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Dendrobium nobile Lindl. alkaloids alleviate Mn-induced neurotoxicity via PINK1/Parkin-mediated mitophagy in PC12 cells

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ABSTRACT

Modern pharmacological studies have demonstrated that *Dendrobium nobile* Lindl. Alkaloids (DNLA), the main active ingredients of *Dendrobium nobile*, is valuable as an anti-aging and neuroprotective herbal medicine. The present study was designed to determine whether DNLA confers protective function over neurotoxicant manganese (Mn)-induced cytotoxicity and the mechanism involved. Our results showed that pretreatment of PC12 cells with DNLA alleviated cell toxicity induced by Mn and improved mitochondrial respiratory capacity and oxidative status. Mn treatment increased apoptotic cell death along with a marked increase in the protein expression of Bax and a decrease in the expression of Bcl-2 protein, all of which were noticeably reversed by DNLA. Furthermore, DNLA significantly abolished the decrease in protein levels of both PINK1 and Parkin, and mitigated the increased expression of autophagy marker LC3-II and accumulation of p62 caused by Mn. These results demonstrate that DNLA inhibits Mn induced cytotoxicity, which may be mediated through modulating PINK1/Parkin-mediated autophagic flux and improving mitochondrial function.

1. Introduction

Epidemiological studies have found that the chronic exposure to high levels of environmental manganese (Mn) causes neurotoxicity and is a potential risk factor for central nervous system disorders [1,2]. Extensive research has shown that the prolonged exposure to environment and occupational Mn causes manganism that is similar to Parkinson's disease (PD) characterized by the death of dopaminergic (DA) neurons [3–5]. The key molecular events underlying the Mn-induced DA neuron death remain not fully elucidated. Due to the complexity of the pathological process of manganism, up to now, no effective therapeutic drugs are available to treat neurotoxicity and significantly improve the clinical symptoms caused by occupational exposure to Mn.

Mitochondria are important cellular targets for Mn neurotoxicity [6,7]. It has been reported that Mn interacts with mitochondria and results in mitochondrial accumulation leading to inhibition of oxidative phosphorylation and production of ROS [8,9]. We recently demonstrated that the dysregulation of autophagy may be a key mechanism involved in the Mn-induced accumulation of ROS and cell injury [10]. In particular, mitophagy selectively eliminates mitochondria by autophagy and plays an important role in controlling mitochondrial quality under physiological and pathological conditions [11]. PINK1 and Parkin are two important proteins related to mitophagy, which coordinates the removal of damaged mitochondria, oxidative damage and some proteins degradation [12]. Following mitochondrial injury, PINK1 stabilizes on the outer membrane of mitochondrial (OMM) and recruits Parkin signal on OMM, allowing it to ubiquitinate mitochondrial proteins and activate mitophagy of PINK1/Parkin pathway preventing the accumulation of defective mitochondria [12]. There is now growing evidence that mitochondrial injury and the defects in PINK1/Parkin regulating mitophagy process are related to Mn-induced neurotoxicity [13,14]. Therefore, modulation of autophagic flux may serve an alternative approach for the intervention and treatment of neurological disorders caused by Mn.

Previous studies have shown that *Dendrobium nobile* Lindl alkaloids (DNLA), effective components extracted from *Dendrobium* DNLA can exert neuroprotective effects, such as improvements of memory and cognitive impairment induced by lipopolysaccharide (LPS) [15,16]. In

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recent years it has been demonstrated that DNLA is able to decrease oxidative stress [17,18] and improve mitochondrial function [19]. There also exists evidence showing the implication of DNLA-mediated activation of autophagy in the mode of action of neuroprotection [20,21]. However, the exact mechanism by which DNLA confers neuroprotection through modulation of autophagic flux remains poorly elucidated. Whether DNLA alleviates Mn-induced neurotoxicity via PINK1/Parkin-mediated mitophagy thus is worthy of full investigation. Therefore, the present study was designed to investigate that DNLA protects Mn induced cytotoxicity by regulating the PINK1/Parkin-mediated mitophagy in PC12 cells, a neuroendocrine cell line that produces neurotransmitter dopamine (DA) and possesses functional DA metabolic pathways.

