SC79, a novel Akt activator, protects dopaminergic neuronal cells from MPP+ and rotenone

Jian-liang Zhu[1] · Yu-ying Wu[2] · Di Wu[3] · Wei-Feng Luo[2] · Zhi-qing Zhang[2,3] · Chun-feng Liu[2]

Abstract

In pathogenesis of Parkinson's disease (PD), mitochondrial dysfunction causes substantial reactive oxygen species (ROS) production and oxidative stress, leading to dopaminergic (DA) neuronal cell death. Mitochondrial toxins, including MPP+ (1-methyl-4-phenylpyridinium ion) and rotenone, induce oxidative injury in cultured DA neuronal cells. The current study tested the potential effect of SC79, a first-in-class small-molecule Akt activator, against the process. In SH-SY5Y cells and primary murine DA neurons, SC79 significantly attenuated MPP+- and rotenone-induced viability reduction, cell death, and apoptosis. SC79 activated Akt signaling in DA neuronal cells. Akt inhibition (by LY294002 and MK-2206) or CRISPR-Cas9-mediated Akt1 knockout completely abolished SC79-induced DA neuroprotection against MPP+. Further studies demonstrated that SC79 attenuated MPP+- and rotenone-induced ROS production, mitochondrial depolarization, and lipid peroxidation in SH-SY5Y cells and primary DA neurons. Moreover, upregulation of Nrf2-dependent genes (*HO1* and *NQO1*) and Nrf2 protein stabilization were detected in SC79-treated SH-SY5Y cells and primary DA neurons. Together we show that SC79 protects DA neuronal cells from mitochondrial toxins possibly via activation of Akt-Nrf2 signaling.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder. It is characterized by impaired motor functions, caused by the progressive depletion of dopaminergic (DA) neurons in the substantia nigra (SN), striatum, and putamen [1, 2]. The typical PD symptoms include distal resting tremor, rigidity, and disturbance in balance [1, 2]. The

Jian-liang Zhu, Yu-ying Wu, and Di Wu have contributed equally to this work.

✉ Zhi-qing Zhang
  zhiqing630@163.com

✉ Chun-feng Liu
  liuchunfeng@suda.edu.cn

[1] Department of Emergency and Intensive Care Unit, The Second Affiliated Hospital of Soochow University, Suzhou, China

[2] Department of Neurology, The Second Affiliated Hospital, Institute of Neuroscience, Soochow University, 1055 Sanxiang Road, Suzhou 215004, China

[3] Institute of Neuroscience, Soochow University, Suzhou, China

pathological mechanisms of PD remain unclear [2–4]. Mitochondrial complex I dysfunction and subsequent oxidative stress play a crucial role in DA neuronal cell injury [1, 2].

Mitochondrial toxins are added to cultured DA neuronal cells to mimic PD-associated oxidative injures [5, 6]. Rotenone can rapidly cross the blood–brain barrier and accumulate in the brain, damaging mitochondrial functions to ultimately induce DA neuronal cell death. MPP+ (1-methyl-4-phenylpyridinium ion), the neurotoxic byproduct of MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine) metabolic oxidation, is selectively taken up by DA neurons, inhibiting complex I of mitochondrial electron transport [5, 6].

Recent studies have characterized a first-in-class small-molecule Akt activator, namely SC79 [7]. It is a novel, efficient, and cell-permeable Akt activator [7]. SC79 physically binds to Akt PH domain, thereby inducing Akt conformational change [7]. SC79 uniquely suppresses Akt membrane translocation, while promoting its activation in the cytosol [7]. SC79 is shown to phosphorylate Akt at both Ser-473 and Thr-308, causing significant Akt activation [8–11].

Researchers have proposed a cytoprotective function of SC79. For instance, Zheng et al. reported that SC79 activated Akt signaling to protect myocardiocytes from oxygen

and glucose deprivation/re-oxygenation [8]. Activation of Akt by SC79 inhibited dexamethasone-induced injury in osteoblasts [9]. Furthermore, the Akt activator attenuated Ultra-violet (UV)-induced retinal pigment epithelium cell apoptosis [10]. The results of the current study show that SC79 protects DA neuronal cells from mitochondrial toxins, rotenone, and MPP$^+$.

Materials and methods

Reagents and antibodies

SC79, MK-2206, and LY294002 as well as rotenone and MPP$^+$ were obtained from Sigma-Aldrich (St. Louis, Mo). The antibodies were all provided by Cell Signaling Tech (Danvers, MA). Cell culture reagents were obtained from Gibco Co. (Suzhou, China).

SH-SY5Y cells

SH-SY5Y DA neuronal cells were purchased from the Cell Bank of Biological Institute of CAS (Shanghai, China). SH-SY5Y cells were cultured and differentiated as previously described [12].

