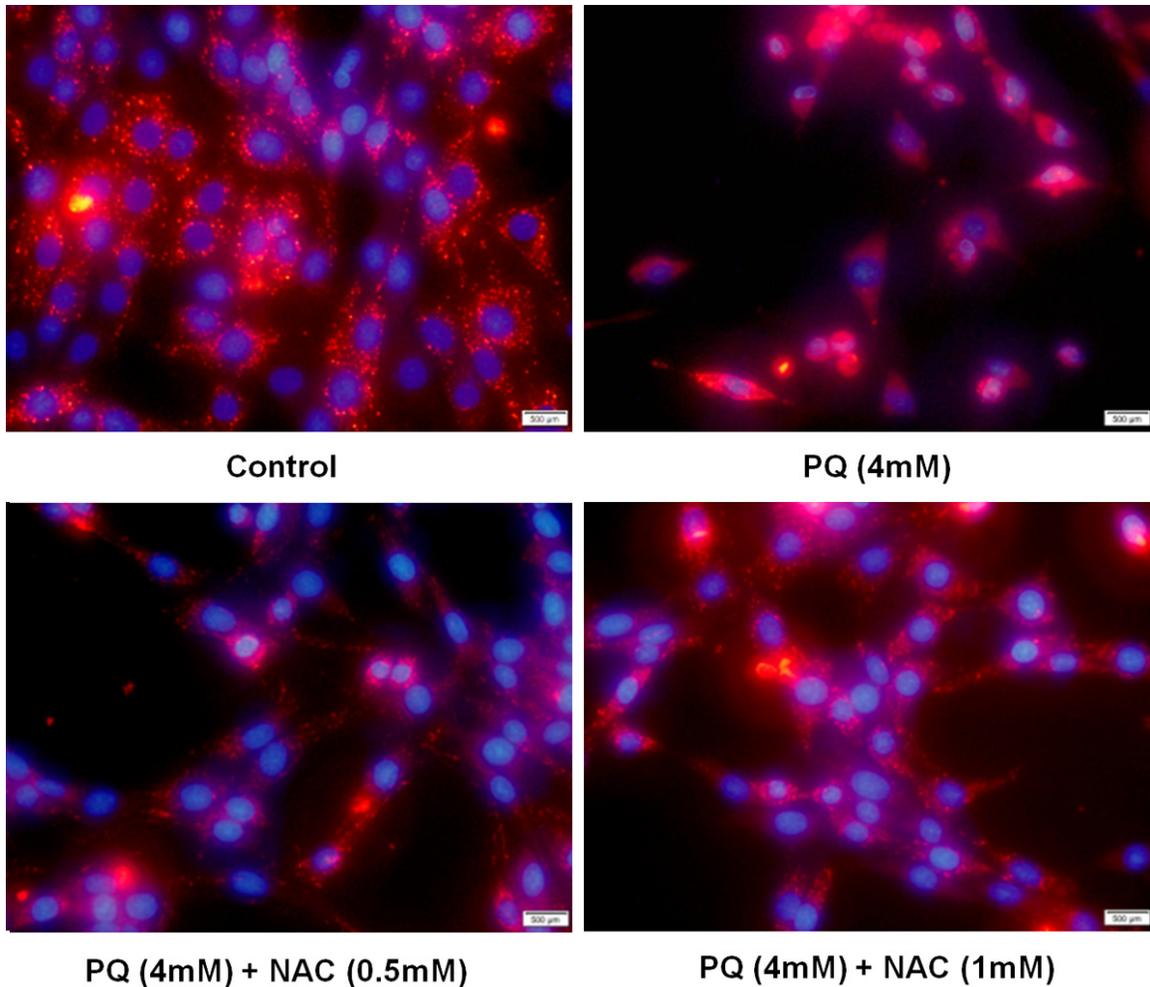
### *Effect of paraquat on autophagy*

Autophagy plays an important role in chemical-induced neurotoxicity. To determine whether autophagy is involved in the mechanism of NAC-mediated protection against paraquat cytotoxicity, autophagy flux was examined in cells treated with paraquat with or without addition of NAC. Treatment with paraquat resulted in higher protein expression of LC3-II, a marker of autophagy, as compared with control. In addition, paraquat treatment also caused significant increase in the protein expression of p62, a ubiquitin-binding scaffold protein, linking ubiquitinated proteins to the autophagic machinery to enable their degradation in the lysosome [16], suggesting an inhibition of autophagy flux by paraquat. Interestingly, pretreatment with NAC further enhanced the expression of p62 while diminished LC3-II expression, as compared with paraquat alone (**Figure 5**), indicating that NAC inhibits autophagy.

### *Modulation of autophagic flux alters sensitivity of PC12 cells to paraquat-elicited cytotoxicity*

To define the role of autophagy in paraquat cytotoxicity, autophagy inhibitor and inducer, chloroquine and rapamycin, respectively, were employed to modulate cellular autophagy flux. Incubation of cells with chloroquine enhanced the protein expression of LC3-II and p62 com-

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**Figure 3.** Effect of paraquat on mitochondrial morphology. Following treatment of cells with PD and NAC, cells were stained with MitoTracker and DAPI as described in the Materials and Methods. Low-level fluorescence of MitoTracker was observed in PQ-treated cells as compared with the control. Addition of NAC reversed PQ-induced decrease in MitoTracker fluorescence intensity.

pared with paraquat alone (**Figure 6**). On the other hand, treatment of cells with rapamycin induced the expression of LC3-II and decreased p62 expression compared with paraquat alone. These results suggest rapamycin induced cellular autophagy, while chloroquine inhibited autophagy in the cells treated with paraquat (**Figure 7**). Assessment of cell toxicity showed that the employment of chloroquine caused increased cytotoxicity of paraquat, while autophagy inducer rapamycin conferred cytoprotective effect on the cells treated with paraquat (**Figure 8**).