2. Material and methods

2.1. Reagents

Dendrobium nobile Lindl alkaloids (DNLA) (alkaloids $\geq$ 79.8%) was purchased from Xintian Traditional Chinese Medicine Industry Co., LTD of Guizhou Province. $MnCl_2$ and Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), horse serum (HS), L-glutamine, penicillin-streptomycin and trypsin were purchased from Sigma-Aldrich (St Louis, MO, USA). The fluorescence probe 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). Antibodies for Parkin, Bcl-2, Bax, β-actin, and GAPDH were purchased from Beyotime Biotechnology (Beijing, China). Antibodies for PINK1, p62 and LC3 were purchased from Abcam (Cambridge, UK).

2.2. Cell culture

PC12 cells were cultured in complete DMEM medium supplemented with 10% HS, 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell culture was kept in a 37 °C humidification incubator with 5% CO_2 and 95% O_2.

2.3. Cytotoxicity assay

The cytotoxicity of $MnCl_2$-induced PC12 cells was determined by MTT. Cells were seeded at a density of 2×10^4 cells/well in 24-well plates. Following 24 h, the cells were treated with $MnCl_2$ at different concentrations (0, 100, 200, 300, 400 and 600 μM) for 24 h. An aliquot of 10 μL of MTT agents was added to each well and incubated for another 2 h. The intracellular punctate purple precipitate was dissolved in DMSO. The optical density was read at 570 nm on a microplate reader, and the result was expressed as a percentage survival rate compared with untreated control.

2.4. Measurement of intracellular ROS

Intracellular ROS was determined flow metrically using fluorescent probe DCFH-DA. Cells (1.5×10^5 cells/well) were seeded on 6-well culture plates and pretreated with DNLA (35 and 350 ng/ml, respectively) for 2 h before exposure to 300 μM $MnCl_2$ for 24 h. Upon the completion of the treatment, cells were rinsed with PBS and then incubated with 10 μM DCFH-DA in a serum-free medium in the dark for 30 min. Fluorescent intensity was analyzed using a Beckman flow cytometer. Fluorescence was measured at an average fluorescence strength of 50,000 cells.

2.5. Annexin V/FITC apoptosis assay

The AnnexinV/PI binding assay was used to detect the apoptosis of PC12 cells. Cells at a density of 1.5×10^5 cells/well were seeded in 6-well plates, and then treated with 300 μM $MnCl_2$ for 24 h following pretreatment with 35 and 350 ng/ml DNLA for 2 h. Upon the completion of the treatment, cells were collected, washed twice, and then incubated with Annexin V/PI (Solarbio, China). Apoptotic analysis was carried out using a Beckman flow cytometer.

2.6. Mitochondrial respiration

Mitochondrial respiration was measured by high-resolution oxygraph with DatLab software (Oxygraph-2k Oroboros Instruments, Austria). After the indicated treatment, PC12 cells were collected by 0.5% trypsin digestion and centrifuged by $100 \times g$ for 3 min at room temperature. Then, the suspended particles were resuspended in serum-free DMEM medium for high resolution respiration. Under the stirring of magnetic field, the oxygen consumption was measured in an airtight chamber with a final concentration of 2×10^6 cells/ml. The oxygen flow rate (pmol O_2/s/10^6 cells) was continuously recorded using DatLab software.

2.7. Protein extraction and Western blot analysis

Western blot was performed as previously described [8]. Upon the completion of the treatments, cells were washed with ice-cold PBS and lysed with ice-cold RIPA buffer containing 1% phenylmethanesulfonyl fluoride. The protein concentration of each sample was determined using a Bicinchoninic Acid Protein Assay Kit (Beyotime Institute of Biotechnology, Beijing, China). A total of 30 μg of protein from each group was separated by SDS-PAGE (Invitrogen, Shanghai, China) and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked 2 h with 5% skim milk and incubated overnight at 4 °C with primary antibodies including rabbit polyclonal anti-PINK1 (1:1000), rabbit polyclonal anti-Parkin (1:1000), rabbit polyclonal anti-Bcl2 (1:1000), rabbit polyclonal anti-Bax (1:1000), rabbit monoclonal anti-GAPDH (1:5000) and mouse monoclonal anti-β-actin (1:2000). The membranes were then washed three times for 10 min with TBST and incubated with the corresponding secondary antibodies at room temperature for 60 min. An enhanced chemiluminescence (ECL) (Qihai Biotec, Shanghai, China) kit was sued to visualize the blots. The density of the protein expression was quantified by a ChemiDoc imager (Bio-Rad). β-actin and GAPDH were used as semi-quantitative internal standards.