Primary murine DA neurons

Isolation and primary culture of murine DA neurons were described in detail previously [11, 13]. Briefly, murine ventral mesencephalon tissues containing DA neurons were dissected from murine embryo (C57/B6) on day-14 of gestation. Single cells were cultured in mixed hormone MEM (MHM) supplemented with 5% FBS. Neurons were plated in poly-L-lysine-coated six-well plates at a density of 1×10^6 neurons per mL. The medium was renewed every 2–3 days. DA neuron cultures were grown for 8–10 days before further experiments. The protocols were approved by the Ethics Board of Soochow University. The animal protocol was approved by the Soochow University Institutional Animal Care and Use Committee (IACUC).

Lactate dehydrogenase (LDH) assay

Cells were cultured in six-well tissue plates. After treatment, LDH levels in the conditional medium and cell lysates were measured by the two-step LDH detection kit (Promega). Medium LDH was normalized to the total LDH (medium LDH plus lysate LDH), reflecting cell death percentage.

Cell viability assay

Cells were cultured onto 96-well tissue culture plates. After treatment, cell viability was tested by CCK-8 assay. CCK-8 optical density (OD) values at 550 nm were recorded.

Western blotting

Total cell lysates were separated by SDS-PAGE gels [14], which were transferred to PVDF blots (Millipore). The blots were blocked (in 10% milk-PBST), followed by incubation with designated primary and secondary antibodies. The protein band was detected via enhanced chemiluminescence (ECL) reagents (Pierce, Shanghai, China) by X-ray films. ImageJ software (NIH) was always utilized to quantify the total gray of each band.

Caspase activity assay

After treatment, the caspase-3 and caspase-9 activities were measured via using the fluorometric caspase assay kits (Beyotime Biotechnology, Suzhou, China) [15]. The caspase-3 and caspase-9 p-nitroaniline (pNA) absorbance was detected at 405 nm.

TUNEL assay

Cells were seeded onto six-well tissue culture plates (2×10^5 cells per well). TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling] In Situ Cell Death Detection Kit (Roche, Shanghai, China) was applied to quantify TUNEL-positive nuclei in apoptotic cells. TUNEL percentage (% *vs.* Hoechst staining) of 200 cells per treatment in five random views (1: 100 magnification) was recorded.

ROS assay

Cells were seeded onto 12-well plates at 3×10^4 cells per well. Following treatment, ROS production was measured via the cell-permeable CM-H2DCF-DA reagent (Life Technologies, Grand Island, NY) according to the protocol. Cells were incubated with 10 μM of CM-H2DCF-DA for 30 min. The DCF-DA fluorescence absorbance was detected by a fluorescent photometer (BD Biosciences, Shanghai, China) with the excitation and emission wavelengths at 485 nm and 538 nm, respectively.

Mitochondrial depolarization assay

After treatment, mitochondrial depolarization ("ΔΨ") was examined by using the mito-dye JC-1. JC-1 will aggregate to form the green monomers following mitochondrial depolarization, and JC-1 fluorescence absorbance was recorded at 550 nm, indicating mitochondrial depolarization.

Lipid peroxidation assay

Following an early protocol [16], cellular lipid peroxidation levels were measured by the TBAR (thiobarbituric acid reactive substances) activities. In brief, following the treatment, 50 μg total cell lysates (TCL) per treatment were mixed with 20% of acetic acid and thiobarbituric acid solution. After heating, the mixture was centrifuged, with the red pigment dye in the supernatant tested by a microplate reader.

CRISPR/Cas9-mediated Akt1 knockout

The small guide RNA (sgRNA) targeting human Akt1 with the targeted DNA sequence, 5′-TCACGTTGGTCCACA TCCTG-3′ (*sgRNA-1*, PAM, CGG) or 5′-GAGCGACGT GGCTATTGTGA-3′ (*sgRNA-2*, PAM, AGG), was inserted into the lenti-CRISPR-GFP plasmid (Addgene). Cells were cultured in six-well plates. Each of the two CRISPR/Cas9 Akt1-knockout construct was transfected to SH-SY5Y cells. Stable cells were selected via FACS sorting of GFP-positive SH-SY5Y cells. Akt1 knockout (KO) in the resulting stable cells was verified by Western blotting assay.

The quantitative real-time reverse transcriptase polymerase chain reaction (qPCR) assay

Following treatment, total RNA was extracted by the TRIzol reagents (Sigma). Reverse transcription and qPCR were performed by the TOYOBO ReverTra Ace qPCR kit (Tokyo, Japan), under the ABI Prism 7600H fast Real-Time PCR system (Foster City, CA). The mRNA primers for *nuclear factor (erythroid-derived 2)-like 2* (*Nrf2*), *heme oxygenase1* (*HO1*), *NAD(P)H:quinone oxidoreductase 1* (*NQO1*), and *GAPDH* were provided by Dr. Di [17]. The melt curve analysis was performed to calculate the product melting temperature. The $2^{-\Delta\Delta Ct}$ method was utilized for mRNA quantification, using *GAPDH* as the reference gene.

Nrf2 KO

The lentiCRISPR-GFP-Nrf2-puro KO construct was provided by Dr. Di [17], transfected to SH-SY5Y neuronal cells.

