Resveratrol Suppresses Rotenone-induced Neurotoxicity Through Activation of SIRT1/Akt1 Signaling Pathway

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ABSTRACT

Rotenone is a common pesticide and has been reported as one of the risk factors for Parkinson disease. Rotenone can cause neuronal death or apoptosis through inducing oxidative injury and inhibiting mitochondrial function. As a natural polyphenolic compound, resveratrol possesses the antioxidant capacity and neuroprotective effect. However, the mechanism underlying the neuroprotective effect of resveratrol against rotenone-induced neurotoxicity remains elusive. Here, we treated PC12 cells with rotenone to induce neurotoxicity, and the neurotoxic cells were subjected to resveratrol treatment. The CCK8 and LDH activity assays demonstrated that resveratrol could suppress neurotoxicity induced by rotenone ($P < 0.01$). The DCFH-DA assay indicated that resveratrol reduced the production of reactive oxygen species (ROS). JC-1 and Hoechst 33342/PI staining revealed that resveratrol attenuated mitochondrial dysfunction and cell apoptosis. Moreover, resveratrol reversed rotenone-induced decrease in SIRT1 expression and Akt1 phosphorylation ($P < 0.05$). Furthermore, when the SIRT1 and Akt1 activity was inhibited by niacinamide and LY294002, respectively, the neuroprotective effect of resveratrol was remarkably attenuated, which implied that SIRT1 and Akt1 could mediate this process and may be potential molecular targets for intervening rotenone-induced neurotoxicity. In summary, our study demonstrated that resveratrol reduced rotenone-induced oxidative damage, which was partly mediated through activation of the SIRT1/Akt1 signaling pathway. Our study

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launched a promising avenue for the potential application of resveratrol as a neuroprotective therapeutic agent in Parkinson disease. Anat Rec, 301:1115–1125, 2018.

Key words: Akt1; neuroprotection; resveratrol; rotenone; SIRT1

Rotenone, a popular pesticide, is still widely used in industry and agriculture. It has been reported that rotenone could produce 1-methyl-4-phenylpyridine (MPP^+) and can cause cell death or apoptosis in dopaminergic neurons (Jackson-Lewis et al., 1995; Lim et al., 2007). Moreover, it has been demonstrated that synthetic heroin containing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) could induce Parkinsonism in humans (Matsubara, 1998). Importantly, Parkinsonism could also be induced in primate animals with MPTP treatment. This implies that environmental toxins may be risk factors for Parkinson disease (PD). Although genetic factors are clearly related with familial PD, mechanisms involving sporadic PD are unclear. Epidemiological studies suggested that exposure to pesticides including rotenone may increase the incidence of PD (Di Monte, 2003; Dhillon et al., 2008). Accordingly, rotenone has been used in numerous studies to produce *in vitro* cellular and animal models of PD (Tamilselvam et al., 2013; Wu et al., 2013; Kang et al., 2017).

Extensive studies have confirmed that rotenone can induce oxidative damage in dopaminergic neurons, which exerts a key role in PD. Additionally, rotenone has also been shown to induce mitochondrial dysfunction (Yee et al., 2017), increase cytosolic free Ca^{2+} levels (Jiang et al., 2016), generate reactive oxygen species (ROS) (Brennan-Minnella et al., 2016), and elevate apoptotic proteins such as, Bax, caspase-3, and caspase-9 (Narasimhan et al., 2016). Furthermore, recent studies have demonstrated that rotenone could inhibit the mTOR signaling pathway (Liu et al., 2016), activate the glycogen synthase kinase-3 (GSK-3) signaling pathway (Giménez-Cassina et al., 2012), and inhibit JNK and p38 MAPK signaling pathways (Park et al., 2014) leading to cell death or apoptosis. However, the exact molecular mechanism needs to be further elucidated.

In recent years, increasing evidences have demonstrated that resverotrol possessed extensive biological effects, including anti-inflammatory, antioxidant, anticancer, and antiaging (Bhat et al., 2001; Anekonda and Reddy, 2005; Tsai et al., 2007). It is therefore reasonable to consider using resveratrol as an antioxidative drug for inhibiting rotenone-induced neurotoxicity. A large number of *in vitro* and *in vivo* experiments have demonstrated that resveratrol could protect neurons against 6-hydroxydopamine (6-OHDA)- (Khan et al., 2010), MPP^+- (Zeng et al., 2017), and β-amyloid peptide-induced damage (Deng and Mi, 2016), and can also improve animal's movement and spatial memory performance (Wang et al., 2017). Resveratrol has also been shown to enhance degradation of α-synuclein and protect SH-SY5Y cells against rotenone-induced autophagic dysfunction (Lin et al., 2014). Our previous study has shown that resveratrol could reduce 6-OHDA induced damage in PC12 cells via the CXCR4 signaling pathway (Zhang et al., 2015). Moreover, studies have shown that resveratrol indeed ameliorates symptoms of MPTP- (Wang et al., 2015) and 6-OHDA-induced (Khan et al., 2010) PD in rats through downregulation of α-synuclein expression and up-regulation of antioxidant enzymes, respectively. Importantly, a more recent study reported that resveratrol has potential therapeutic capacity for rotenone-induced PD in rats through inhibiting endoplasmic reticulum stress-mediated apoptosis (Gaballah et al., 2016). However, the underlying molecular mechanisms are unknown. Thus, the present study aims to gain deeper insight into the molecular mechanism involved in the neuroprotective effect of resveratrol against rotenone-induced neurotoxicity.