2.8. Statistical analysis

All data were analyzed using SPSS 19.0 software. The statistical significance of the differences among different groups was analyzed with one-way ANOVA, and post hoc multiple comparisons was conducted by Tukey's test. Data were expressed as the mean ± SD of three or more independent experiments. A value of $p < 0.05$ was considered as statistical significance.

3. Results

3.1. Protective effect of DNLA on $MnCl_2$ induced cytotoxicity in cultured PC12 cells

PC12 cells were incubated with various concentrations of $MnCl_2$ (0, 100, 200, 300, 400 and 600 μM) for 24 h, and then subjected to MTT assay to determine the cell viability. As shown in Fig. 1A, the treatment of cells with $MnCl_2$ resulted in a dose-dependent loss of cell viability. On the basis of this result, 300 μM $MnCl_2$ was chosen to test the protective effect of DNLA on Mn toxicity. Cells were pretreated with DNLA (3.5, 35 and 350 ng/ml, respectively) for 2 h before exposure to $MnCl_2$ (300 μM) for 24 h. Mn exposure decreased cell viability to 60.8 ± 4.8% of control. However, the Mn induced loss in the cell survival was reduced when pretreated with DNLA (350 ng/ml), with a cell viability of 69.6 ± 5.7%,

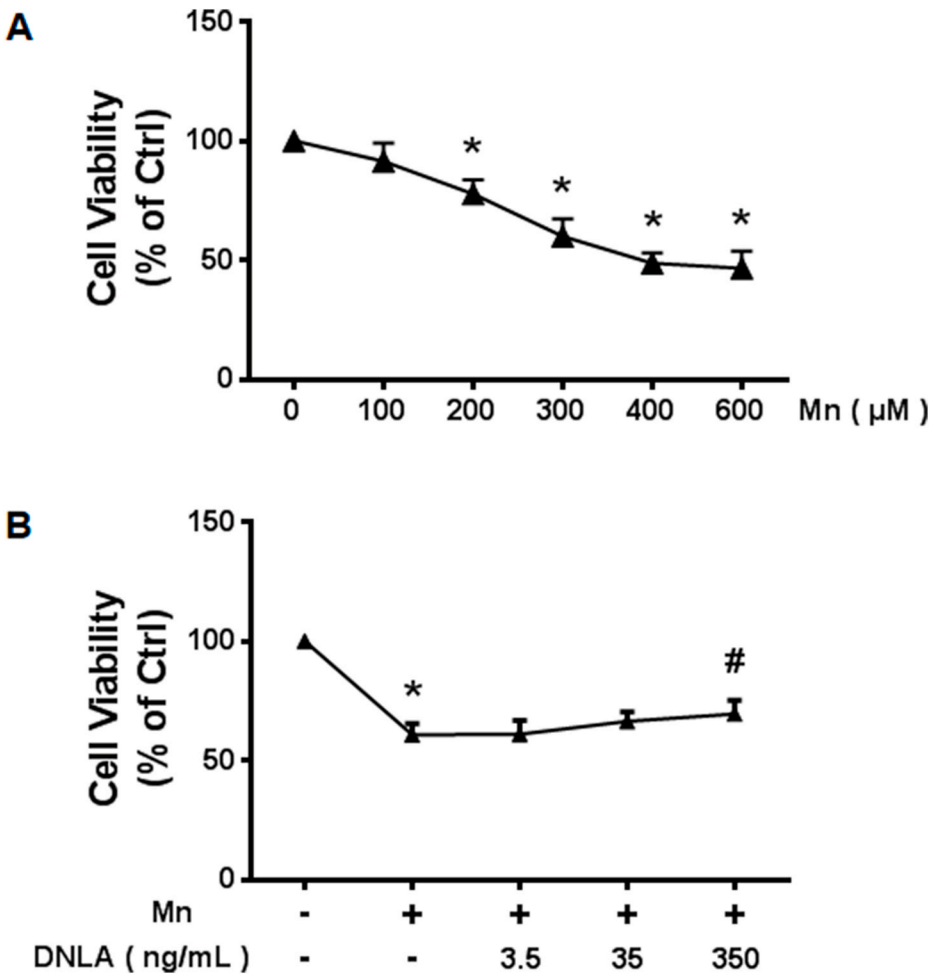

Fig. 1. Protective effect of DNLA on $MnCl_2$-induced cytotoxicity in PC12 cells. The cells were incubated with the indicated chemicals for 24 h, and the relative cell viability was determined using MTT assay. (A) $MnCl_2$ induced cytotoxicity. (B) DNLA-mediated cytoprotective effect on $MnCl_2$-induced cytotoxicity. Values represent mean ± S.D. of 3–5 individual experiments. *$p < 0.05$ compared with control; #$p < 0.05$ compared with Mn alone.

as shown in Fig. 1B.

3.2. Effects of DNLA on cellular ROS generation and mitochondrial respiration

Mn-induced oxidative stress was assessed by flow cytometry using fluorescence probe DCFH-DA. As shown in Fig. 2A and B, the production of ROS in cells treated with 300 μM $MnCl_2$ was increased to about 2-fold of the control, while pretreatment with DNLA 3.5, 35 and 350 ng/ml effectively decreased the production of ROS to 128%, 125.5% and 112.3% of the control, respectively. The cellular oxygen consumption of mitochondria was measured by Oxygraph-2K. As shown in Fig. 2C, the mitochondrial oxygen consumption of normal cells was 43.4 ± 4.6 pmol O_2/s, while the treatment of cells with $MnCl_2$ decreased oxygen consumption to 25.8 ± 3.1 pmol O_2/s. On the contrary, cells pretreated with DNLA 350 ng/ml exhibited an improvement in cellular respiration (31.2 ± 4.7 pmol O_2/s) compared to the cells treated with Mn only.

