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Original Research Article

Ruscogenin regulates endogenous antioxidation in dopamine neurons by activating Keap1/Nrf2/HO-1 pathway

Kai Shi, Bing Wang*
Department of Neurology, Huai'an Hospital of Traditional Chinese Medicine, Huai 'an City, Jiangsu Province 223001, China

For correspondence:* ***Email:* *wangbing28028@163.com;* ***Tel:*** *+86-13813347793*

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Abstract

Purpose: *To investigate the effect of ruscogenin (RUS) on cell viability, lipid peroxidation and mitochondrial dysfunction in a Parkinson's disease (PD) model.*
Methods: *The neuroblastoma cell line SH-SY5Y was modified with 1-methyl-4-phenylpyridine (MPP+) to establish a PD model. RUS (1 or 10 μM) was used to treat MPP+ induced SH-SY5Y cells. Cell viability was assessed using CCK-8 assay. The concentrations of lactate dehydrogenase (LDH), superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and glutathione peroxidase (GPx) were determined by enzyme-linked immunosorbent assay (ELISA). ATP production, Ca^{2+} concentration and JC-1 were quantified using commercial kits. The expression levels of tyrosine hydroxylase (TH), Keap1, Nrf2 and HO-1 were evaluated by western blot analysis.*
Results: *RUS protected cell viability, reduced LDH production, and elevated TH expression in MPP^{+}-modified SH-SY5Y cells. RUS promoted the release of SOD, CAT and GPx, but suppressed MDA production. Furthermore, RUS enhanced ATP metabolism, decreased Ca^{2+} leakage and maintained mitochondrial function. RUS also repressed Keap1 expression but increased Nrf2 and HO-1 levels.*
Conclusion: *RUS enhances cell viability while alleviating cytotoxicity, lipid peroxidation and mitochondrial dysfunction in dopamine neurons through the activation of Keap1/Nrf2/HO-1 signaling pathway. Thus, RUS is a promising therapeutic candidate for PD treatment.*

Keywords: *Antioxidant stress, Dopamine neurons, Parkinson's disease, Ruscogenin, Keap1/Nrf2/HO-1*

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INTRODUCTION

Globally, Parkinson's disease (PD) impacts around 2 % of individuals aged 60 and above. Its main symptoms include rest tremor, bradykinesia, rigidity, cognitive impairment, and sleep disturbance [1]. There is evidence that genetic and environmental factors are implicated in PD progression [2]. However, its pathogenesis is yet to be fully clarified, and clinical treatment is also a huge challenge. There is therefore the urgent need to develop effective drugs for PD management. The primary neuropathological characteristic of PD is the depletion of dopaminergic neurons in *substantia nigra compacta* [3]. In addition, lipid peroxidation and mitochondrial dysfunction substantially contribute to the demise of dopaminergic neurons [4,5].

As one of the well-known neurotoxins, 1-methyl-4-phenylpyridine (MPP+) is transported into dopaminergic neurons and then segregated into synaptic vesicles or enriched in mitochondria [6]. Since dopaminergic neurons are highly sensitive to these compounds. MPP+ is widely used to establish different *in vitro* and *in vivo* PD models [7]. RUS is derived from traditional Chinese herb, *Radix Ophiopogon japonicus*. It has been widely used for treating chronic and acute inflammation, and has a protective effect against brain injury [8]. RUS alleviates blood-brain barrier dysfunction by inhibiting TXNIP/NLRP3 inflammasome and Mitogen-activated protein kinase (MAPK) pathway [9]. In an experimental stroke mouse model, RUS reduced cerebral ischemic injury through NF-κB-mediated inflammatory pathway [10]. In addition, RUS also plays an antioxidant role by activating Keap1/Nrf2/HO-1 pathway [11].

However, the effect of RUS on MPP+ induced PD model remains to be further investigated. This study established a PD model in SH-SY5Y cells induced by MPP+, and examined the effect of RUS on cell viability, dopaminergic neuronal cytotoxicity, lipid peroxidation levels, mitochondrial dysfunction and Keap1/Nrf2/HO-1 pathway in this PD model.