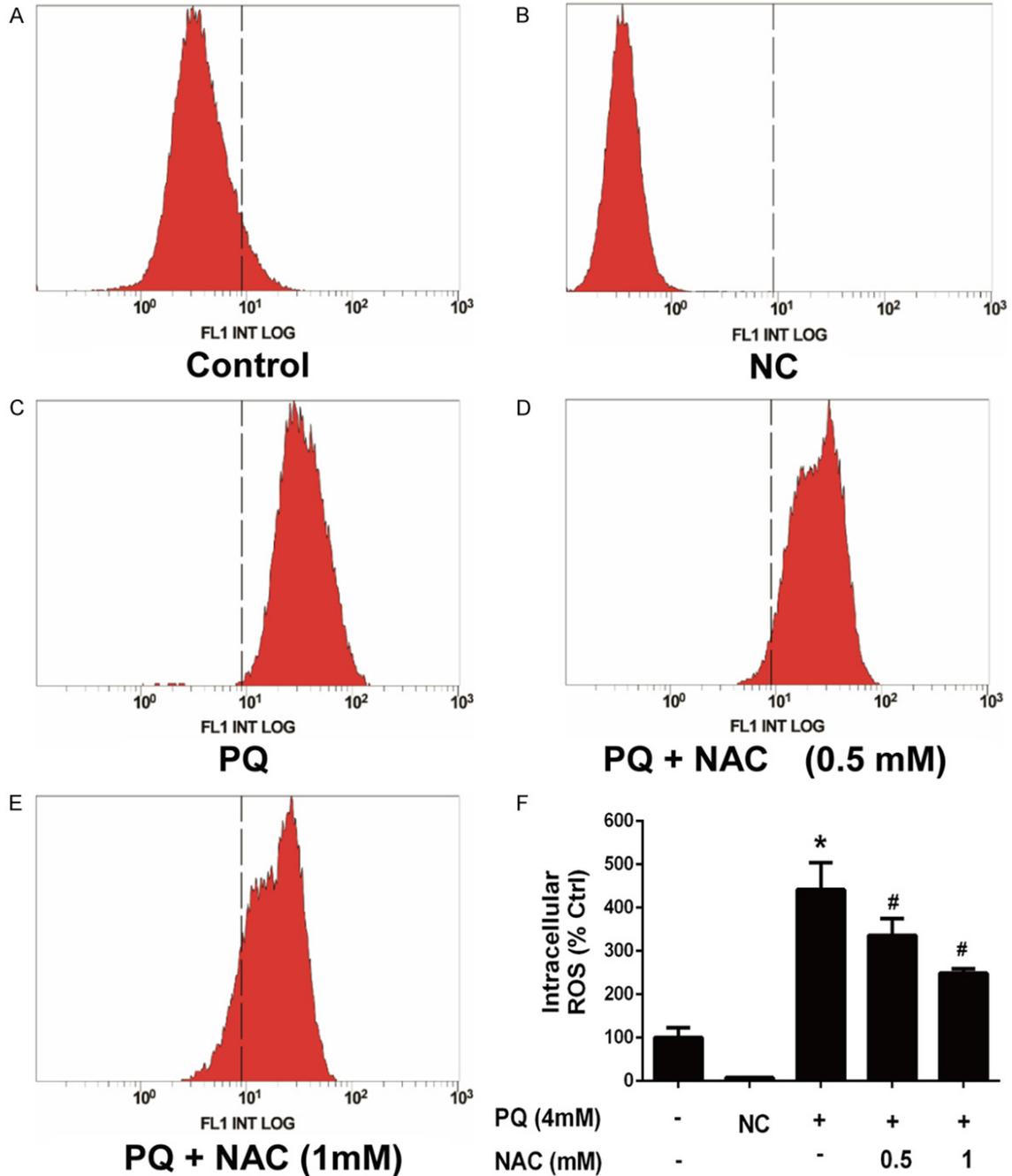
### Discussion

PQ is one of the most widely used herbicide in the world. A large body of epidemiologic studies

has suggested that the exposure to PQ is an environmental factor linking to increased risk of PD [4, 5]. Despite extensive research, the exact mechanism by which PQ causes dopaminergic cell death has not been fully elucidated. In the present study, we have shown that inhibition of basal autophagy is involved in the mechanism of PQ induced cytotoxicity in PC12 cells, and activation of autophagic flux confers cellular protection against PQ cytotoxicity.

Autophagy is a conserved catabolic process in which it maintains cellular homeostasis and protect cells from varying insults by degrading and removing misfolded proteins and other damaged organelles including malfunctioning mitochondria. The increasing evidence indicates that not only are autophagy pathways

## Paraquat cytotoxicity

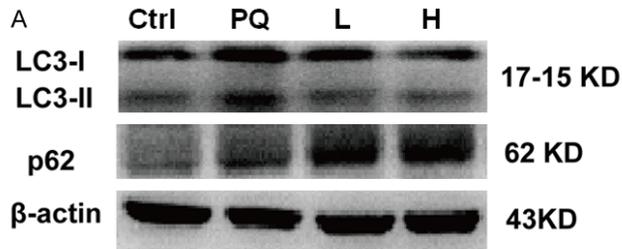


**Figure 4.** PQ increases ROS generation of PC12 cells. Determination of cellular ROS generation was determined by flow cytometry as described in Materials and Methods. A: Control; B: NC, negative control, cells not loaded with the probe; C: PQ, cells treated with 4 mM paraquat; D and E: Cells treated with 4 mM PQ plus pretreatment with 0.5 mM, or 1.0 mM NAC, respectively; F: Quantification of ROS generation. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with paraquat alone.