3.3. Effect of DNLA on apoptosis caused by Mn in PC12 cells

Mn-induced apoptosis was analyzed using flow cytometry with Annexin V-FITC/PI double staining. It was found that DNLA at 35 and 350 ng/ml markedly inhibited $MnCl_2$ induced apoptosis compared to the control group (Fig. 3A and B). The detection of the expression of two apoptosis-related proteins Bax and Bcl-2 showed that $MnCl_2$ treatment caused a marked increase in the expression of Bax, and down-regulated the expression of Bcl-2. In contrast, pre-treatment with DNLA significantly increased the protein level of Bcl-2, and concomitantly decreased the Bax/Bcl-2 ratio (Fig. 3C–E). These results indicated that DNLA has an inhibitory effect on apoptosis.

3.4. Protective effect of DNLA on $MnCl_2$-induced mitophagy by PINK1/parkin pathway in PC12 cells

We next determined protein levels of the two key mitophagy marker proteins, LC3-II and p62 by Western blotting. LC3-II is a marker of autophagy formation, which is associated with autophagy membrane. As shown in Fig. 4, $MnCl_2$ treatment increased the level of LC3-II to about two-fold of the control, while pretreatment with DNLA 350 ng/ml significantly decreased the expression of LC3-II to 1.10-fold of the control. p62 protein is a ubiquitin-binding scaffold protein that binds ubiquitin to autophagy for degradation in lysosomes, and the accumulation of p62 thus indicates damage or inhibition of autophagy flux. After treatment with $MnCl_2$, the p62 expression was increased to 1.42-fold of the control, indicating inhibition of autophagy flux by Mn. However, the p62 protein level was significantly decreased in the presence of DNLA (350 ng/ml). These results demonstrated that DNLA was capable of enhancing autophagosome degradation, suggesting a role in the DNLA-mediated cytoprotective effect over Mn-induced cytotoxicity. We further investigated whether DNLA protects Mn-induced neurotoxicity by activating PINK1/Parkin-mediated mitophagy. After exposure to 300 μM $MnCl_2$ for 24 h, the expression levels of

