Neuroprotective effects of 5-(4-hydroxy-3-dimethoxybenzylidene)-thiazolidinone in MPTP induced Parkinsonism model in mice

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A B S T R A C T

Parkinson’s disease (PD) is a neurological disorder characterized by degeneration of nigrostriatal dopaminergic (DAergic) system. Present treatment targeting to DAergic system solely ameliorated the symptoms but failed to retard the DAergic neuron degeneration, therefore new therapeutic methods aiming at preventing or delaying the neurodegenerative process are urgently needed. In the present study, we found that 5-(4-hydroxy-3-dimethoxybenzylidene)-2-thioxo-4-thiazolidinone (RD-1), a compound derived from rhodanine, protected DAergic neurons from neurotoxicity of MPTP/MPP+. Firstly, RD-1 significantly improved the locomotor ability in the MPTP mice model, and elevated the tyrosine hydroxylase (TH) positive cell numbers in substantianigra pars compacta (SNpc) and the integrated optical density (IOD) of TH-positive nerve fibers in striatum respectively. Since mitochondrial dysfunction plays an important role in pathogenesis of PD, thereby we investigated the molecular mechanisms of RD-1 against MPTP/MPP+ neurotoxicity, focusing on its effects on the mitochondrial dysfunction. Immunoblotting analysis showed that RD-1 significantly elevated the Parkin and Miro2 expression levels in acute MPTP treated mice, and improved mitochondrial membrane potential and ATP synthesis in MPP+-treated Neuro-2a cells. Moreover, RD-1 attenuated impaired mitochondrial transport and vesicle release dysfunction evoked by MPP+ cytotoxicity in cultured primary mesencephalic neurons. Taken together, these results indicate that improving the mitochondrial dysfunction may be a good choice to delay the neurodegenerative progression commonly associated with PD.

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1. Introduction

Parkinson’s disease (PD) is one of the common progressive neurologic diseases with a high and increasing prevalence in elderly population (Lees et al., 2009; Hirsch et al., 2013). Resting tremor, postural instability, bradykinesia and rigid are the cardinal signs of the disease. PD was characterized by remarkable selective degeneration of mesencephalic dopaminergic (mDA) neurons in the substantia nigra pars compacta (SNpc) that project their axons to the striatum. mDA neuron loss in the SNpc was associated with consequent dopamine depletion in striatum (Dauer et al., 2003; Hirsch et al., 2013). Dopamine precursor substance L-3,4-dihydroxyphenylalanine (L-dopa), in combination with a peripheral dopamine decarboxylase inhibitor (Benserazide or Carbipoda), is the optimal dopamine substitution therapy. However this kind of DAergic targeting therapeutic strategy solely ameliorated the symptoms but failed to retard DA neuron degeneration (Cranwell-Bruce, 2010). Therefore, it is crucial to develop new effective therapeutic drugs and strategies that can reverse or alleviate the pathological process in PD patients.

The molecular mechanisms leading to mDA cell loss are not entirely clear. Mitochondria are well known to be vital organelles with multiple functions for neuronal survival and activity. Mitochondrial dysfunction can lead to a decline in energy production, generation of reactive oxygen species and consequently mitochondrial-induced apoptosis. Several studies indicate that mitochondrial dysfunction might be a defect that occurs early in PD...
pathogenesis and appears to be a widespread feature in both sporadic and inherited forms of PD (Park et al., 2009; Morais et al., 2010; Xie et al., 2010). Thereby, improving the mitochondrial dysfunction may be alternative strategy for PD therapy.

Rhodanines, thiazolidine-2,4-diones and pseudotrihydantoins are becoming popular in drug discovery and represent very interesting class of heterocyclic compounds (Tomasic and Masic, 2009). Chemical modifications of these heterocycles constantly result in compounds with a wide spectrum of pharmacological activities. 5-(4-hydroxy-3-dimethoxybenzylidene)-2-thioxo-4-thiazolidinone (RD-1) was synthesized and screened in our lab. In preliminary experiment, results showed that RD-1 protected PC12 cells from the neurotoxicity of MPP+, which spurred us to further investigate the anti-parkinism effects and the underlying mechanism.