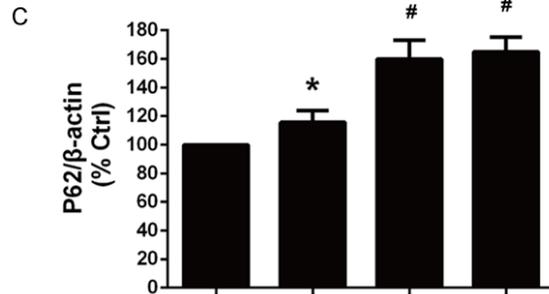
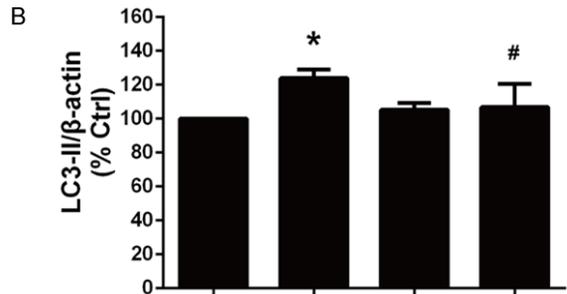
essential for neural function, but they have a direct impact on developmental and neurodegenerative processes of neuronal cells [17]. Yet, it is still unknown what factors determine whether cellular autophagy activation is protec-

tive or pathogenic during neurodegeneration. In agreement with literature that paraquat accumulates in mitochondria and induces superoxide and other ROS generation [18-20], in the present study, treatment of PC12 cells with

## Paraquat cytotoxicity

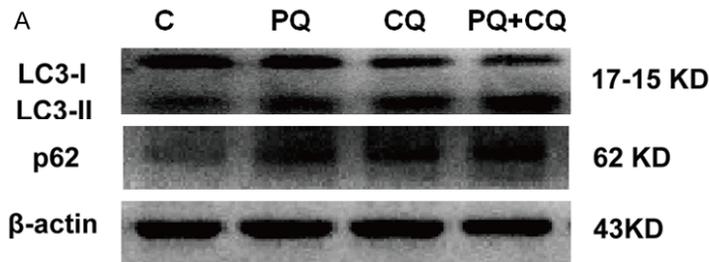


**Figure 5.** Effect of paraquat and NAC on basal autophagy. PC12 cells exposed to 4 mM of PQ for 24 h. Cells were pretreated with 0.5 mM (L) or 1.0 mM (H) NAC before exposure to PQ. A: Representative blots of LC3-I/II, and p62. B, C: Quantitative analysis of LC3-II and p62 expressions in cells treated with PQ with or without pretreatment of NAC. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with paraquat alone.

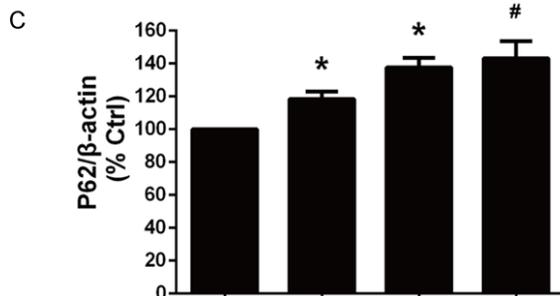
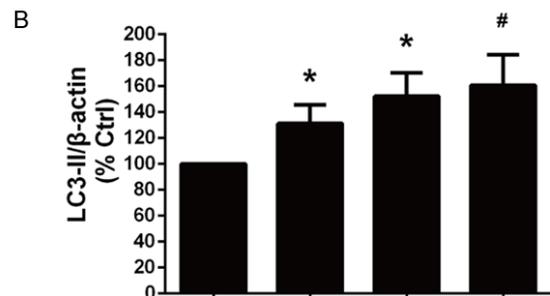


PQ (4mM)	NAC (mM)
-	-
+	-
+	0.5
+	1

PQ (4mM)	NAC (mM)
-	-
+	-
+	0.5
+	1



**Figure 6.** Effect of autophagy inhibitor chloroquine (CQ) on autophagy flux. A: Representative blots of LC3-I/II and p62. B, C: Quantitative analysis of LC3-II and p62 expressions in cells treated with PQ with or without 2 h pre-incubation with CQ. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with paraquat alone.