GFP-positive stable cells were sorted by FACS. Single cells were cultured onto 96-well plate to generate the monoclonal cells. Nrf2 KO was confirmed by Western blotting.

Statistics

Data were presented as mean ± standard deviation (SD). Statistical differences were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparisons performed with post hoc Bonferroni test (SPSS). Values of $P < 0.05$ were considered statistically significant.

Results

SC79 protects dopaminergic neuronal cells from MPP+ and rotenone

To establish the cytotoxic effect of MPP+ in DA neuronal cells, differentiated SH-SY5Y cells were treated with different concentrations of MPP+, from 0.3–10 mM. CCK-8 assay was performed to test cell viability, and results show that MPP+ (at 3–10 mM, treatment for 48 h) significantly inhibited survival (CCK-8 OD) of SH-SY5Y cells (Fig. 1a). Further experimental results showed that MPP+ dose-dependently induced LDH release to the medium, indicating cell death (Fig. 1b). Significantly, co-treatment of the Akt activator SC79 (10 μM) potently attenuated MPP+ (3–10 mM)-induced viability reduction and LDH release (Fig. 1a and b). These results suggest that SC79 can protect SH-SY5Y cells from MPP+.

SC79′s dose response was studied next. As shown, SC79 dose-dependently inhibited MPP+ (3 mM, 48 h)-induced viability reduction (Fig. 1c) and LDH release (Fig. 1d) in SH-SY5Y cells. The Akt activator was effective at 1–10 μM, but not at 0.3 μM (Fig. 1c and d). Among the tested concentrations, SC79 at 10 μM demonstrated highest efficiency in protecting SH-SY5Y cells (Fig. 1c–d). Importantly, rotenone (300 nM, 48 h)-induced SH-SY5Y cell viability reduction (Fig. 1e) and death (Fig. 1f) were attenuated by SC79 (10 μM) co-treatment as well (Fig. 1e and f). SC79 single treatment, at the tested concentrations, did not affect SH-SY5Y cell functions (Fig. 1a–d).

The activity of SC79 was also tested in the primary cultured murine DA neurons. As shown, SC79 (10 μM) largely inhibited MPP+-induced viability reduction (Fig. 1g) and LDH release (Fig. 1h) in DA neurons. Furthermore, rotenone-induced neuronal death was significantly alleviated by SC79 co-treatment (Fig. 1i). SC79 alone was ineffective on neuronal functions (Fig. 1g and h). Collectively, these results show that SC79 protects DA neuronal cells from MPP+ and rotenone.
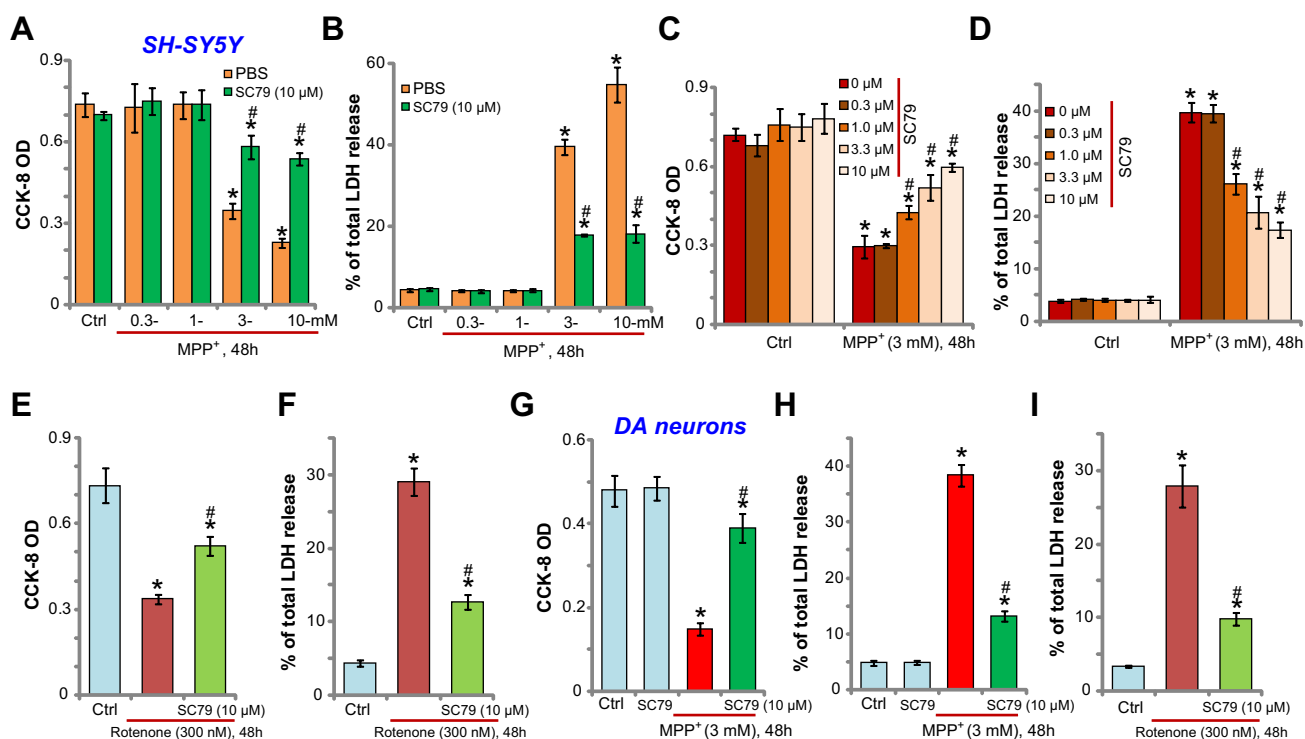
Fig. 1 SC79 protects dopaminergic neuronal cells from MPP$^+$ and rotenone. SH-SY5Y cells (**a–f**) or the primary murine dopaminergic neurons ("DA neurons," **g–i**) were treated with MPP$^+$ (0.3–10 mM), rotenone (300 nM), or together with applied concentration of SC79, cells were further cultured in the medium for 48 h, and cell survival and cell death were tested by CCK-8 assay and LDH release assay, respectively. "Ctrl" stands for untreated control group (same for all figures). "PBS" stands for SC79's vehicle PBS. Bars stand for mean ± standard deviation (SD, $n = 5$). *$P < 0.05$ vs. "Ctrl" cells. #$P < 0.05$ vs. MPP$^+$ or rotenone single treatment. Experiments in this figure were repeated five times, with the similar results obtained