Sirtuin-1 (SIRT1), a prototypical member of the class III histone deacetylases (HDACs) and a NAD^+-dependent histone deacetylase, can be activated by resveratrol (Borra et al., 2005). SIRT1 plays an important role in a variety of biological functions, such as, embryonic development, cell differentiation, and apoptosis (McBurney et al., 2003; Finkel et al., 2009; Wu et al., 2015). The serine/threonine-protein kinase, Akt1, is a downstream molecule of SIRT1, which exerts major influences on the regulation of cell proliferation, cell survival and protein synthesis (Franke, 2008). Our previous study also suggested that resveratrol can activate SIRT1 and increase Akt1 phosphorylation against β-amyloid protein induced neuronal damage (Feng et al., 2013). Recently, Price et al. have reported that SIRT1 is essential for moderating doses of resveratrol to enhance mitochondrial function (Price et al., 2012). SIRT1 overexpression improves cell metabolism and extends lifespan, and reduces incidence of neurodegenerative diseases (Sansone et al., 2013). Moreover, in the absence of Akt1, mouse showed more developmental issues than wild-type in life span and organ size (Chao et al., 2010). In addition, SIRT1 is known to deacetylate Lysine residues of K14 and K20 situated in the pleckstrin homology domain of Akt, which presents in lipid-binding proteins and is necessary for Akt1 phosphorylation (Sundaresan et al., 2011). SIRT1 can regulate multiple cellular processes that are also regulated by Akt (Finkel et al., 2009). Previous study demonstrated that SIRT1 coimmunoprecipitates with the full-length Akt (Sundaresan et al., 2011). The above studies implied that SIRT1/Akt1 may have an essential role in the neuroprotective effect of resveratrol against rotenone-induced cytotoxicity, and if so, the function of SIRT1/Akt1 in rotenone cellular model of PD needs to be deeply addressed. To our knowledge, only a few studies have investigated this signaling mechanism.

To test our hypothesis, we cultured the rat pheochromocytoma clonal cell line PC12. PC12 cells can secrete

neurotransmitter dopamine (Bethea and Kozak, 1984) and express dopamine transporter (DAT) (Alyea et al., 2008), which is widely used as a cell model of dopaminergic neurons. Studies have suggested that grafted PC12 cells have potential for treating PD in animal models (Tatard et al., 2004). We damaged PC12 cells with rotenone to produce a cellular model of PD. First, the neuroprotective effect of resveratrol against rotenone-induced cytotoxicity was investigated; second, real time PCR and western blotting were employed to detect the expression of SIRT1 and Akt1; and finally, functional analysis of SIRT1/Akt1 pathway was performed by selectively blocking SIRT1 and Akt1 with nicotinamide and LY294002, respectively. Moreover, we also used MK-2206, an Akt1 inhibitor, to further block Akt1 activity in order to confirm its specific role in the protective effect of resveratrol against rotenone-induced neurotoxicity. Our study will provide a new understanding of the molecular mechanisms involved in rotenone-induced neuronal damage, and the potential application of resveratrol in PD treatment.

MATERIALS AND METHODS

Chemicals and Reagents

Rat adrenal pheochromocytoma cells (PC12 cell line) were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences (Shanghai, China). Rotenone, nicotinamide and DMSO were obtained from Sigma–Aldrich (St. Louis, MO). LY294402 and MK-2206 were provided by Selleckchem Company. Resveratrol was purchased from Aladdin Industrial Corporation (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were provided by Hyclone Company (Logan, UT). Anti-SIRT1 polyclonal antibody, Anti-Akt1 and *p*-Akt1 polyclonal antibodies and Anti-GAPDH monoclonal antibody were purchased from Cell Signaling Technonlogy (USA).

Cell Culture

PC12 cells were cultured in DMEM medium containing 10% FBS at 37°C with humidified 5% CO_2 and passaged with 0.25% trypsin treatment and centrifuged at 1,200 r min^{-1} for 5 min. And then the harvested cells were seeded in 96-well plates or other plates at a concentration of $2 \times 10^4/cm^2$. Resveratrol and rotenone were dissolved in DMSO, and the final concentration of DMSO in medium was <5%. The cultured cells were divided into the following groups: (1) Control group: with normal culture medium; (2) Injury group: rotenone was added into culture medium at 0.1, 0.5, 1.0, 5.0, and 10 μM of concentrations for 24 h *in vitro* culture; (3) Resveratrol group: cells were pretreated by resveratrol for 2 hr at concentrations of 12.5, 25, 50, and 100 μM and were subsequently treated with rotenone for 24 h *in vitro*. Finally, the following detection assays were performed.

CCK-8 Assay

To evaluate the cellular viability of cultured neurons treated with rotenone or resveratrol, we performed CCK-8 assay according to the previous report (Peng et al., 2015). Briefly, cells were seeded in 96-well plate with 100 μL DMEM medium containing PLAC8 protein as indicated in the manufacturer's protocol (Dojindo, Japan) at 37°C, 5% CO_2. After 1.5 hr culture, 10 μL of cell counting kit was added into the plates and the cell viability was detected by a microplate reader (Multiskan MK3, Thermo Labsystems, Philadelphia, PA) at an absorbance of 450 nm.

LDH Assay

LDH release of damaged cells was further detected to measure the cell cytotoxicity. PC12 cells were treated similarly as in CCK-8 assay. According to the instructions of commercial reagent kit (Nanjing Jiancheng Biology Engineering Institute, China), each sample containing 20 μL supernatant was measured at an absorbance of 450 nm using a microplate reader. The extracellular LDH release rate was then quantified based on the optical density (OD) values.

ROS Measurement

The cellular ROS was detected using DCFH-DA as previously described (Zhang et al., 2014). In short, cells were cultivated in 24-well plates and treated with rotenone or resveratrol for 24 h. Then the cells were collected and incubated in medium with 10 μM DCFH-DA for 30 min in the dark at 37°C. After washing with PBS, intracellular fluorescence was observed under fluorescence microscopy and analyzed by Image-Pro Plus 6.0 software. The fluorescence intensity represented the ROS levels.

Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential (MMP) was assessed by JC-1 staining kit (Beyotime Company, Shanghai, China) according to the previous report (Yu et al., 2017). JC-1 is a fluorescence probe, and is frequently used to detect MMP. The PC12 cells in different groups were washed with PBS, after 24 hr *in vitro* culture. Subsequently, 250 μL of JC-1 solution with a concentration of 5 μM fluorescent dye and 250 μL culture medium were added into each well. Cells were incubated for 30 min in the dark at 37°C and washed twice with cold JC-1 staining buffer. Finally, fluorescence intensity of cultured cells was captured by fluorescence microscopy. MMP changes were analyzed by Image-Pro Plus 6.0 software based on the ratio of red/green fluorescence intensity.