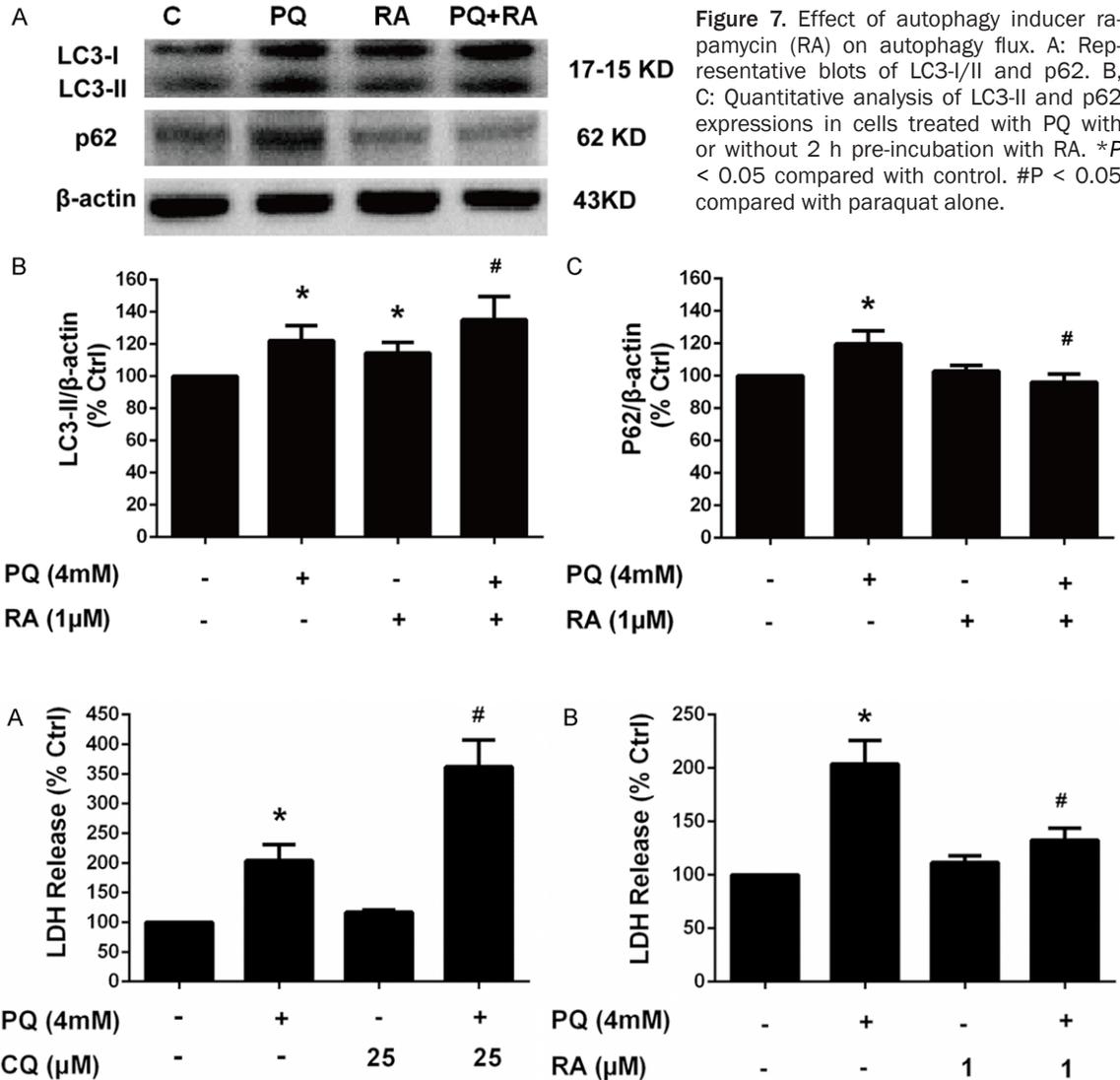
PQ (4mM)	CQ (25 $\mu$ M)
-	-
+	-
-	+
+	+