SC79 inhibits MPP$^+$- and rotenone-induced apoptosis in dopaminergic neuronal cells

MPP$^+$ disrupts mitochondrial functions, leading to substantial ROS production and oxidative stress, which shall induce the release of pro-apoptosis proteins, causing cell apoptosis [18]. Since SC79 significantly inhibited MPP$^+$-induced DA neuronal cell death, we next tested its effect on cell apoptosis. As shown, treatment with MPP$^+$ (3 mM, 16 h) increased the activities of caspase-3 (Fig. 2a) and caspase-9 (Fig. 2b) in SH-SY5Y cells. Co-treatment with SC79 (10 μM) potently inhibited caspase-3/-9 activation by MPP$^+$ (Fig. 2a and b). Western blotting analyses in SH-SY5Y cells demonstrated that SC79 significantly attenuated MPP$^+$-induced cleavages of caspase-3, caspase-9 and poly (ADP-ribose) polymerase (PARP) (Fig. 2c). MPP$^+$-induced activation of caspases induces DNA damages, causing accumulation of Histone-bound single-strand DNA (Fig. 2d), again inhibited by SC79 (Fig. 2d). To further evaluate the anti-apoptosis activity of SC79, TUNEL staining assay was performed. Results show that SC79 co-treatment significantly attenuated MPP$^+$-induced increases of TUNEL-positive SH-SY5Y

cells (Fig. 2e). SC79 single treatment had no effect on caspase-PARP activation nor cell apoptosis (Fig. 2a–e).

In the primary DA neurons, SC79 inhibited MPP$^+$-induced Histone-bound DNA accumulation (Fig. 2f) and TUNEL-nuclei ratio increase (Fig. 2g). Rotenone treatment similarly induced Histone-bound DNA accumulation in SH-SY5Y cells (Fig. 2h) and primary DA neurons (Fig. 2i), again attenuated by SC79 (Fig. 2h and i). These results show that SC79 potently inhibited MPP$^+$- and rotenone-induced DA neuronal cell apoptosis.