2. Materials and methods

2.1. Reagents

RD-1 (CAS: 99988-74-6) was entrusted synthesized by Beijing Youshiwanzong Pharmaceutical Corporation with a purity more than 98% (Fig. 1). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium (MPP+) (Sigma), 3,4-Dihydroxy-β-phenylalanine (β-dopa), Benserazide hydrochloride and poly-β-lysine were purchased from Sigma. Rabbit anti-tyrosine hydroxylase (TH) and mouse anti-Parkin antibodies were supplied by Millipore. Rabbit anti-RH2T/Miro2 antibody was obtained from Proteintech Group (Wuhan, China). Mouse anti-β-actin antibody was transfected by TransGen Biotech (Beijing, China). Polink-2 plus polymer HRP detection system for primary antibody was acquired from Zhongshan Biotechnology (Beijing, China). Neurobasal medium and B-27 Supplement were purchased from Gibco. Rhodamine (TRITC) AffiniPure Goat Anti-Rabbit IgG (H + L), FITC-conjugated AffiniPure Goat Anti-Mouse IgG were obtained from Jackson Immuno Research. MitoTracker Red CMXRos, MFI-43 molecular probe was supplied by Invitrogen. X-tremeGENE HP DNA transfection reagent was from Roche. JC-1 Mitochondrial Membrane Potential Detection Kit was purchased from Biotium. ATP assay kit was obtained from Beyotime in China.

2.2. Animal grouping and treatment

Adult male C57/BL6J mice were supplied by Weitonglihua Experimental Animal Centre (Beijing, China). They were randomly housed in groups of five in polypropylene cages with wood shavings as bedding and maintained in a temperature controlled room under a 12/12-h light/dark cycle with ad libitum access to food and water. Animal treatment and maintenance were carried out in accordance with the guidelines established by the National Institutes of Health for the care and use of laboratory animals and were approved by the Animal Care Committee of the Peking Union Medical College and Chinese Academy of Medical Sciences.

After 3 days' acclimation, the mice were randomly divided into six groups containing 10 animals in each group: Control, MPTP (MPTP 60 mg/kg), β-dopa (MPTP 60 mg/kg + L-dopa20 mg/kg + Benserazide 5 mg/kg) as positive control, RD-1-L (MPTP 60 mg/kg + RD-125 mg/kg), RD-1-M (MPTP 60 mg/kg + RD-150 mg/kg), and RD-1-H (MPTP 60 mg/kg + RD-1100 mg/kg). All the animals except the mice in the Control group were subjected to acute MPTP treatment described by Schöber (2004). In brief, mice received intraperitoneal injection of MPTP-HCl (15 mg/kg in saline, four injections at 2 h interval in one day). Control mice received saline only. From the first day following the fourth injection of MPTP, RD-1 (stably suspended in 0.5% CMC-Na solution) was intragastrically administered once daily for successive 4 weeks with the dose of 25, 50 or 100 mg/kg respectively. Meanwhile, β-dopa and Benserazide were orderly and intraperitoneally injected for 4 consecutive weeks. The mice in the Control and MPTP groups received equivalent administration of 0.5% CMC-Na solution. Mice were subjected to the Beam-Walking test at the end of treatment phase. All the animals were terminally sacrificed after 2 days' behavioral test. Mice brain tissues were processed for further investigation of biochemical parameters and immunohistochemistry studies.

2.3. Beam-walking test

The beam used for this test was 80 cm long, 1 cm wide and 1 cm tall, which was suspended 50 cm above the ground by wooden supports at either end. The wooden supports at the "starting" end of the beam formed a sheer drop while a platform was located at the other end, next to which was placed the home cage of the mouse being tested. Chips were placed beneath the beam for animals in case of a fall. During a test the mouse was placed at starting zone facing its home cage and a stopwatch started immediately upon release of the animal. The stopwatch was then stopped when all four feet were placed entirely upon the finishing platform at the opposite end of the beam. The maximum time allowed for the task was 2 min. A testing session consisted of five trials on the beam, recording the time of last trial for each animal (Albutt and Henderson, 2007).