EXPERIMENTAL

Cell culture

The neuroblastoma cell line SH-SY5Y (CRL-2266, ATCC) was cultured in Dulbecco's Modified Eagle Medium (Gibco, CA, USA) with 10 % fetal bovine serum (Gibco) in an incubator set at 5 % CO_2 and 37 °C.

Cell viability assay

The SH-SY5Y cells treated with 1, 5, 10 or 50 μM RUS (no. 472-11-7, Sigma-Aldrich) were cultured in fresh medium supplemented with 10 μl CCK-8 (Glpbio, CA, USA) for 5 days. Absorbance was determined at 450 nm wavelength daily, and cell viability ratio calculated.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of LDH, SOD, MDA, CAT and GPx in the cell medium were evaluated using ELISA kits of LDH (MBS720560, MyBioSource), SOD (MBS034842, MyBioSource), MDA (MBS263626, MyBioSource), CAT (ab190523, Abcam) and GPx (MBS2516156, MyBioSource). An aliquot (100 μl) was added to ELISA well and incubated for 2 h followed by 100 μl Conjugate sulotion for 1 h. Then each well was reacted with enzyme working reagent, TMB reagent, and terminated by stop solution. Absorbance was read spectrophotometrically at 450 nm.

Evaluation of ATP and Ca^{2+}

The levels of ATP in the SH-SY5Y cells were measured using ATP assay kit (ab83355, Abcam) according to the manufacturer's manual guide. Ca^{2+} concentration was measured with Fluo-8 Calcium Flux Assay Kit (ab112129, Abcam). SH-SY5Y cells were seeded into 96-well plate and supplemented with 100 μL Fluo-8 dye-loading solution for 30 min in a cell incubator and for another 30 min at room temperature. The fluorescence intensity was monitored at 488 nm, and the Ca^{2+} concentration was computed.

JC-1 staining assay

The SH-SY5Y cells were seeded on pre-coated glass coverslips, washed by PBS and incubated with JC-1 dye (M34152, Thermo Fisher Scientific, USA) at the final concentration of 2 μM at 37 °C, 5 % CO_2 for 30 min. Then the cells were stained with DAPI for 5 min. The cells were washed twice with PBS and finally observed in a fluorescence microscopy.

Western blot

The whole cellular protein was extracted using lysis buffer (25 mM Tris-HCl pH 7.4, 250 mM NaCl, 50 mM KCl, 10 % Glycerol, 0.5 % NP-40). The samples were then subjected to gel separation and transferred to Nitrocellulose membrane followed by overnight incubation at 4 ℃ with primary antibodies against TH (25859-1-AP, ProteinTech, IL, USA), Keap1 (10503-2-AP, ProteinTech), Nrf2 (16396-1-AP, ProteinTech), HO-1 (10701-1-AP, ProteinTech) and β-actin (20536-1-AP, ProteinTech). The mixture was then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (B900210, ProteinTech) at room temperature for 1 h. The target bands were visualized with ECL reagents (Solarbio, Beijing, China). The relative intensity of each band was assessed by ImageJ software and normalized to β-actin [12].

Statistical analysis

Data are presented as mean ± standard deviation (SD) from three replicates. Differences between any two groups were calculated by unpaired t-tests, while multiple group comparisons were carried out using ANOVA.

RESULTS

RUS protects the viability of SH-SY5Y cells

The structure of RUS is shown in Figure 1 a. To evaluate the effect of RUS on the proliferation of SH-SY5Y cells, an increasing dose of RUS (1, 5, 10 and 50 μM) was administered to SH-SY5Y cells, and the data showed that RUS at doses of 1, 5 and 10 μM did not affect cell viability, although there was a significant decrease in the 50 μM RUS group (Figure 1 b). Thus, 1 and 10 μM RUS were used for subsequent experiments. In MPP+-treated SH-SY5Y cells, MPP+ reduced cell viability, while 1 or 10 μM RUS significantly increased cell viability, when compared to the MPP+ group (Figure 1 c). LDH was assessed as the indicator for cellular anaerobic metabolism, and the results showed that MPP+ induced the release of LDH, while 10 μM RUS significantly alleviated LDH production compared to the MPP+ group (Figure 1 d). Western blot results showed that MPP+ reduced the expression of TH, while 1 or 10 μM RUS significantly increased TH level compared to the MPP+ group (Figure 1 e). Thus, RUS enhanced the cell viability of SH-SY5Y in a concentration-dependent manner.