Cell Apoptosis Measurement

To analyze the cell apoptosis, Hoechst 33342 and propidium iodide (PI) double-staining was performed according to our previous study (Feng et al., 2013). Briefly, PC12 cells were cultured in 24-well plate and treated with rotenone or resveratrol. After 24-hr culture *in vitro*, cells were washed twice and incubated in Hoechst 33342/PI double-stained dye at a concentration of 10 mg mL^{-1} for 15 min at room temperature. Then cells were washed with PBS again and observed under a LSM 780 laser scanning confocal microscopy (Carl Zeiss SAS, Jena, Germany). Hoechst positive cells showed blue fluorescence, while PI positive cells were red fluorescent. Apoptotic cells were Hoechst positive and

demonstrated characteristic features of apoptosis, such as, condensed or fragmented nuclei. The staining was analyzed by the morphology and fluorescence.

Real Time Quantitative PCR

Total RNA was extracted from cultured cells using Trizol reagent (Sangon Biotech, Shanghai, China). RNA integrity was examined and cDNA was synthesized using reverse-transcription kit. Real time quantitative PCR was performed on Mastercycler ep realplex (Eppendorf, Hamburg, Germany). The amplification conditions were as follows: melting temperature of 94°C for 15 min, annealing temperature of 60°C for 1 min, and extension temperature of 72°C for 50 min, total 40 cycles. The primers of SIRT1 and Akt1 were as follows: SIRT1, forward primer: 5′-CCAGAAACAATTCC TCCACCT-3′; reverse primer: 5′-CAG-CAAGGCGGC ATAAATAC-3′; Akt1, forward primer: 5′-CCTCAAGAAGGA GGTCATCG-3′, reverse primer: 5′-GTCGTGGGTCTGGAA TGAGT-3′. GAPDH was used as the internal control. The expression quantity of the target genes was calculated by the $2^{-\Delta\Delta CT}$ method.

Western Blot Analysis

The cultured cells were washed with ice-cold PBS and lysed by RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Shanghai, China) on ice. The plastic cell scraper was used to scrape the adherent PC12 cells. Cell suspension was agitated and centrifuged at 12,000 rpm for 20 min. Total protein was extracted. A small volume of lysate was removed and the protein concentration was determined with BCA kit. The protein samples of different groups were loaded into the wells of 10% SDS-PAGE gel together with the molecular weight marker (ThermoFisher, Shanghai, China). Next, proteins were electrotransferred onto nitrocellulose membranes. Blots were blocked with 5% defatted milk containing 0.1% Tween-20 for 2.5 h. And then the membrane was incubated with rabbit anti-SIRT1 monoclonal antibody (1:1,000, Santa Cruz Biotechnology, CA) and anti-Akt antibody monoclonal antibody (1:1,000, Santa Cruz Biotechnology, CA) at 4°C overnight. Subsequently, the membrane was washed three times with TBST solution and then incubated with anti-rabbit secondary antibody (1:5,000) for 1 hr at room temperature and developed with ECL reagent (Millipore Corporation, Billerica, MA). The protein expression was analyzed by Image J software.

Statistical Analysis

All data were expressed as mean ± SD and processed with the one-way analysis of variance (ANOVA), and the Tukey test. $P < 0.05$ was considered statistically significant.

RESULTS

Resveratrol Inhibited Rotenone-induced Neurotoxicity

PC12 cells grew well in culture medium with long neurites (Fig. 1A). When treated with rotenone, the cellular morphology changed gradually following the increase in rotenone concentration. No obvious damage effects were observed in PC12 cells with 0.1 μM rotenone treatment (Fig. 1B), while 1 μM rotenone strongly destroyed the neurites (Fig. 1C) and 10 μM rotenone made all neurites disappeared and most cells died with condensed or fragmented nuclei (Fig. 1D). Considering the damage effects, 1 μM rotenone was chosen as the suitable concentration in the following experiments. To assess the protective effect of resveratrol, the cultured cells were pretreated with different concentrations of resveratrol for 2 h, and then exposed to 1 μM rotenone. The results showed that concentrations of 12.5, 25, and 50 μM resveratrol all had neuroprotective effects. The concentration of 25 μM resveratrol rescued damaged cells significantly and promoted extension of short neurites (Fig. 1E–H).

To further detect the cell viability, CCK8 and LDH release assays were performed and the data showed that rotenone significantly reduced OD value of CCK8 and increased LDH release (Fig. 1I,J). When given resveratrol, the cell viability increased and LDH release significantly decreased (Fig. 1K,L). Especially, 25 μM resveratrol could increase the cell viability from 55.40% to 79.40%, indicating the protective effect of resveratrol against rotenone. Therefore, the concentration of 25 μM resveratrol was used in the following experiments.

Resveratrol Suppressed Rotenone-induced Oxidative Stress and Improved Mitochondrial Function

ROS could oxidize DCFH to DCF, which stimulates the green fluorescence and indicates ROS levels. Compared with the control group (Fig. 2A), ROS production significantly increased in the rotenone injury group (Fig. 2B). While resveratrol obviously reduced ROS levels (Fig. 2C). JC-1 staining was further performed to determine the damage of mitochondria. The changes of fluorescence from red to green indicated the decline in mitochondrial membrane potential ($\Delta\psi m$). JC-1 aggregated in normal mitochondria as polymers and exhibited red fluorescence, whereas, JC-1 monomer exhibited green fluorescence represented the low $\Delta\psi m$. The data of JC-1 staining showed that red/green fluorescence intensity distinctly decreased in the rotenone injury group (Fig. 2F) compared with the control group (Fig. 2E), while resveratrol could reverse this decrease and protect mitochondrial function (Fig. 2G). Statistical analysis showed that rotenone increased the ROS levels and decreased the $\Delta\psi m$ ($P < 0.01$), whereas resveratrol reversed these changes ($P < 0.05$).