2.4. Tissue processing

At the end of behavioral test, 4 mice were randomly selected for the immunohistochemistry analysis and 6 mice for the biochemical determination. For immunohistochemistry analysis, mice were anesthetized via pentobarbital sodium and transcardially perfused firstly with 0.1 M PBS then 4% paraformaldehyde (pH 7.4). The brains were removed and postfixed in the same 4% paraformaldehyde solution at 4 °C for 2 days. After that they were transferred to PBS containing 15%, 20% and 30% sucrose at 4 °C, respectively. Coronal sections (35 μm) were cut using a cryostat. For biochemical determination, 6 animals in each group were decapitated. Striatum and mesencephalic tissues were immediately isolated, frozen in liquid nitrogen and stored at −80 °C for further investigations.

2.5. Immunohistochemistry analysis

Free-floating brain slices were washed in PBS to remove the cryopreservative and incubated for 30 min at room temperature in 3% H2O2 solution to reduce endogenous peroxidase activity, and then additionally washed in PBS. After the bovine serum blocking process for 30 min at room temperature, these slices were incubated overnight at 4 °C with rabbit anti-TH-antibody (1:1000). Then the slices were washed with PBS and incubated with polymer Helper (Polink-2 plus polymer HRP detection system for rabbit primary antibody, Beijing Zhongshan Biotechnology Co., China) for 15 min at room temperature, washed again and incubated with poly-HRP anti-rabbit IgG for 15 min at room temperature. To develop color, the slices were incubated briefly in DAB substrate kit. After a final set of washes in PBS, the slices were mounted on slides, dehydrated, cleared, and coverslipped with mounting medium. Immunostaining process for all the experimental groups was concomitantly performed to minimize the possible background differences between animals. Images were captured using a CCD camera (Olympus, Japan). Four sections of each animal were stereologically selected to estimate the number of D4ergic cells in SNpc, one at the rostral, two at medium and one at caudal level. The TH+ cell counting was performed for the whole extension of the evaluated regions within each section. Additionally, integrated optical density (IOD) was used to assess TH+ nerve fiber with the similar methods for choosing sections in striatum. In each section, the IOD values were evenly distributed throughout the areas of interest were analyzed. The number of TH+ cells and IOD of TH+ fibers were calculated by Image-Pro Plus (IPP) 6.0 image analysis software.

2.6. Mitochondria isolation and Western blotting assay

Using real-time quantitative PCR (RT-qPCR) method, we found that RD-1 treatment significantly elevated the Parkin and Miro2 mRNA expression in striatum compared with MPTP group (Data not shown). To investigate the effect of RD-1 on the corresponding protein expression, mitochondrial and cytoplasmic proteins were extracted using Mitochondria Isolation Kit (Appygen Technologies Inc. Beijing, China). The brain tissues were homogenized with ice-cold Mitto-Cyto isolation buffer. The homogenate was then centrifuged at 800 × g for 10 min at 4 °C. The supernatants were collected in a new tube, and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant and pellet were saved as cytosolic fraction and intact mitochondria, respectively. The intact mitochondria were lysed with RIPA Buffer (Appygen Technologies) to extract mitochondrial protein. The alteration of Miro2 and Parkin protein expression in mitochondria and cytoplasm were analyzed by Western blotting.

For immunoblotting experiments, protein concentration in the lysate was quantified by a BCA Protein Assay Kit (Pierce). Protein extracts were separated by a SDS-polyacrylamide gel electrophoresis and then transferred onto a PVDF membrane. The membranes were blocked with 5% milk in Tris-buffered saline and then blotted with primary antibodies for overnight at 4 °C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Proteins were detected using a Super Enhanced Chemiluminescence Detection Kit (Appygen Technologies). The antibodies used in the study were anti-Parkin (1:1000), anti-Miro2 (1:1000) and anti-β-actin (1:10000). The total protein content was normalized using mouse anti-β-actin antibody.