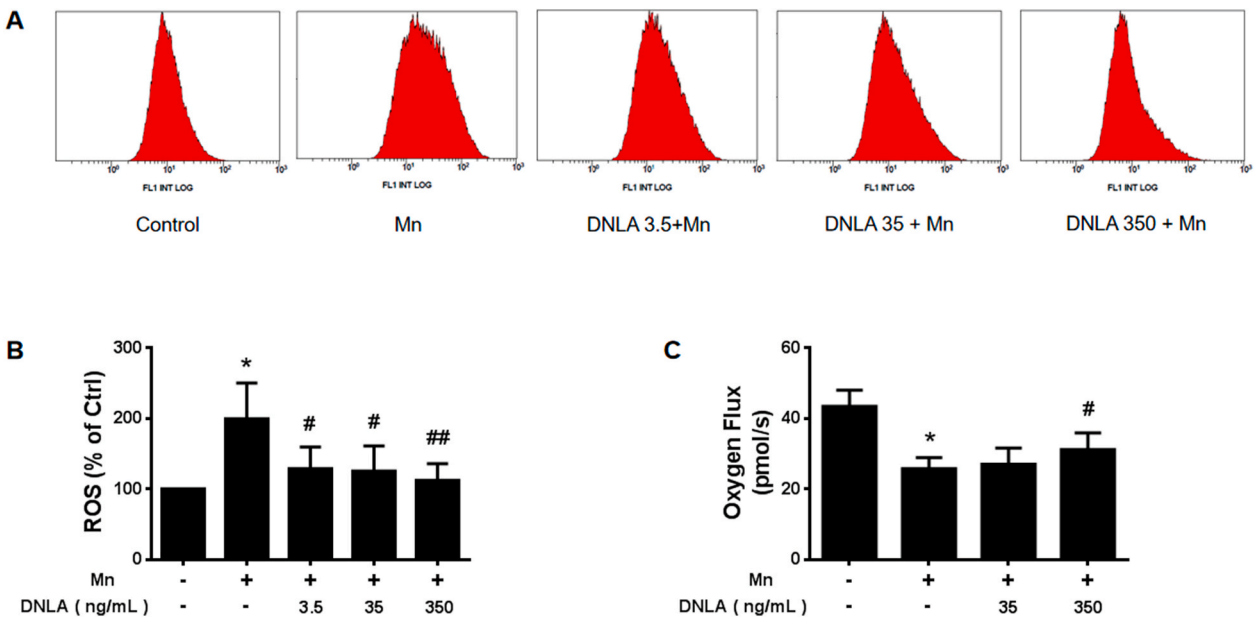

Fig. 2. Effect of DNLA on cellular ROS generation and respiration in $MnCl_2$-treated PC12 cells. (A) Determination of cellular ROS level was carried out by flow cytometry. (B) Quantification of intracellular ROS levels. (C) Oxygen consumption measured by Oxygraph-2k high-resolution oxygraphy. Values were calculated from six independent experiments and presented as mean ± S.D. $*p < 0.01$ as compared with control; $\#p < 0.05$, $\#\#p < 0.01$ as compared with Mn alone, respectively.