Resveratrol Inhibited Rotenone-induced Cell Apoptosis

To determine the protective effect of resveratrol against rotenone induced cellular apoptosis, double staining of Hoechst 33342 (blue)/PI (red) was performed in this study. In the control group, most cells emitted dark blue fluorescence with normal nuclei were Hoechst positive (Fig. 3A–C). When exposed to 1 μM rotenone for 24 hr, a few cultured cells emitted red fluorescence and stained PI positive (dead cells), and some cells exhibited bright blue color with nuclear chromatin condensation, karyopyknosis, and nuclear fragmentation, indicating obvious apoptosis (Fig. 3D–F). When pretreated with 25 μM resveratrol and then exposed to rotenone, cellular apoptosis was attenuated and only a few cells showed

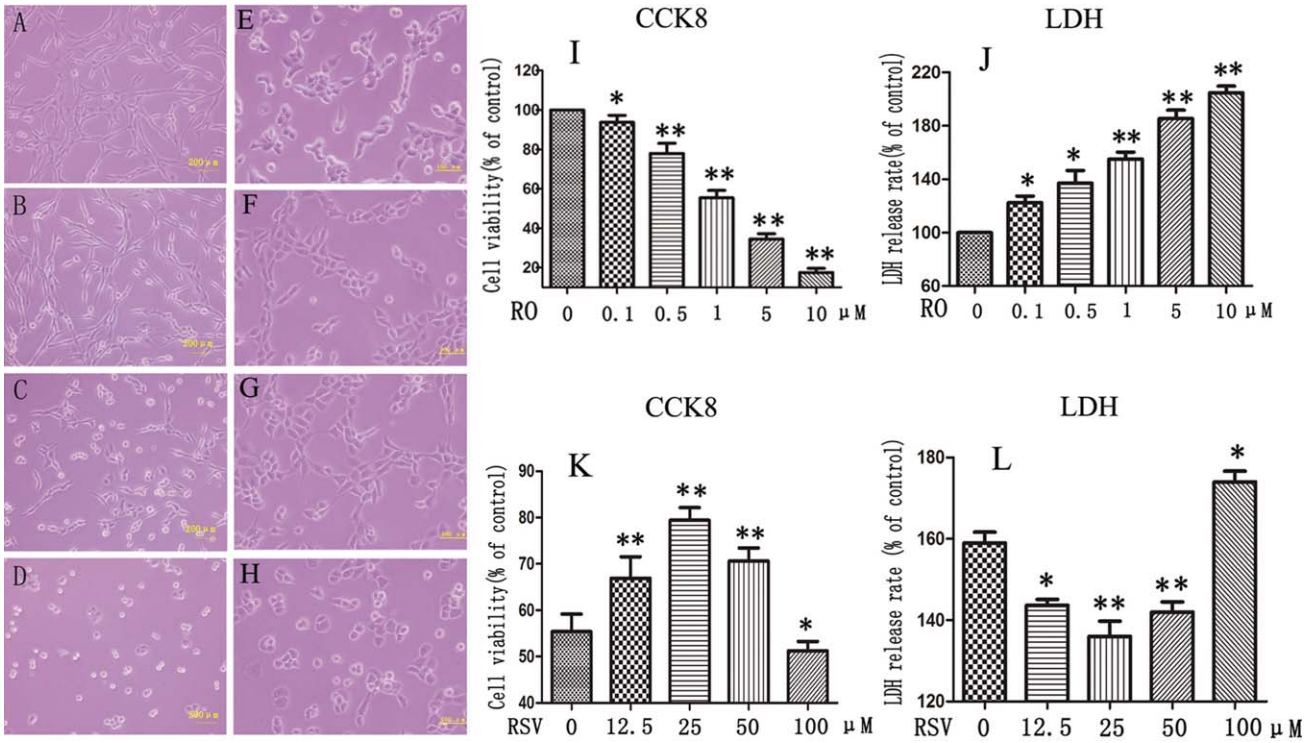

Fig. 1. Resveratrol inhibited rotenone-induced neurotoxicity. The morphological changes of PC12 cells treated with different concentrations of rotenone (0, 0.1, 1, 10 μM) for 24 hr *in vitro* culture, respectively (**A–D**). Following the increase in rotenone concentration, neurites of cells disappeared with condensed or fragmented nuclei. When treated with 12.5, 25, and 50 μM resveratrol, cells neurotoxicity was attenuated (**E–G**). The concentration of 100 μM resveratrol demonstrated no protection (**H**). Cellular viability was further determined by CCK8 and LDH release assays. Rotenone significantly decreased cell viability and increased LDH release (**I**, **J**). While resveratrol increased cell viability and decreased LDH release (**K**,**L**), which rescued cell viability damaged by rotenone. * $P < 0.05$ and ** $P < 0.01$ vs. control ($n = 3$ independent experiments; five wells/time). Bars: A–D, 200 μm; E–H, 100 μm.

red fluorescence (Fig. 3G–I). Statistical analysis indicated that the apoptotic cells and dead cells increased significantly in the rotenone injury group compared with the control group (Fig. 3J, $P < 0.01$ and $P < 0.001$, respectively). Resveratrol remarkably reduced the rate of cell apoptosis and the rate of cell death (Fig. 3K, $P < 0.01$).

Resveratrol Upregulated SIRT1 Expression and Activated Akt1 Phosphorylation, While Blocking SIRT1/Akt1 Pathway Attenuated Resveratrol Protective Effect

To analyze the molecular mechanisms involving the protective effect of resveratrol, the expression levels of SIRT1 and Akt1 were examined. The data of real-time PCR showed that SIRT1 and Akt1 expressions significantly decreased when treated with 1 μM rotenone (Fig. 4A–D; $P < 0.01$). Moreover, western blotting also indicated that rotenone suppressed the protein levels of SIRT1 and p-Akt1 (Fig. 4E,F; $P < 0.01$). The concentration of 25 μM resveratrol reversed the decreased expressions of both SIRT1 and p-Akt1, though the expressions remained still lower than the control group (Fig. 4A–F).

To further reveal the protective mechanism of resveratrol against rotenone-induced cytotoxicity, we used nicotinamide and LY294002 to block SIRT1 and Akt1 activities, respectively. After adding 5 mM nicotinamide or 10 μM LY294002 into culture medium together with resveratrol, Western blotting was performed to detect the changes in SIRT1 and Akt1 expressions at protein level. The data showed that nicotinamide inhibited SIRT1 expression and the Akt1 phosphorylation, respectively (Fig. 5A,B). The CCK8 assay showed that the protective effect of resveratrol was significantly attenuated (Fig. 5C). Interestingly, we found that LY294002 not only inhibited Akt1 activation but also inhibited SIRT1 expression. An Akt1 inhibitor, MK-2206, was used to confirm the experiment. Consistent with above results, after blocking Akt1 by MK-2206, the cell apoptotic rate in the rotenone group was higher than that in the resveratrol group (Fig. 5D–F,I), and the SIRT1/p-Akt1protein expressions were also reduced significantly (Fig. 5G–H). These data suggested that the SIRT1/Akt1 pathway indeed participated in the process of resveratrol protection against rotenone-induced cytotoxicity.