Fig. 1. The chemical structure of 5-(4-hydroxy-3-dimethoxybenzylidene)-thiazolidinone.
2.7. JC-1 mitochondrial membrane potential analysis in Neuro-2a cells

To investigate the effect of RD-1 on the mitochondrial membrane depolarization, mitochondrial membrane potential (MMP, ΔΨm) was assessed by the lipophilic cationic probe JC-1. Neuro-2a neuroblastoma cells were obtained from Cell Resources Center of Institute of Basic Medical Sciences of Chinese Academy of Medical Sciences and cultured in DMEM supplemented with 2 mM glutamine, 100U penicillin and 100U streptomycin per ml, and 10% fetal bovine serum at 37°C in an atmosphere of 95% air and 5% CO2. Neuro-2a cells were treated with MMP (final concentration of 1 mM) in presence or absence of 10 μM RD-1 at 37°C for 6 h. RD-1 stock solutions (10 mM) was dissolved in DMSO/Ethanol (1:1,v/v) and stored at −20°C before use. MMP stock solution (10 mM) was prepared using PBS freshly and protected from the light. The control cultures were supplemented with the same amount of the appropriate vehicle. Then cells were labeled with JC-1 dye for 15 min at 37°C according to the protocol described by the manufacturer. The images were captured using a fluorescent microscope and counted without bias. Data are expressed as percentage of cells with high mitochondrial membrane potential (ΔΨm) in terms of JC-1 red fluorescence.

2.8. Measurement of ATP level in Neuro-2a cells

Intracellular ATP levels were determined using a bioluminescence ATP assay kit (Beyotime, China). The process was performed according to the manufacturer’s instructions. MMP (1 mM) and/or RD-1 (10 mM) were added to Neuro-2a cells.After incubation for 6 h at 37°C, harvested cultured cells were lysed with lysis buffer, and centrifuged at 12,000g for 5 min at 4°C. Finally, 50 μL of the supernatant were mixed with 100 μL luciferin-luciferase reagent and shaken vigorously for 3s. Luminescence values were measured on a Modulus™ Microplate Reader (Turner Biosystems, USA) and normalized to the protein content of each sample.

2.9. Mitochondria mass measurement in Neuro-2a cells

To determine whether ATP changes are due to the increase of mitochondria mass, mitochondria were stained with Mito Tracker Red dye according to manufacturer’s instruction. MMP (1 mM) and/or RD-1 (10 μM) were added at final concentrations of 1 μM and 10 μM, respectively. The cells were stained with 100 nM dye for 30 min at 37°C at 6 h after the addition of reagents. Cells were then washed with PBS solution, counterstained with 4,6-Diamino-2-phenylindole (DAPI, Sigma) and observed under a laser confocal microscope. Relative fluorescence intensity was analyzed with IPP 6.0 software.

3. Results

3.1. RD-1 attenuated the locomotor deficit induced by acute MPTP administration

The beam-walking test is a reliable index for assessment of motor coordination of animal (Allbutt and Henderson, 2007). MPTP-treated animals spent longer time in the beam-walking performance test than that of the control group, indicating an impaired locomotor activity induced by MPTP administration. RD-1 (50 mg/kg and 100 mg/kg) significantly shortened total time crossing the beam and improved motor ability of MPTP model mice. L-dopa neurons at a sampling rate of one frame every 5 s for 5 min, with the CCD exposure at 50 ms exposure and 2 × 2 binning. For each experiment, a population of neurons was imaged for a 5 min time-lapse recording at 4 h after addition of reagents. Quantification of moving mitochondria was done by simply counting the number of moving mitochondria in each 5 min time-lapse sequence. A moving mitochondrion was defined as one that moved more than a distance of twice its length over the 5 min period. The velocity of mitochondrial transport was assessed using Velocity Demo software.