Akt inhibition abolishes SC79-mediated neuroprotection against MPP$^+$

Next, we tested the potential effect of SC79 on Akt activation. In SH-SY5Y cells, SC79 dose-dependently induced phosphorylation of Akt at Ser-473 and Thr-308 (Fig. 3a). Total Akt1 level was unchanged (Fig. 3a). SC79-induced SH-SY5Y cell protection against MPP$^+$ was completely nullified by the Akt inhibitors (Fig. 3b). LY294002 and MK-2206 also enhanced MPP$^+$-induced viability reduction (Fig. 3b), indicating that basal Akt activation is cytoprotective in MPP$^+$-treated SH-SY5Y cells.
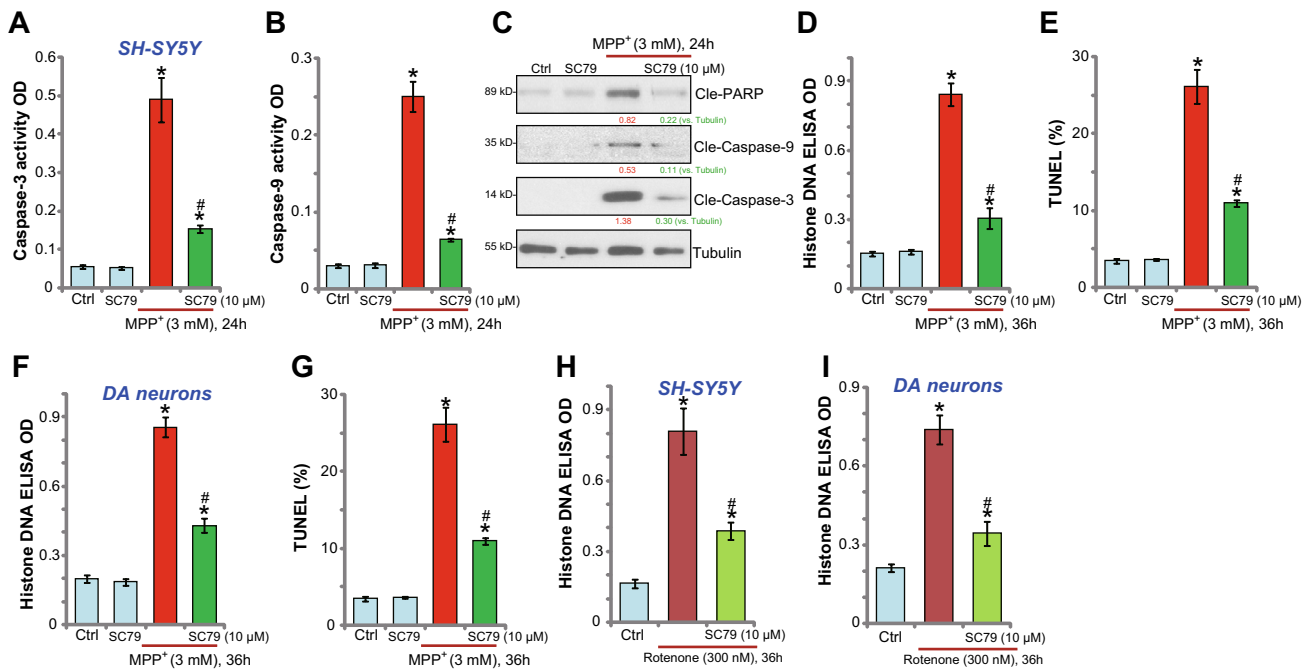
Fig. 2 SC79 inhibits MPP$^+$- and rotenone-induced apoptosis in dopaminergic neuronal cells. SH-SY5Y cells (**a**–**e** and **h**) or the primary murine dopaminergic neurons ("DA neurons," **f**–**g** and **i**) were treated with MPP$^+$ (3 mM), rotenone (300 nM), or together with SC79 (10 μM), cells were further cultured for indicated time periods, and cell apoptosis was evaluated by the assays mentioned in the text. Expression levels of the listed proteins were quantified, normalized to the loading control (Tubulin) **c**. Bars stand for mean ± standard deviation (SD, $n=5$). *$P < 0.05$ versus "Ctrl" cells. $^{\#}P < 0.05$ versus MPP$^+$ or rotenone single treatment. Experiments in this figure were repeated four times, with the similar results obtained

To rule out the possible off-target toxicities of the Akt inhibitors, CRISPR/Cas9 gene-editing method was employed to knockout Akt1 in SH-SY5Y cells. Each of the two applied lenti-CRISPR Akt1 knockout constructs, containing non-overlapping sgRNA sequences (see Methods), led to complete Akt1 knockout (KO) in SH-SY5Y cells (Fig. 3c). SC79-induced Akt activation, or Ser-473 and Thr-308 phosphorylation, was blocked in Akt1-KO cells ("L1/L2," Fig. 3c). Importantly, in Akt1-KO SH-SY5Y cells, SC79 was unable to inhibit MPP$^+$-induced viability reduction (Fig. 3d) and cell death (Fig. 3e). This genetic evidence further supported that Akt activation is required for SC79-mediated DA neuroprotection against MPP$^+$. This is further supported by the fact that Akt1-KO cells were more vulnerable to MPP$^+$ (Fig. 3d and e).

In the primary murine DA neurons, SC79 induced potent Akt activation or increased phosphorylation of Akt at Ser-473 and Thr-308 (Fig. 3f). Significantly, LY294002 and MK-2206 abolished SC79-mediated neuroprotection in MPP$^+$-treated primary neurons (Fig. 3g). Akt inhibitors also intensified MPP$^+$-induced viability reduction in DA neurons (Fig. 3g). These results suggest that Akt activation mediates SC79-induced DA neuroprotection against MPP$^+$.

SC79 inhibits MPP$^+$- and rotenone-induced oxidative injury in dopaminergic neuronal cells

Rotenone and MPP$^+$ are known to disrupt mitochondrial respiratory chain, leading to ROS production and oxidative stress, eventually causing DA neuronal cell death. In SH-SY5Y cells, MPP$^+$-induced ROS production, mitochondrial depolarization (ΔΨ), and lipid peroxidation, which were evidenced by increases of DCF-DA intensity (Fig. 4a), JC-1 intensity (Fig. 4b), and TBAR activity (Fig. 4c), respectively. Significantly, co-treatment of SC79 potently inhibited MPP$^+$-induced oxidative injury in SH-SY5Y cells (Fig. 4a–c). Similarly, rotenone-induced ROS production was attenuated by SC79 (Fig. 4d).