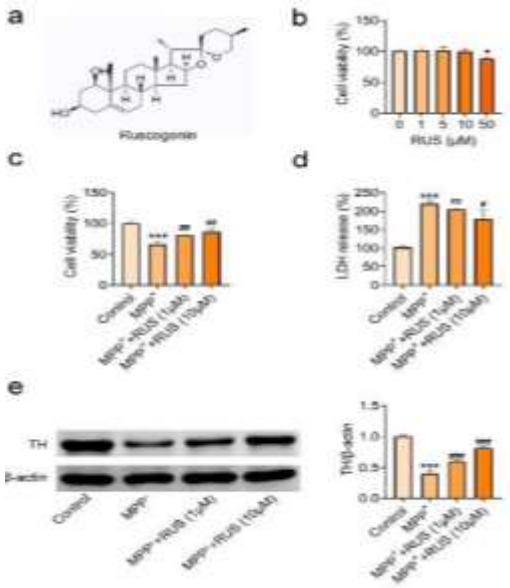

Figure 1: RUS enhanced the viability of SH-SY5Y cells. (a) Structure of RUS; (b) Viability of SH-SY5Y cells in 1, 5, 10 and 50 μM RUS; (c) Viability of SH-SY5Y cells with MPP+ induction and 1 or 10 μM RUS treatment; (d) LDH release in SH-SY5Y cells treated with 1 or 10 μM RUS; (e) Expression of TH. The data from three repeated experiments were used for the statistical analysis. Error bar, mean ± SD; */***compared to Control, #/###/#### compared to MPP+; ns, non-significant; */#$p < 0.05$, **/##$p < 0.01$, ***/###$p < 0.001$

RUS inhibited MPP+-induced lipid peroxidation

SOD, CAT and GPx levels were reduced in MPP+ treated cells, but significantly increased when treated with 10 μM RUS. However, 1 μM RUS had no significant effect on SOD, CAT and GPx. In the MPP+ group, there was an elevation in MDA production; however, the cells treated with 1 or 10 μM RUS exhibited a significant reduction in MDA production within the MPP+-treated cells (Figure 2). Therefore, RUS lowered MPP+-induced lipid peroxidation.

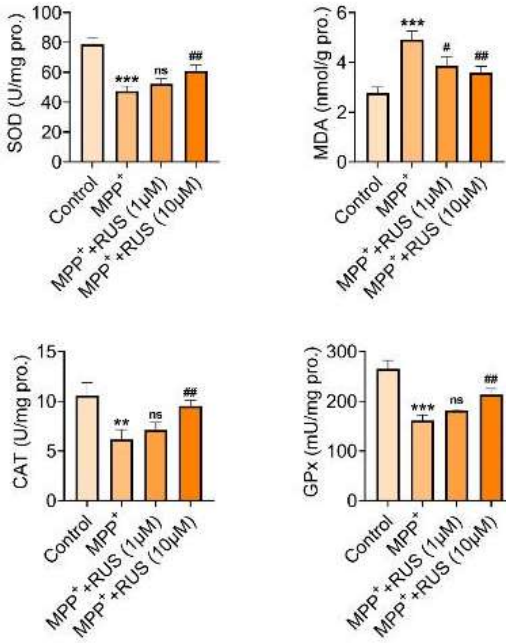

Figure 2: RUS inhibited MPP+-induced lipid peroxidation. Activities of CAT and GPx were significantly increased by 10 μM RUS when compared to MPP+ group. MDA concentration was lower in 1 or 10 μM RUS groups than in MPP+ group. All the experiments were carried out at least three times. **/***Compared to control, #/### compared to MPP+; ns, non-significant; # $p < 0.05$, **/## $p < 0.01$, *** $p < 0.001$