PQ (4mM)	CQ (25 $\mu$ M)
-	-
+	-
-	+
+	+

paraquat caused a robust increase in ROS generation. In addition, mitochondrial respiration rate was significantly suppressed by paraquat, accompanying PQ cytotoxicity in a concentration-dependent manner. This severe oxidative stress and mitochondrial dysfunction did not activate autophagy, as evidenced by increased expression of p62 protein (Figure 5), a ubiquitin-binding scaffold protein linking ubiquitinated

proteins to the autophagic machinery to enable their degradation in the lysosome [16], indicating an inhibition of autophagic flux. These results suggest that the inhibition of autophagic flux might be involved in the mode of action of paraquat-elicited cytotoxicity. Although the exact mechanism behind paraquat-mediated inhibition of autophagy is not fully investigated, the insufficient clearance of

## Paraquat cytotoxicity



**Figure 8.** Effect of chloroquine and rapamycin on cytotoxicity caused by paraquat. A: Autophagy inhibitor chloroquine enhances PQ-induced cytotoxicity. B: Autophagy inducer rapamycin diminishes cytotoxicity by PQ. \**P* < 0.05 compared with control. #*P* < 0.05 compared with paraquat alone.

dysfunctional mitochondria because of suppressed basal autophagy may contribute to the cytotoxicity caused by paraquat. Accumulation of dysfunctional mitochondria may exacerbate cell injury through different mechanisms such as excess generation of ROS by damaged mitochondria in the cell. The protective role of the activation of autophagy flux in PQ cellular toxicity was confirmed by the utilization of autophagy inducer and inhibitor, rapamycin and chloroquine, which attenuated and increased PQ toxicity, respectively. The inhibition of autophagy interferes with clearance of PQ induced dysfunctional mitochondria, which in turn triggers apoptotic cell death. Indeed, an increased

apoptosis was observed in the cells treated with paraquat (data not shown). On the other side, induction of mitophagy by rapamycin robustly enhanced resistance to PQ induced cell toxicity. These results clearly demonstrate a protective effect of activation of autophagy on PQ cell toxicity.

NAC confers a protection over PQ cytotoxicity, however, it also inhibited cellular autophagy flux as evidenced by decreased expression of LC3-II and increased expression of p62 when compared with PQ alone (Figure 5). We speculate that as an antioxidant NAC may directly act on mitochondria to prevent PQ induced mitochon-

drial damage, which in turns diminished production of damaged mitochondria blocking the trigger of autophagy activation. Indeed, it was found that the expression of LC3-II returned to the control level in the presence of NAC (**Figure 5**). Thus, the NAC-induced protection over paraquat is not achieved through mediating the autophagic flux, rather by serving as an antioxidant and maintaining mitochondrial functions and homeostasis, thus preventing activation of autophagy.

There are some major limitations with this study concerning the relevance of autophagy regulation to the development of PQ induced PD disease. First, the cell model used in the present study may not reflect the mechanism of cellular autophagy activation in an animal model of PD. Secondly, the cell line used in the present study is differentiated rat adrenal pheochromocytoma PC12 cells, thus the phenomenon observed in PC12 cells may be different from a typical neuronal cell. Further studies with primary neuronal cells on the regulation of autophagy activation may yield more insight into the mechanism of PQ cytotoxicity.

In conclusion, our results suggest that inhibition of autophagy is involved in the mechanism of PQ induced cytotoxicity, and the manipulation of autophagic flux modulates cellular sensitivity to PQ induced cell death. Further, antioxidant NAC mediated cell protection acts mainly through maintaining mitochondrial function rather than regulating autophagic flux. Thus, multiple mechanisms may be implicated in the toxicity mechanism of PQ, which requires further investigation to gain the necessary insight to modify these pathways to treat neurodegenerative diseases.

### Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant 81460548).

### Disclosure of conflict of interest

None.

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