3.2. TH immunostaining of cultured mesencephalic neurons

To assess the neuroprotection of RD-1 on TH+-positive neurons injured by MPP+ in cultured mesencephalic cells, MMP and/or RD-1 were added at 10 μM and 1 μM, respectively (final concentration) on the seventh day after cell seeding. After further cultured for 24 h, cells were performed for fluorescence immunostaining. Briefly, cells were incubated with primary anti-TH antibody and counterstained with DAPI. Immunostained cells were visualized under the fluorescent microscope (Leica, Wetzlar, Germany) and the TH+ neuron numbers were counted.

3.3. Fluorescent time-lapse recordings were performed on the Live Cell Station (Olympus, Japan). For imaging of mitochondrial transport, we typically recorded neurons at a sampling rate of one frame every 5 s for 5 min, with the CCD exposure at 50 ms exposure and 2 × 2 binning. For each experiment, a population of neurons was imaged for a 5 min time-lapse recording at 4 h after addition of reagents. Quantification of moving mitochondria was done by simply counting the number of moving mitochondria in each 5 min time-lapse sequence. A moving mitochondrion was defined as one that moved more than a distance of twice its length over the 5 min period. The velocity of mitochondrial transport was assessed using Velocity Demo software.

3.4. Statistical analysis

Data were statistically analyzed using one-way analysis of variance (one way ANOVA) followed by Student–Newman–Keuls post hoc test. Summarized data were expressed as mean ± SEM. Statistical significance was set at P < 0.05. All Statistical analysis was undertaken using SPSS v11.5.
combined with Benserazide treatment also improved the beam-walking performances of the mice compared with that of MPTP treated mice (Fig. 2).

3.2. RD-1 attenuated the decrease of TH-positive neuron numbers in SNpc and TH-positive fibers IOD in striatum of MPTP mouse model

The DAergic nigrostriatal system is a key component of regulating the motor performance. Cell bodies of dopaminergic neurons are located in the SNpc and their axons are projected along the nigrostriatal tract to the striatum (Kozina et al., 2010). Tyrosine hydroxylase (TH) is key enzyme in DA production and the marker of DAergic neuron (Bowling et al., 2008, Calvo et al., 2011). In the present study, immunohistochemistry method was used to assess the neuroprotective effects of RD-1 on the DAergic cells in SNpc and TH never fibers in striatum. Changes of TH-positive neuron numbers in SNpc were identified using the unbiased stereological counting method. As shown in Fig. 3A and B, the number of TH+ neurons in SNpc was significantly decreased by MPTP administration, whereas RD-1 treatment markedly increased number of TH+ neurons in MPTP mice. Meanwhile, the IOD of TH-positive nerve fibers in the striatum in MPTP group decreased most obviously (Fig. 3C and 3D) and RD-1 treatment (50 mg/kg and 100 mg/kg) significantly elevated the relative IOD of TH-positive nerve fibers. However in l-dopa group, there was no obvious difference in TH+ neuron and never fibers in two brain regions compared with the MPTP group, though l-dopa improved the locomotor ability in MPTP induced animals (Fig. 3B and 3D).

3.3. RD-1 elevated cytosolic Parkin expression of and mitochondrial Miro2 in mesencephalon of MPTP-intoxicated mice

The qRT-PCR method was applied for screening target genes of RD-1 against MPTP neurotoxicity. Our comparative analysis indicated that RD-1 exerted obvious effect on Parkin and Miro2 mRNA level (data not shown) among all tested genes that positively or negatively modulating mitochondrial function and activity (Wang et al., 2011a, 2011b; Rana et al., 2013). Subsequently, Protein levels of Parkin and Miro2 among groups were further evaluated. Firstly, we extracted mitochondrial and cytosolic proteins respectively to perform the Western blotting analysis. The results indicated that the cytosolic Parkin and mitochondrial Miro2 protein levels in mesencephalon of MPTP treated group were significantly lower than Control group. RD-1 significantly elevated the Parkin and Miro2 proteins in the mesencephalon of MPTP mice (Fig. 4A and B). Furthermore, the Parkin protein levels in both cytosolic and mitochondrial of striatum were decreased after MPTP treatment, whereas RD-1 also attenuated these pathological changes. On the contrary, we did not observe any significant difference among groups with regard to the expression of Miro2 in striatum (Fig. 4C and D).