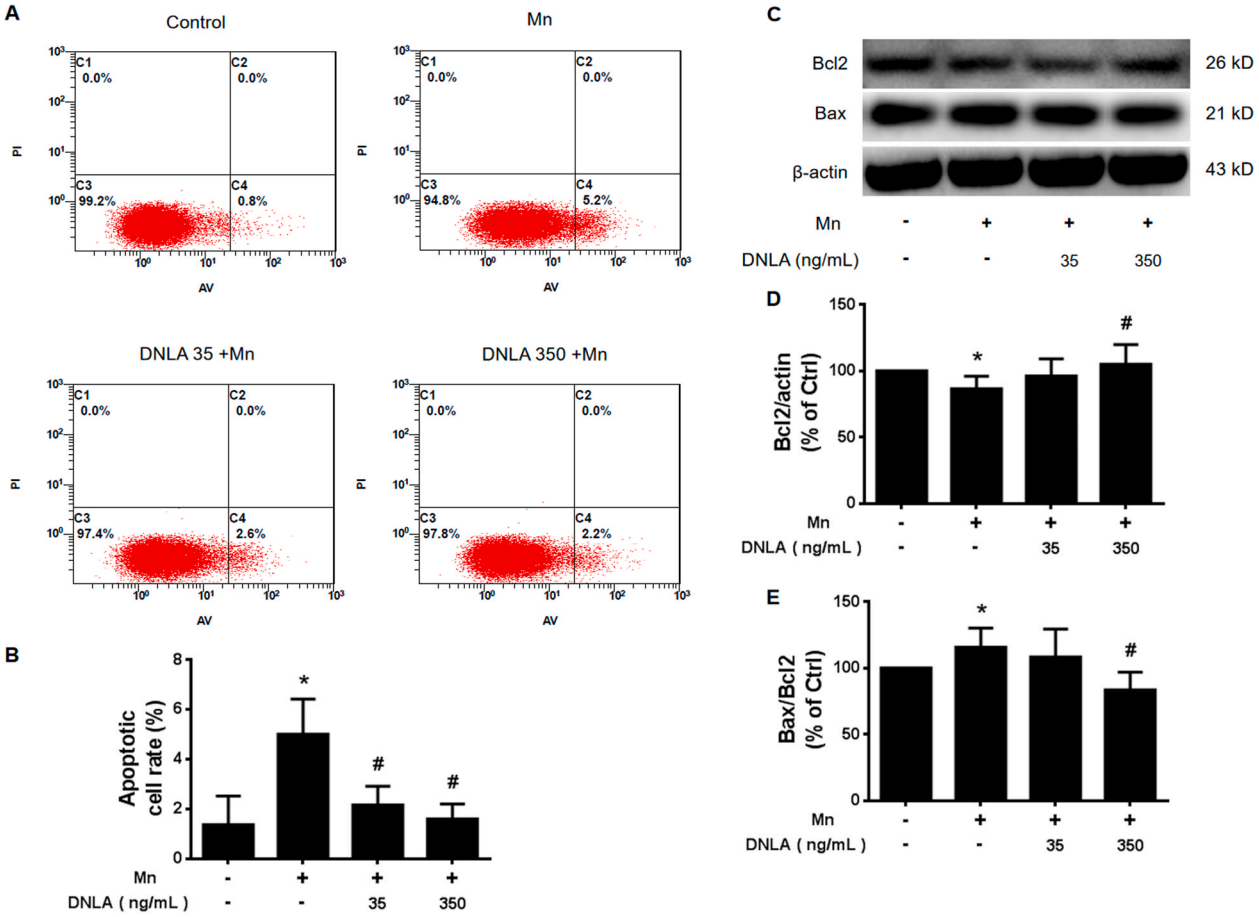

Fig. 3. Effect of DNLA on $MnCl_2$-induced apoptosis in PC12 cells. (A) The apoptotic cells were measured by flow cytometry. (B) Quantitative analysis of apoptosis. (C) Representative Western blots of Bax and Bcl-2. (D and E) Quantification of Bcl-2 expression and Bax/Bcl-2 ratio, respectively. Values represent mean ± S.D. of five individual experiments. $*p < 0.05$ compared with control; $\#p < 0.05$ compared with Mn alone.