DISCUSSION

In the present study, we found that resveratrol significantly reversed rotenone-induced decrease in SIRT1 expression and Akt phosphorytation and showed obvious neuroprotective effect against rotenone-induced

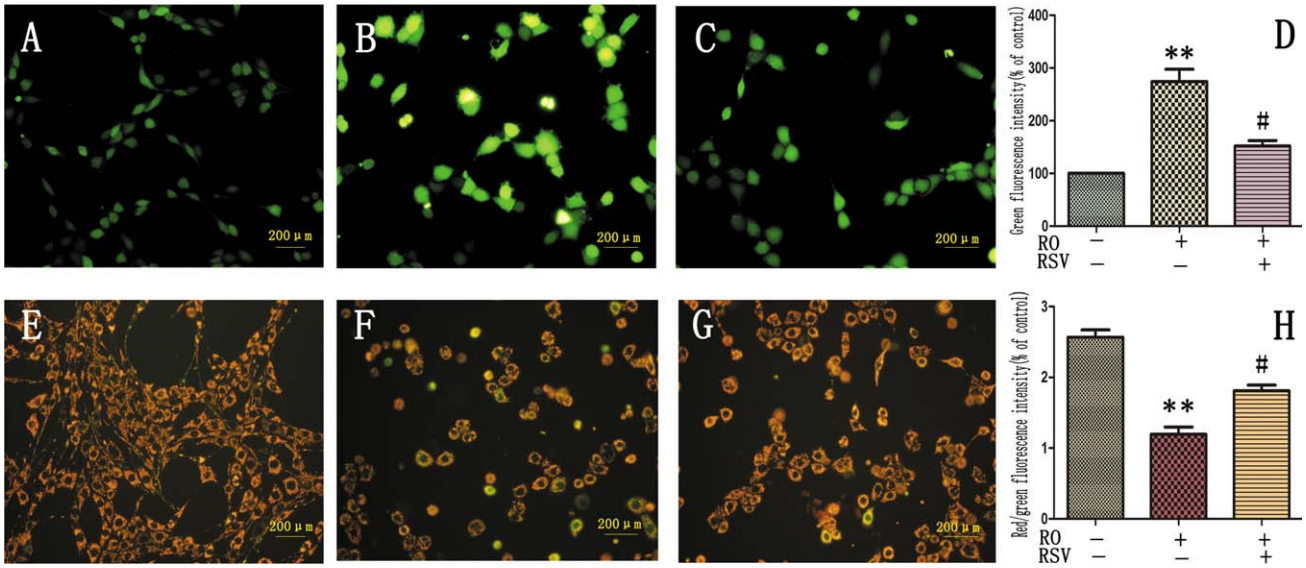

Fig. 2. Resveratrol reduced rotenone-induced oxidative stress and improved mitochondrial function. (**A**–**D**) indicated ROS assay and (**E**–**H**) indicated JC-1 assay. (A, E) In control group, cultured cells showed the normal ROS level and $\Delta\psi$m. (B, F) PC12 cells exposed to 1 μM rotenone showed stronger green fluorescence and lower red/green fluorescence intensity than those in the control group, which suggested excessive ROS gathered in cultured cells and reduced $\Delta\psi$m (C, G). When pretreated with resveratrol, the cells exhibited weaker green fluorescence and the red/green fluorescence intensity obviously decreased than those in the rotenone group (D, H). * $P < 0.05$ and ** $P < 0.01$ vs. control. # $P < 0.05$ vs. rotenone group ($n = 3$ independent experiments). Bars: A–C, E–G, 200 μm.