Nrf2 signaling is a key cellular defensive mechanism against oxidative stress, which could be activated following Akt activation [9, 19, 20]. qPCR assay results show that SC79 dose-dependently increased mRNA and protein expression of Nrf2-dependent genes, including *HO1* and *NQO1* (Fig. 4e and f). Furthermore, Nrf2 protein was stabilized in SC79-treated SH-SY5Y cells (Fig. 4f), with *Nrf2 mRNA* levels unchanged (Fig. 4e). Importantly, CRISPR-Cas69-mediated KO of Nrf2 blocked SC79-induced expression of HO1 and NQO1 in SH-SY5Y cells (Fig. 4g).
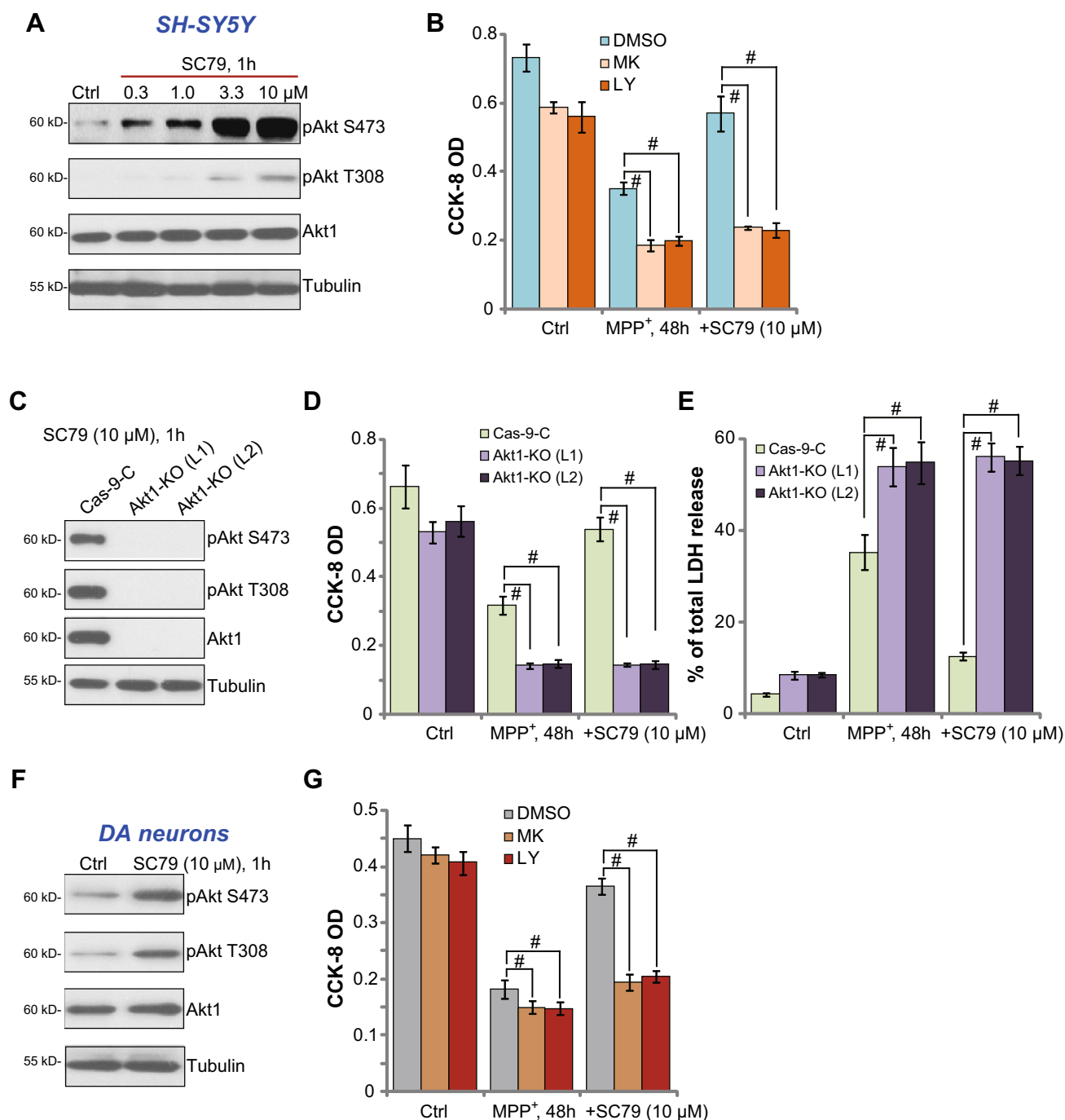
Fig. 3 Akt inhibition abolishes SC79-mediated neuroprotection against MPP$^+$. SH-SY5Y cells (**a**) or the primary murine dopaminergic neurons ("DA neurons," **f**) were treated with SC79 for 1 h, and the listed proteins in total cell lysates (TCL, 30 μg per treatment) were analyzed by Western blotting assays. SH-SY5Y cells (**b**) or the primary murine dopaminergic neurons (**g**) were treated with SC79 (10 μM) or plus MK-2206 ("MK," 5 μM, 30 min pre-treatment)/ LY294002 ("LY," 2 μM, 30 min pre-treatment), followed with MPP$^+$ (3 mM) treatment for applied time, and cell viability was tested by CCK-8 assay. SH-SY5Y cells with the lenti-CRISPR-Cas9-Akt1 KO construct (two different sgRNAs, "L1/L2") or lenti-CRISPR-Cas9 control ("Cas-9-C") were treated with MPP$^+$ (3 mM) or plus SC79 (10 μM), and Akt signaling proteins were analyzed by Western blotting assay (**c**); cell viability and death were tested by the CCK-8 assay (**d**) and LDH release (**e**), respectively. Bars stand for mean ± standard deviation (SD, $n = 5$). *$P < 0.05$ versus "Ctrl" cells. #$P < 0.05$. Experiments in this figure were repeated three times, with the similar results obtained