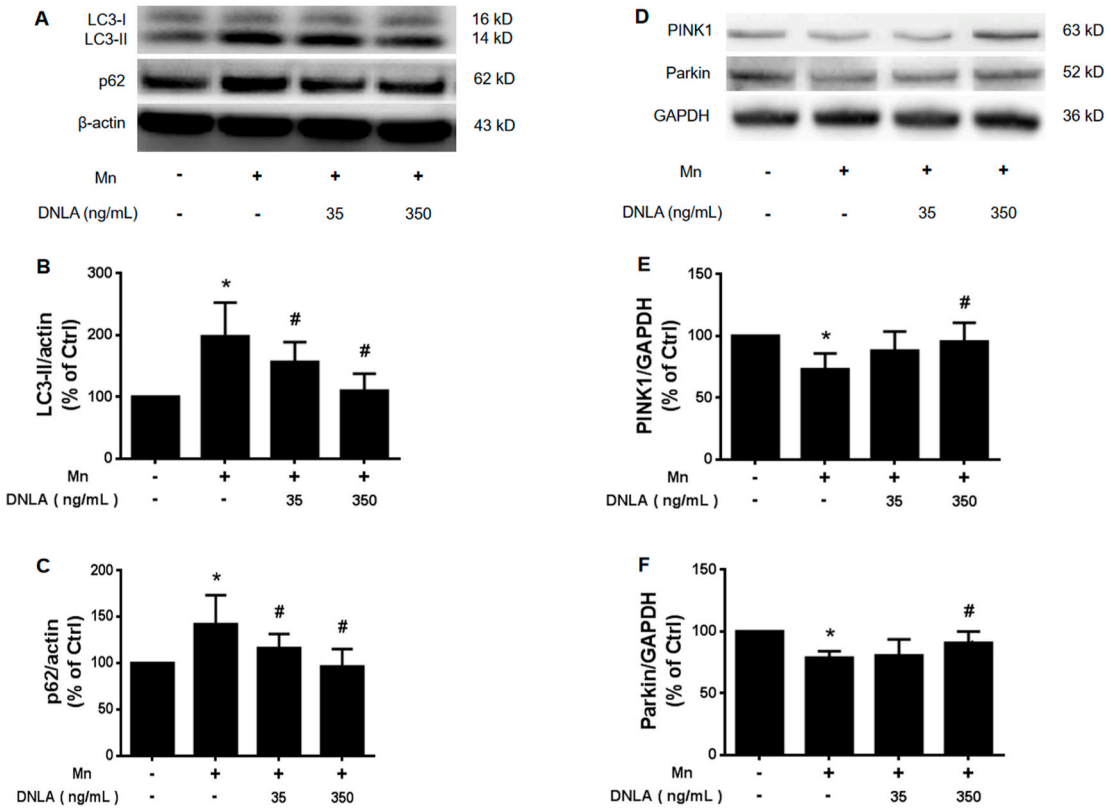

Fig. 4. Expression levels of LC3-II, p62, PINK1 and Parkin proteins in $MnCl_2$-treated PC12 cells. (A) Representative Western blots of LC3-II and p62. (B and C) Quantification of LC3-II and p62protein expression, respectively. (D) Representative Western blots of PINK1 and Parkin. (E and F) Quantification of PINK1 and Parkin protein expression, respectively. Data were calculated from six independent experiments and presented as mean ± S.D. $*p < 0.01$ as compared with control; $\#p < 0.01$ as compared with Mn alone.

PINK1 and Parkin proteins were significantly reduced to 73% and 79% of the control, whereas pretreatment with DNLA 350 ng/ml significantly increased protein expression PINK1 and Parkin to 95% and 91%, respectively, in relative to the control. Taken together, these results suggest that DNLA protects $MnCl_2$-induced neurotoxicity by activating PINK1/parkin pathway.

4. Discussion

The excess Mn exposure remains a risk to human health, and currently the treatment of neurotoxicity is still a challenging issue [22,23]. In the present study, we demonstrated that DNLA confers protective effect on Mn-induced neurotoxicity and revealed a role of the activation of PINK1/Parkin mediated autophagy in the protective mechanism of DNLA.

Mitophagy is an important protective mechanism, which can eliminate damaged mitochondria and protect healthy mitochondrial population. Previous studies have shown that DNLA possesses neuroprotective effects [15,16]. In this study we investigated beneficial effects of DNLA on Mn-induced neurotoxicity through improvement of cellular oxidative stress and mitochondrial function by modulating mitophagy. Our results showed that LC3-II and p62 levels were up-regulated in cells treated with Mn. The increase of LC3-II protein is a marker of elevated autophagosome formation, whereas the increase of p62 protein may indicate a decrease in autophagic turnover [24]. Of note, mitophagy is a dynamic process consisting of autophagosomes formation, phagocytosis of its own substances and fusion with lysosomes, and then degradation [11]. Accordingly, the increase of the number of autophagosomes may be due to an increase in formation or a decrease in degradation of autophagosomes [10]. Thus, autophagy flux is the key to evaluate the integrity of the whole process. The increased expression level of p62 protein suggests that Mn has an inhibitory effect on autophagic flux. It is well known that Mn interferes with mitochondria, leading to the collapse of biological energy and increased oxidative stress, which causes additional damage to mitochondria, affecting cell function [8,9]. Therefore, the prevention or elimination of the accumulation of Mn-induced mitochondrial dysfunction is one of the mechanisms to maintain cellular function under the challenging of Mn. Our results showed that pretreatment of cells with DNLA reduced LC3-II and p62 levels, suggesting that DNLA promotes autophagic flux and enhances autophagosome degradation, thereby ameliorates Mn-elicited toxicity.