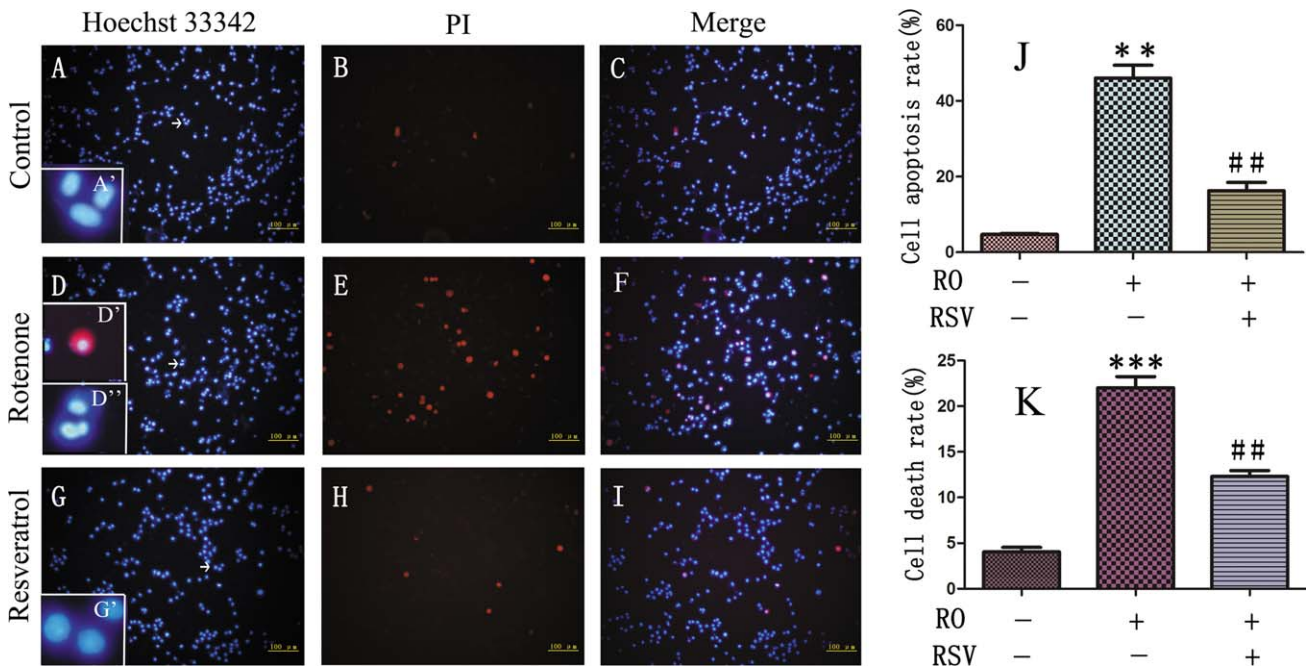

Fig. 3. Resveratrol prevented rotenone-induced cell apoptosis. In control group, the cells exhibited normal nuclear morphometry, with few PI positive cells which were red fluorescence (**A**–**C**). **A**' indicated a normal cell. When treated with 1 μM rotenone (**D**–**F**), cells showed clear apoptosis, karyopyknosis, nuclear fragmentation, and more dead cells (**D**"). **D**' and **D**" indicated a dead cell and an apoptotic cell, respectively. When pretreated with 25 μM resveratrol, the quantity of normal cells and nuclear morphometry was improved (**G**–**I**). **G**' indicated a cell rescued by resveratrol. The statistical analysis showed that resveratrol remarkably attenuated cellular apoptotic rate and death rate (**J**, **K**). ** $P < 0.01$ compared to control and ## $P < 0.01$ vs. rotenone group (n = 3 independent experiments). Bars: A–C, E–G, 200 μm.

neurotoxicity. Moreover, when the SIRT1/Akt1 signaling pathway was inhibited, the neuroprotective effect of resveratrol was remarkably attenuated, which implied that SIRT1 and Akt1 could mediate neuroprotection and possibly were the potential molecular targets for intervening rotenone-induced neurotoxicity.

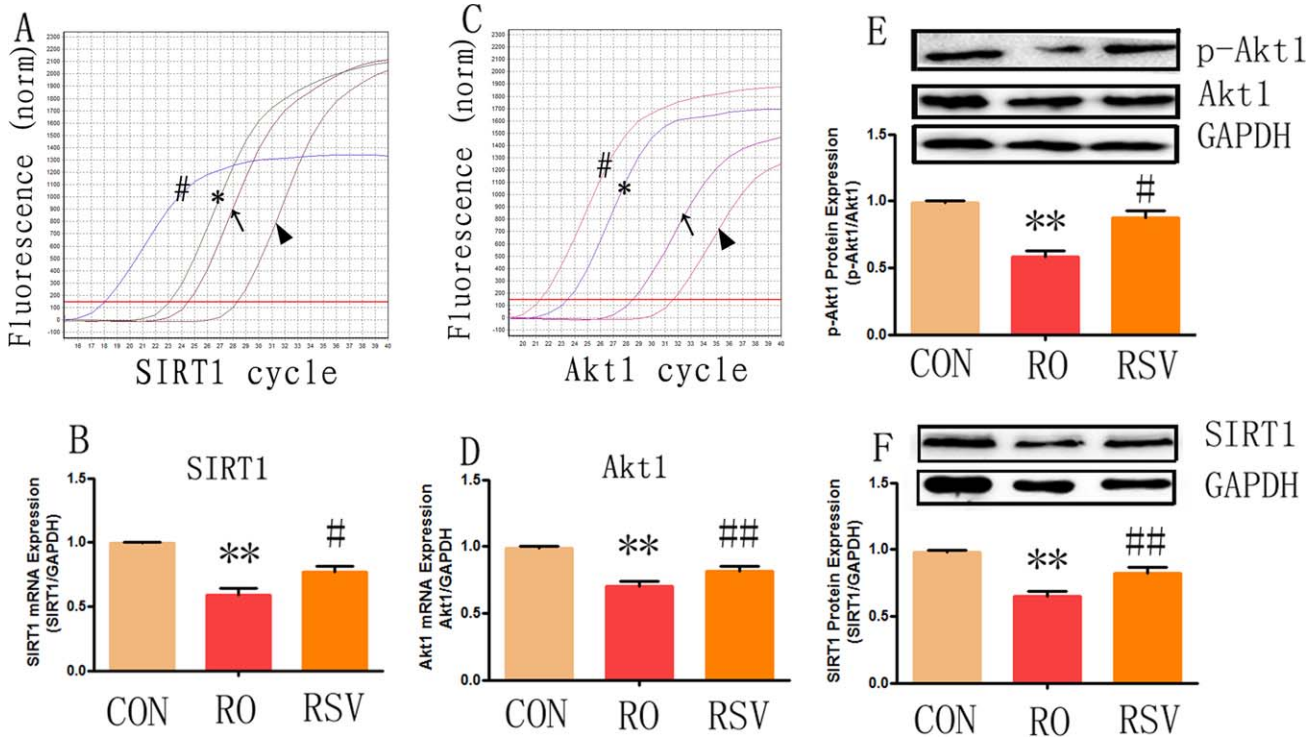

Fig. 4. Resveratrol reversed the decrease of SIRT1 and p-Akt1 caused by rotenone. The real-time PCR (**A–D**) and western blotting (**E–F**) were performed to detect the expression of SIRT1 and p-Akt1 at the mRNA and protein levels, respectively. Compared with the control group, 1 μM rotenone significantly decreased the expression of SIRT1 (A,B) and Akt1 (C,D). Moreover, western blotting data also indicated that rotenone suppressed SIRT1 (E) and p-Akt1 (F). After adding 25 μM resveratrol into the culture medium, the expression levels of SIRT1 and p-Akt1 were reversed (E,F). * $P < 0.05$, ** $P < 0.01$, vs. the control group; # $P < 0.05$, ## $P < 0.01$ vs. the rotenone group. In the panel A and C, arrow heads indicated injury group, arrows indicated the protective group, stars indicated the control group, and the hash key indicated the GAPDH (n = 3 independent experiments).