3.4. RD-1 elevated mitochondrial membrane potential in MPP+-injured Neuro-2a cells

We employed JC-1 probe to evaluate the effect of RD-1 on MMP against early event during mitochondrial dysfunction. Red fluorescence represents the mitochondrial aggregate form of JC-1 (JC-1

![Fig. 3. Effects of RD-1 on TH-positive neurons and fibers in brains of MPTP-induced mice. (A,C) Representative immunohistochemical images of TH labeling in SNpc and striatum respectively. (B,D) Quantification of TH-positive cells and IOD (integrated optical density) of TH-positive fibers in different groups (magnification 20x, Scale bar = 200 μm). Each value represented the mean ± SEM (n = 4 in each group, *P < 0.05, **P < 0.01 versus Control group; #P < 0.01 versus MPTP group; $P < 0.05, $$P < 0.01 versus MPTP + L-dopa group; one-way ANOVA followed by Student–Newman–Keuls post hoc test).]
polymers), which indicates the intact mitochondrial membrane potential. Green fluorescence represents the monomeric form of JC-1 (JC-1 monomers), which reflects the dissipation of mitochondrial transmembrane potential. As shown in Fig. 5, significant low percentage of cells with high ΔΨm was observed in MPP⁺ injured Neuro-2a cells as compared to controls. Furthermore, RD-1 treatment markedly reversed the decrease of ΔΨm evoked by MPP⁺.

3.5. RD-1 attenuated MPP⁺-induced ATP depletion and mitochondria decrease in Neuro-2a cells

The above result indicates that MPP⁺ evoked mitochondrial depolarization in Neuro-2a cells, which reflect the early stage of mitochondrial dysfunction. RD-1 significantly rescued the decreased MMP. Then we speculated that RD-1 could attenuate ATP depletion induced by MPP⁺. Disability of mitochondria to provide sufficient energy has emerged as a hallmark of aging and various age-related neurodegenerative diseases (Rangaraju et al., 2014; Rawson et al., 2014). Using luciferase-based chemiluminescence ATP monitoring kit, we found that MPP⁺ impaired ATP synthesis, whereas 10 μMRD-1 markedly attenuated ATP depletion in MPP⁺ injured Neuro-2a cells (Fig. 6), confirming the neuroprotective effects of RD-1. Meanwhile, confocal images revealed that RD-1 treatment facilitate to increase the mitochondria mass as supported by enhanced relative fluorescence intensity (Fig. 7).

3.6. RD-1 attenuated the MPP⁺-induced neurotoxicity in cultured mesencephalic TH⁺ neurons

To verify the effect of RD-1 on MPP⁺-induced TH expression in primary mesencephalic neuron, cells were challenged with MPP⁺...
and then treated with or without RD-1. We found that MPP\(^+\) seri-
ously decreased the number of TH-positive neurons. As shown in
Fig. 8, RD-1 treatment increased the TH\(^+\)-cells number against
MPP\(^+\) neurotoxin in primary cultured mesencephalic neurons.