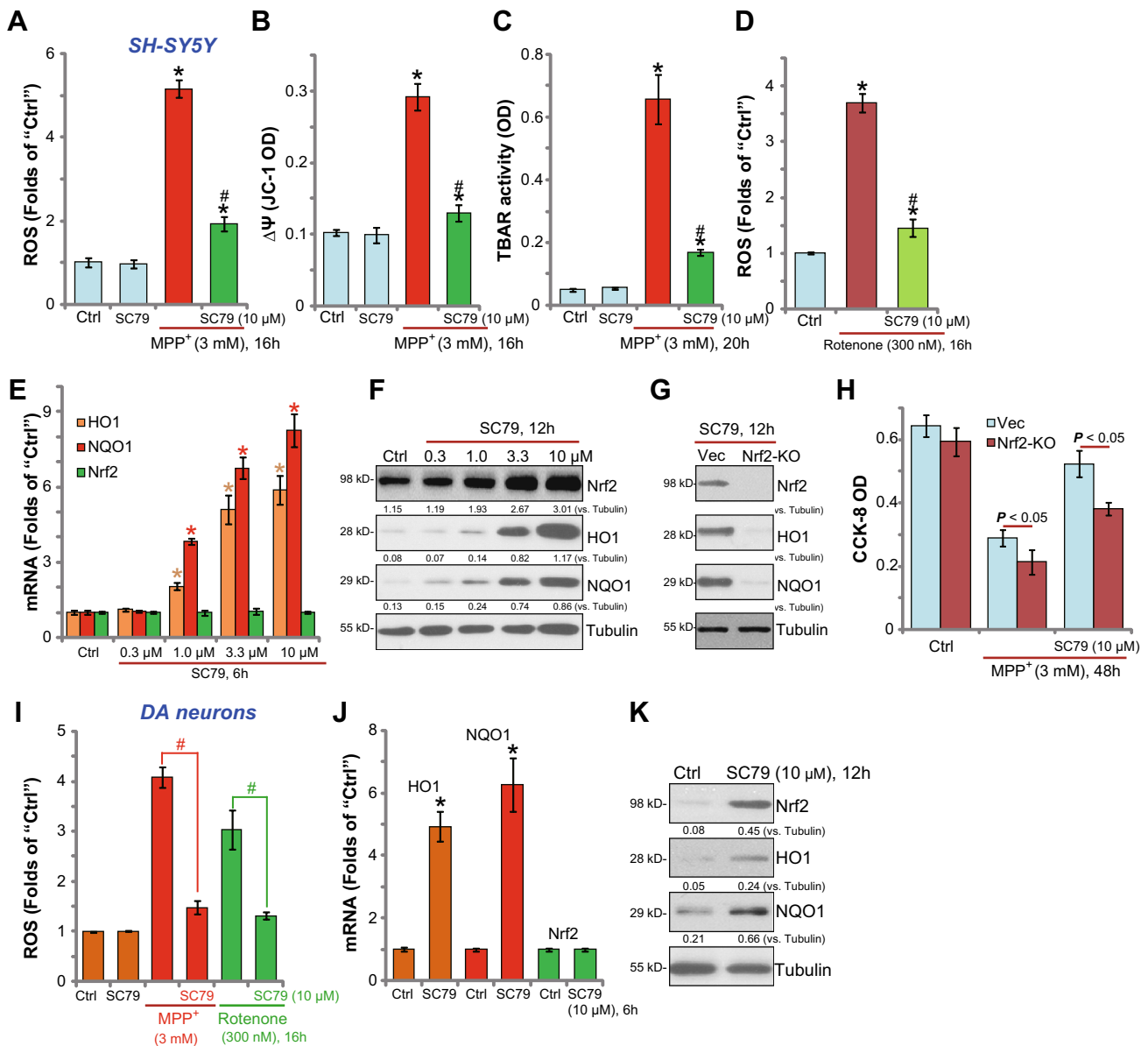
Fig. 4 SC79 inhibits MPP$^+$- and rotenone-induced oxidative injury in dopaminergic neuronal cells. SH-SY5Y cells (**a–d**) or the primary murine dopaminergic neurons ("DA neurons," **i**) were treated with MPP$^+$ (3 mM), rotenone (300 nM), or together with SC79 (10 μM), cells were further cultured for applied time periods, and ROS production (**a**, **d**, and **i**), mitochondrial depolarization (**b**), and lipid peroxidation (**c**) were tested by the assays mentioned in the text. SH-SY5Y cells (**e** and **f**) or the primary murine DA neurons (**j** and **k**) were treated with SC79 (at applied concentration) for indicated time period, and expression levels of Nrf2 pathway genes were analyzed by qPCR and Western blotting assays. Stable SH-SY5Y cells with lentiCRISPR-GFP-Nrf2-puro KO construct ("Nrf2-KO") or empty vector ("Vec") were treated with MPP$^+$ (3 mM) or plus SC79 (10 μM), and listed proteins were shown (**g**); cell viability was tested (**h**). Expression of the listed proteins were quantified, normalized to the loading control (Tubulin) (**f**, **g** and **k**). Bars stand for mean ± standard deviation (SD, $n = 5$). *$P < 0.05$ versus "Ctrl" cells. #$P < 0.05$ versus MPP$^+$/rotenone single treatment. Experiments in this figure were repeated three times, with the similar results obtained