SIRT1 is a NAD^+-dependent histone deacetylase, which can be activated by resveratrol (Finkel et al., 2009; Baur et al., 2010). Previous studies have demonstrated that resveratrol could reduce neuron damage and alleviate neurodegeneration (Jackson-Lewis et al., 1995; Matsubara, 1998). It is therefore reasonable to assume that SIRT1 can play an important role in resveratrol protection against rotenone-induced neurotoxicity. Our data indicated that rotenone significantly decreased SIRT1 and p-Akt levels, while resveratrol obviously reversed these changes and p-Akt was almost rescued to normal level. To further test our hypothesis, the functional analysis was performed. Blocking SIRT1 activity or Akt1 phosphorylation aggravated rotenone-induced cell damage and attenuated the protective effect of resveratrol. This indicated that the SIRT1/Akt1 signaling pathway was indeed involved in mediating resveratrol protection against rotenone toxicity (Fig. 6). Previous studies have suggested that activation of SIRT1 can enhance Aβ clearance and increases the cell viability (Lee et al., 2015). In contrast, decreasing SIRT1 in mouse led to impaired cell survival and neurite outgrowth in primary cultured neurons (Guo et al., 2011). Additionally, it has been demonstrated that activating Akt could inhibit alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid induced neuronal cell damage (Kim and Han, 2005). Also, blocking Akt1 activity through intracerebroventricular injection of LY294002 has been shown to aggravate the neural damage in the hippocampus (Yang et al., 2015). Our previous study also suggested that Akt1 plays an important role in amyloid β-protein-induced neurotoxicity (Zhang et al., 2014). All these studies suggested that SIRT1/Akt1 signaling pathway can mediate the protective effect of resveratrol against rotenone-induced neuronal toxicity.

Interestingly, we found SIRT1 and Akt1 exhibited a bi-directional regulation relationship. It is well known that inhibition of SIRT1 could decrease Akt1 phosphorytation. However, the present study showed that blocking Akt1 activity could also decrease SIRT1 expression. Moreover, an Akt1 inhibitor MK-2206 was used in this experiment. MK-2206 inhibited both Akt1 activation and SIRT1 expression. Previous studies have indicated that Akt1 is a downstream molecule of SIRT1 and could be activated by SIRT1 mediated deacetylation, and even other members of Sirtuins, such as, SIRT3 and SIRT6 can also regulate Akt activation (Pillai et al., 2014; Pinton et al., 2016). Previous studies have also suggested that SIRT1 and Akt can coimmunoprecipitate with each other, and SIRT1 can increase Akt phosphorylation at Thr^{308} and Ser^{473} sites in pleckstrin homology domain (Sundaresan et al., 2011). Our data implied that the relationship between SIRT1 and AKT1 is a complex two-way regulation. Possibly, SIRT1 directly deacetylates Akt1, while activated Akt1 can also regulate SIRT1 by other signaling pathways. Previous studies supported