3.7. RD-1 improved impaired axonal mitochondrial transport
induced by MPP\(^+\)

It is well known that most of the energy supplying the brain is
generated by mitochondria in mammal body (Lovas and Wang,
2013). Many studies have demonstrated that many newly gener-
ated mitochondria in cell body must be moved the distal area for
supplying sufficient energy. Dynamic transport of mitochondria
along axons is crucial for the maintenance of neuronal fate.
Several studies showed that impaired mitochondrial axonal
transport played an important role in a variety of neurodegener-
avative disorders, including PD (Shidara et al., 2010; Saxton et al.,
2012; Sheng et al., 2012). Here we investigated the effects of
RD-1 on the mitochondrial axonal transport in cultured mesen-
cephalic neurons. The statistical results showed that MPP\(^+\) treat-
ment decreased mitochondrial speeds moving in the anterograde
direction to some extent but significantly increased that moving in
the retrograde direction (Fig. 9B). Moreover, MPP\(^+\) treatment
significantly decreased the percent of anterograde moving mito-
chondria, but increased the percent of stationary mitochondria
(Fig. 9C). In normal physiological condition, the percent of
different moving state of mitochondrion was maintained in suit-
able proportion (Sheng and Cai, 2012). The increased percent of
stationary mitochondria might reflect the existing mitochondria
dysfunction owing to MPP\(^+\) intoxicated. Treatment with RD-1
showed obvious neuroprotective effects on mitochondrial
motility, including mean motile speed and percent of moving
mitochondria, which subsequently influence the neuronal activity
based on energy.
3.8. RD-1 promoted the neurotransmitter release of synapse vesicle in primary mesencephalic neuron culture

In neurons, the synaptic terminals are sites of high energy demand and synaptic transmission requires high levels of cellular ATP for numerous energy consuming processes (Cavallucci et al., 2013), including the maintenance of synaptic membrane potential and reloading of synaptic vesicles with neurotransmitters (de Castro et al., 2010a). Then its dysfunction may influence energy-based functional activity, consequently generating pathological changes (de Castro et al., 2010b). However, the relationship between mitochondrial dysfunction and vesicle release has been questioned based on weak experimental support. Here, to further investigate the secondary effect of mitochondrial damage observed in above-mentioned experiment, FM1-43 was employed to directly measure synaptic vesicle release in cultured mesencephalic neurons. Our results showed that mitochondrial complex I inhibitor MPP+ 4 h exposure significantly decreased the efficacy of neurotransmission compared with the control neuron, which may reflect an early consequence of mitochondrial dysfunction (Fig. 10). RD-1 treatment significantly elevated the efficacy of neurotransmission, providing another evidence for its improving neuronal terminal activities.

4. Discussion

In this study, we investigated the protective effects of RD-1 on movement disorder and mitochondrial dysfunction using an acute MPTP mouse model in vivo and the MPP+ -intoxicated Neuro-2a cells and primary cultured mesencephalic neurons in vitro. L-dopa, the most commonly used medication for treating the motor symptoms, was used as positive control in vivo experiment. The results indicated that L-dopa combined with Benserazide treatment exhibited no obvious effect on the number of TH+ cells in SNpc as well as striatal TH+ fibers although it could improve the locomotor ability. Thereby, these results again show that L-dopa, as dopamine supplement therapy, can only provide the symptomatic benefits for PD but hardly have an effect on the neurodegenerative process and DAergic cell loss.

In recent years, increasing attention has been paid to the relationship between mitochondrial dysfunction and PD (Schapira, 2008; Lee et al., 2009; Gaweda-Walerych et al., 2013; Subramaniam et al., 2013; Sanders et al., 2014). Neuronal mitochondrial function and behavior in physiological conditions are the key processes for high energy demanding synaptic activity by rapidly providing energy. Therefore, the high energy demand of neurons increases the risk of mitochondrial oxidative stress. Free radicals,
mitochondrial DNA (mtDNA) mutation, impaired mitochondrial respiration and unbalanced calcium homeostasis are widely observed in many neurodegenerative diseases such as Alzheimer’s disease (AD) (Swerdlow, 2009), PD and so on. In addition, experiments performed in animal and cellular models of AD, PD and HD suggested the benefit of improvement in the mitochondrial function for attenuating and delaying neurodegeneration (Jauslin et al., 2003; Liu et al., 2005; Kasparova et al., 2006; Cleren et al., 2008; Du et al., 2008; Martin, 2010). The therapeutic strategy by targeting mitochondrial dysfunction for the treatment of neurodegenerative diseases have been widely investigated (Bogaerts et al., 2008; Reddy, 2008; Beal, 2009; Du et al., 2010). For example, progressive improvement was witness with coenzyme Q10 (CoQ10) plus vitamin E treatment in PD clinical trials, indicating the promise of mitochondrial medicine applications for the treatment of neurodegenerative disease.