It has been established that the PINK1/Parkin pathway is a typical mitophagy regulation mechanism in most mammals [25,26]. In mitochondrial injury, PINK1 accumulates in depolarized mitochondria and recruits Parkin into mitochondria from the cytoplasm as part of the ubiquitin-associated process that selectively removes mitochondria [13,14]. It has been demonstrated that mutations in PINK1 or Parkin lead to mitochondrial dysfunction and may be directly involved in the pathogenesis of PD [13,27]. We hypothesized that DNLA exerts its protective effect on Mn-induced neurotoxicity through modulation of the PINK1/Parkin pathway. Indeed, our results demonstrated that paralleling to the loss of cell survival was the decrease in the expression of PINK1 in PC12 cells, accompanied by an inhibition of mitophagy caused by Mn challenging. On the contrary, the pretreatment of cells with DNLA significantly increased the expression of PINK1 and Parkin proteins, and a resultant increase in cell survival. These results indicated that DNLA activates PINK1/Parkin-mediated mitophagy and thus exerts neuroprotective effect on cytotoxicity induced by Mn.

We also demonstrated in this study that DNLA is able to improve mitochondrial respiration and inhibit Mn-induced apoptosis. Numerous studies have shown that mitochondria are the target of Mn poisoning *in*

vivo and *in vitro* [8]. An important consequence of mitochondrial dysfunction is the increased production of ROS, which leads to oxidative stress and intracellular macromolecular damage, and induces apoptotic cell death [28,29]. Our results showed that Mn caused significant inhibition of oxygen consumption, and a resultant increase in ROS generation accompanied by an induction of apoptosis. However, the presence of DNLA obviously improved the mitochondrial function and inhibited Mn-induced apoptosis. Mechanistically, we showed that this DNLA-mediated inhibition may at least partially be related to the down-regulation of Bcl-2, which needs to be further investigated. It has been well established that there exists an interplay between autophagy and apoptosis. In general, apoptosis-associated caspase activation shuts off the autophagic process, whereas autophagy blocks the induction of apoptosis [30]. Interestingly, we demonstrated that Mn treatment caused inhibition of autophagic flux and the induction of apoptosis, both of which were reversed by pretreatment of cells with DNLA. These results suggest a concerted dual action of DNLA in the protective mode of action on Mn induced neurotoxicity. We recently demonstrated that DNLA inhibits the carbon tetrachloride-induced apoptosis in liver in a Nrf2-dependnet manner [31]. In fact, it has been well described that there exists a link between Nrf2 signaling and autophagy [32,33]. Whether an interaction between Nrf2 signaling and activation of autophagy is involved in the mode of action of DNLA-mediated protection over Mn neurotoxicity has yet to be defined. In this regard, further exploration of the molecular events linking DNLA-modulated regulations of autophagy and apoptosis may be valuable in terms of the potential application of DNLA in neuroprotection.

In conclusion, in the present study we demonstrate that DNLA is capable of improving cellular mitochondrial respiration function and reducing cell injury caused by Mn exposure. The activation of PINK1/Parkin-mediated mitophagy may be an important cellular event by which DNLA executes its cytoprotective mode of action over Mn-induced toxicity. These findings provide a potential therapeutic strategy of DNLA for the intervention of Mn associated neurological disorders.

Declaration of competing interest

The authors declare no competing financial interest.

Author statement

SZ conceived the experiments. XF, SC, XW, YS, RZ, QW, YL and JS performed the experiments. XF, SC and SZ analyzed the data. XF wrote the manuscript, SZ revised the manuscript. All authors read and approved the present manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100877.

Transparency document

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