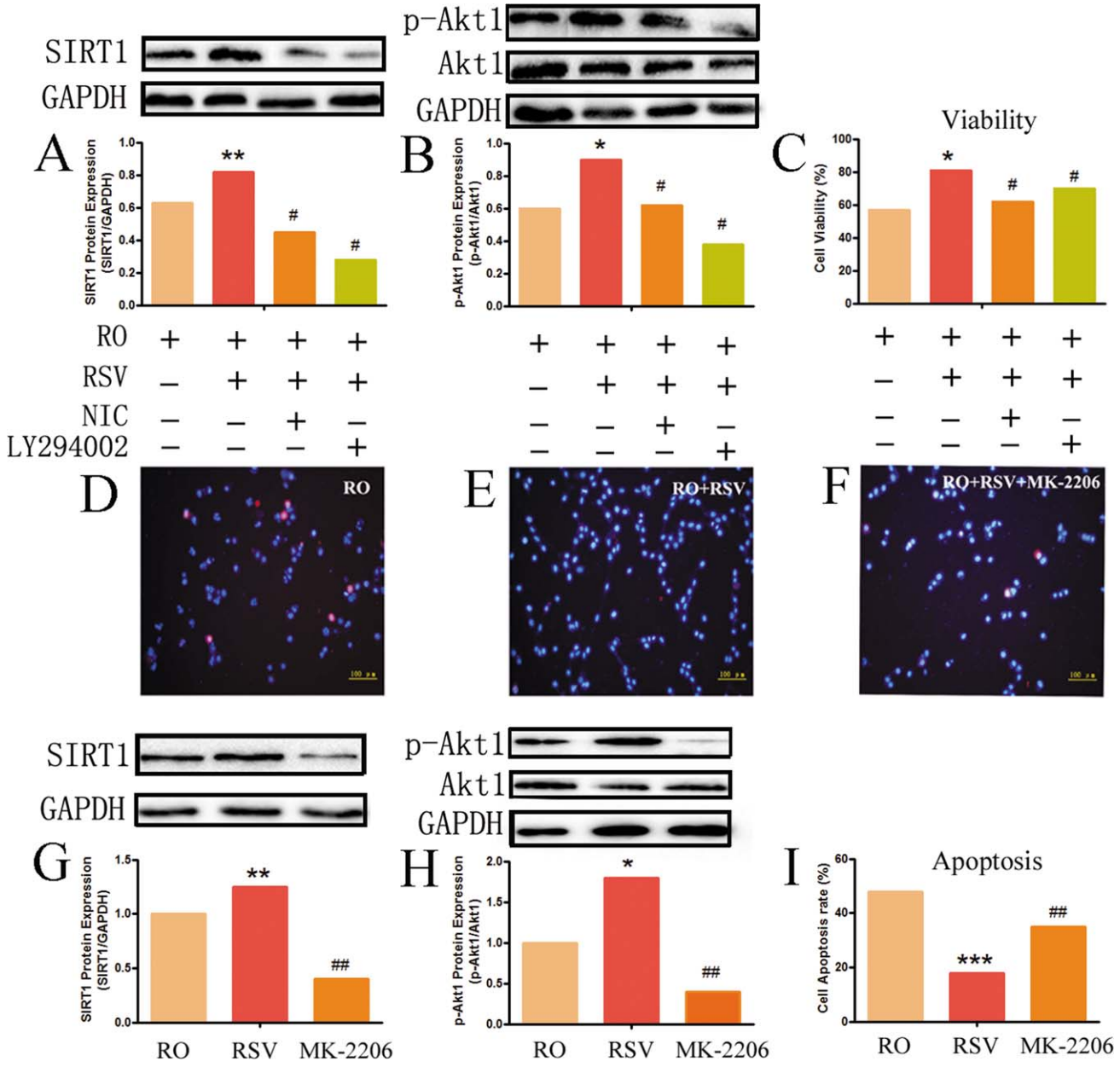

Fig. 5. The SIRT1/Akt1 signaling pathway participated in the protective effect of resveratrol against rotenone induced neurotoxicity. The SIRT1/Akt1 pathway was blocked by nicotinamide and LY294002, respectively. When 5 mM nicotinamide or 10 μM LY294002 were added into the culture medium together with resveratrol, SIRT1 (**A**) and p-Akt1 (**B**) protein levels decreased obviously and the cellular viability (**C**) was weakened. An Akt1 inhibitor, MK-2206, was used in the experiment to verify the results. Hoechst/PI double staining demonstrated that MK-2206 attenuated cell apoptosis (**D–F**, **I**) and decreased the expression of SIRT1 and p-Akt1 (**G–H**). * $P < 0.05$ and ** $P < 0.01$ vs. control. # $P < 0.05$ and ## $P < 0.01$ vs. the rotenone group (n = 3 independent experiments). Bars: E–G, 100 μm.

our assumption. SIRT1 and Akt can cooperate to regulate p53 activity. SIRT1 deacetylates p53 to render its inactivation. Meanwhile, Akt is involved in this process via phosphorylating associated protein (Gottlieb et al., 2002; Tang et al., 2008).

To our knowledge, it is a significant finding that resveratrol can protect against rotenone-induced oxidative damage. Rotenone is a risk factor for PD. Although the role of resveratrol for treating PD has been described previously, the exact mechanism needs to be further explored. Resveratrol is a natural polyphenolic compound, which is rich in red wine, polygonum cuspidatum, and grapes (Pervaiz, 2003). In this study, the data of CCK8 and LDH released assays showed that resveratrol significantly protected PC12 cells from rotenone-induced neurotoxicity. However, we found that the protective effect of resveratrol was not completely dose-dependent. At the concentration of 25 μM, resveratrol exerted optimal protective effect, whereas higher concentration (100 μM) of resveratrol exhibited damaging

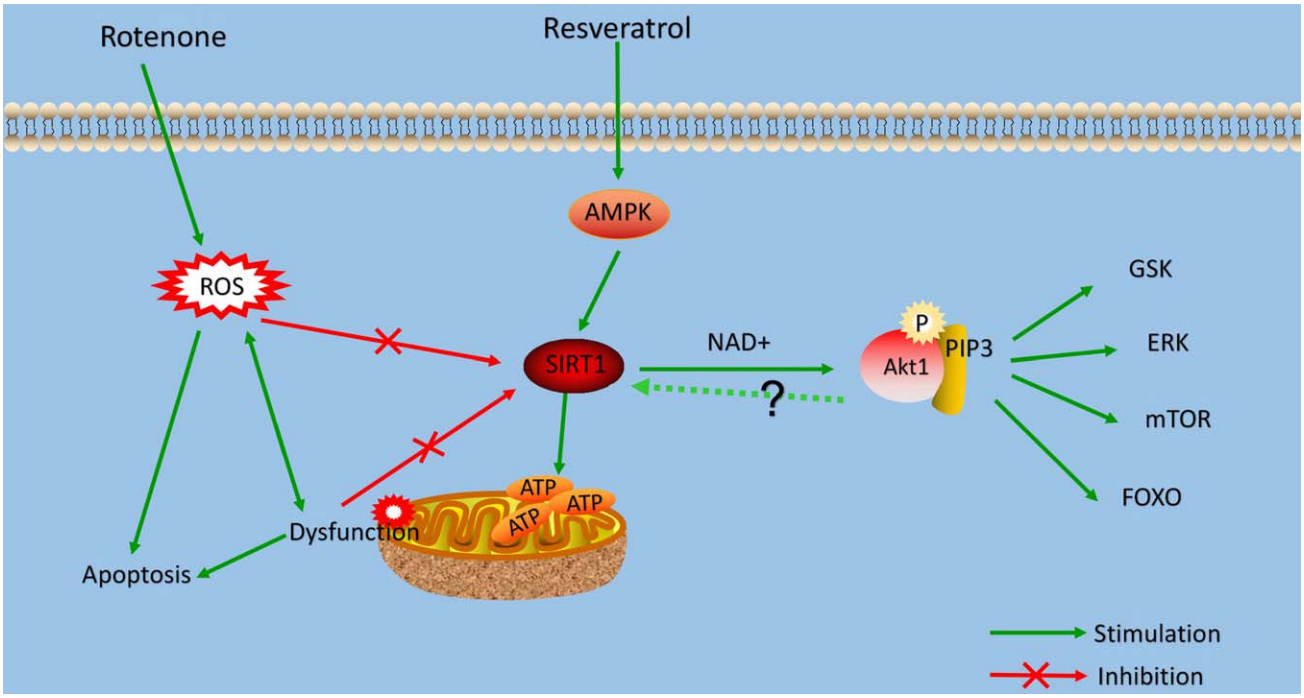

Fig. 6. Schematic diagram of the mechanism involved in the protective effect of resveratrol against rotenone induced cytotoxicity. Rotenone causes cells to produce a large amount of ROS, gathering in the mitochondria, which can lead to mitochondrial dysfunction and cell apoptosis. Whereas resveratrol can indirectly induce SIRT1 overexpression to enhance mitochondrial function and generate abundant ATP. Simultaneously, SIRT1 deacetylates Akt1 to phosphorylated Akt1 depending on NAD^+, and then activates the downstream targets of Akt1. Solid lines in the figure mean a direct effect.

effect. Recently, it has been demonstrated that 100 μM resveratrol can exert negative effect on the mitochondrial stability and cell viability in C2C12 myoblasts (Bosutti and Degens, 2015). We speculated that PC12 cells, as a kind of tumor cell lines, may be more sensitive to oxidative stress in higher doses of resveratrol, which potentially become the reason of resveratrol antitumor effect. More work need to be done to make further explorations.

In conclusion, we found that resveratrol significantly reduced rotenone induced neuronal damage. The SIRT1/Akt1 signaling pathway played an essential role in this process. Our study not only analyzed the mechanism involved in the protective effect of resveratrol, but also provided an evidence for potential application of resveratrol in treating PD.

CONFLICT OF INTEREST

The authors have declared that they have no conflict of interest.

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