To uncover the potential action of RD-1, multiple genes related with mitochondrial biogenesis, transport, dynamics and quality control were analyzed with real-time PCR and Western blotting technology. We found that Miro2 and Parkin expression levels were regulated by RD-1 treatment. Miro2, also termed as Rhot2, a RHO family GTPase that locates outer membrane of mitochondria (Birsa et al., 2013). Mutation of Miro gene in D. melanogaster results in impaired anterograde mitochondria in distal synaptic area (Guo et al., 2005; Russo et al., 2009), Parkin, the cytosolic E3 ubiquitin ligase (Kitada et al., 1998), its mutation impairs the UPS of protein degeneration, leading to the accumulation of toxic or aggregated protein. In recent years, studies have shown that Parkin, together with its functional upstream PINK1, a Ser/Thr kinase, which are mutated in autosomal recessive PD, have been found to work in the same pathway to regulate mitochondrial function and morphology (Greene et al., 2003; Clark et al., 2006; Park et al., 2006; Lim et al., 2012), which mediated the clearance of dysfunctional mitochondria via mitophagy. Parkin overexpression extends the lifespan of DAergic neurons, demonstrating its newly protective effect (Rana et al., 2013). Since these three genes are involved in the mitochondrial regulation, we further investigated the effects of RD-1 on the mitochondrial function.

Mitochondria are important modulators of neuronal viability during toxic chemical and neurotoxin exposure, wherein mitochondrial membrane potential (MMP) reflects early stage of mitochondrial dysfunction or different apoptotic stimuli and ATP depletion may be a common pathway of cell death (Wang et al., 2001). It was proposed that MPTP/MPL+− induced mitochondrial dysfunction by inhibiting complex I, altering membrane potential, consequently decreasing ATP level in Neuro-2a cells. Our results showed that RD-1-attenuated the changes in the mitochondrial membrane potential decline, impaired ATP synthesis and consequently ATP-dependent cellular processes, which reasonably protected neuronal cells against neurotoxin injury.

As mentioned above, impaired mitochondrial transport influences the distribution of mitochondria and thus consequential local energy supply. Recently, the regulatory mechanisms of mitochondrial transport were widely studied (Misko et al., 2010; Morotz et al., 2012; Chen et al., 2013). The investigation showed that MPL+− specifically impaired mitochondrial transport in DAergic axons (Kim-Han et al., 2011). In present study, our results indicated that MPL+− significantly decreased the speed of anterograde-moving mitochondrion and increased retrograde speed, which in line with the previous report (Kim-Han et al., 2011). Meanwhile, MPL+− elevated the percent of stationery mitochondrion and inhibited the percent of anterograde-moving mitochondrion. RD-1 markedly attenuated these changes induced by MPL+−. Given that RD-1 reversed the impaired mitochondrial transport, sufficient energy supply might be warranted in the distal synapses. Therefore, we utilized FM1-43 dye to further characterize the effect of RD-1 on neurotransmitter vesicle release, which reflects neuronal functional output. Indeed, a significant increase in vesicle release efficacy in RD-1-treated mesencephalic neurons was observed, which constituted the molecular basis underlying the improvement in the motor function.

5. Conclusion

Taken together, present studies demonstrated that RD-1 effectively improved movement disorder induced by MPTP. Attenuation in the mitochondrial dysfunction and secondary DAergic...
neurodegeneration underlies the mechanism of RD-1 treatment, indicating a promising candidate for PD treatment.

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