Moreover, SC79-induced SH-SY5Y cell protection against MPP$^+$ was alleviated in the Nrf2-KO cells (Fig. 4h).

In the primary cultured murine dopaminergic neurons, co-treatment of SC79 also significantly inhibited MPP$^+$- and rotenone-induced ROS production (DCF-DA intensity increase, Fig. 4i). Nrf2 protein stabilization and increased expression of Nrf2-dependent genes (*HO1* and *NQO1*) were detected in SC79-treated primary DA neurons (Fig. 4j and k). These results suggest that SC79 activates Nrf2 signaling, inhibiting MPP$^+$- and rotenone-induced oxidative stress in DA neuronal cells.

Discussion

PD pathogenesis is multi-factorial, including genetic and environmental factors [1, 2]. DA neuronal dysfunction and death in PD progression involve several biochemical factors, including free radicals, mitochondrial dysfunction, abnormal protein aggregation, excitotoxicity, and inflammation [1, 2]. Of particular relevance, mitochondrial dysfunction and substantial ROS production are the main causes of DA neuronal cell death [1, 2]. Akt is a key pro-survival signaling. Akt phosphorylates its substrates, including mTOR, BAD, CREB, and the Forkhead transcription factors, to promote DA neuronal cell survival [21–23] and to protect neuronal cells from oxidative stress [24–26].

In the current study, we show that SC79, a first-in-class small-molecule Akt activator, significantly inhibited MPP$^+$- and rotenone-induced viability reduction, cell death, and apoptosis in SH-SY5Y cells and the primary murine DA neurons. SC79 activated Akt in DA neuronal cells, which is required for DA neuroprotection against mitochondrial toxins. Akt inhibitors or CRISPR-Cas9-mediated Akt1 knockout abolished SC79-induced DA neuroprotection against MPP$^+$. Therefore, activation of Akt by SC79 protects DA neuronal cells from mitochondrial toxins.

Activation of Nrf2 signaling is an established strategy to protect DA neuronal cells from oxidative injury (*i.e.,* by mitochondrial toxins) [27–29]. Recent studies have implied that activated Akt could lead to Nrf2 signaling activation in different cells. For example, Lee et al. showed that sulforaphane-induced Nrf2 activation is the downstream PI3 K-Akt activation [30]. Xu et al. demonstrated that PI3 K-Akt activation is required for pyocyanin-induced Nrf2 signaling activation [31]. Furthermore, Zhang et al. showed that Salvianolic acid A (Sal A) activated Akt-dependent Nrf2 signaling in human retinal pigmentation epithelial (RPE) cells [32].

In the current study, SC79 induced Nrf2 protein stabilization and increased expression of Nrf2-dependent genes (*HO1* and *NQO1*) in SH-SY5Y cells and primary DA neurons, confirming Nrf2 cascade activation. SC79 potently attenuated MPP$^+$- and rotenone-induced ROS production, mitochondrial depolarization, and lipid peroxidation in DA neuronal cells. Therefore, we implied that SC79 activated Akt-Nrf2 signaling to inhibit MPP$^+$- and rotenone-induced oxidative injury in DA neuronal cells. This is further supported by the fact that Nrf2 KO alleviated SC79-induced SH-SY5Y cell protection against MPP$^+$. The underlying mechanisms of Nrf2 signaling activation by SC79 in DA neuronal cells and how SC79-activated Nrf2 signaling inhibits oxidative stress by mitochondrial toxins warrant further studies.

Conclusion

Together we show that SC79 protects DA neuronal cells from mitochondrial toxins possibly via activation of Akt-Nrf2 signaling.

Compliance with ethical standards

Conflict of interest None of the authors have any competing interests.

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