RUS mitigated MPP+-induced mitochondrial dysfunction

ATP concentration was reduced in SH-SY5Y cells treated with MPP+, but 10 μM RUS significantly increased ATP production, when compared to MPP+ group (Figure 3 a). MPP+ treatment elevated Ca^{2+} levels, but lowered by 1 or 10 μM RUS (Figure 3 b). When the SH-SY5Y cells were treated with MPP+, there was a decrease in the red fluorescence. Correspondingly, 1 or 10 μM RUS increased the density of the red fluorescence (Figure 3 c). Overall, RUS mitigated mitochondrial dysfunction induced by MPP+.

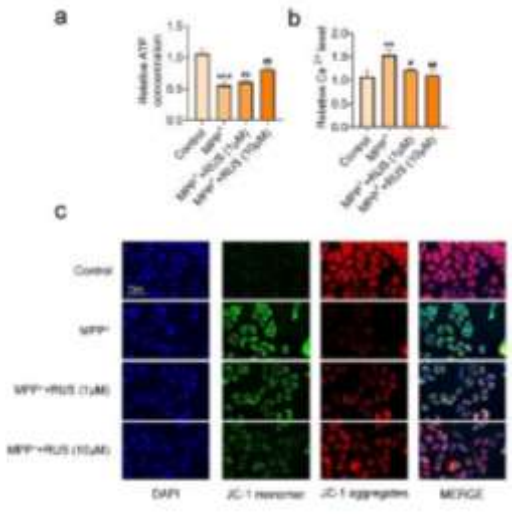

Figure 3: RUS mitigates MPP+-induced mitochondrial dysfunction. (a) ATP concentration was reduced in SH-SY5Y cells treated with MPP+, while 10 μM RUS significantly increased ATP production compared to MPP+ group; (b) MPP+ treatment elevated Ca^{2+} level, but reduced by 1 or 10 μM RUS; (c) 1 or 10 μM RUS enhanced the density of red fluorescence. Three repeated experiments were used for analysis. Error bar, mean ± SD. **/***Compared to control, #/###compared to MPP+; ns, non-significant; #$p < 0.05$, **/##$p < 0.01$, ***$p < 0.001$

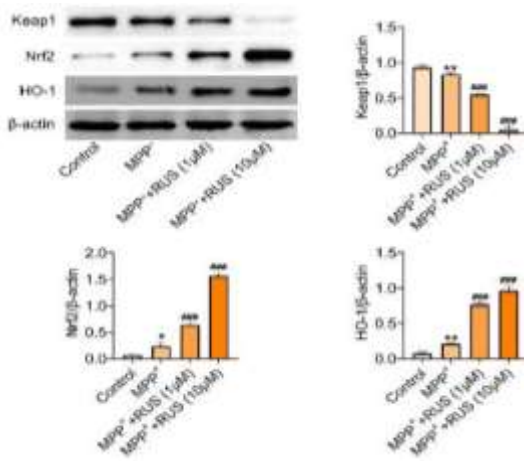

Figure 4: RUS activates Keap1/Nrf2/HO-1. 1 or 10 μM RUS significantly reduced Keap1, but increased Nrf2 and HO-1 levels compared to MPP+ group. Three repeated experiments were used for analysis. Error bar, mean ± SD; */**compared to control, ###compared to MPP+; *$p < 0.05$, **$p < 0.01$, ###$p < 0.001$

RUS activated Keap1/Nrf2/HO-1

Compared to the control group, MPP+ lowered Keap1 level and elevated but Nrf2 and HO-1 levels, meaning that Keap1/Nrf2/HO-1 signaling was activated in the PD model. On the other hand, 1 or 10 μM RUS significantly reduced Keap1 level, but increased Nrf2 and HO-1 levels compared to the MPP+ group (Figure 4). These data suggest that RUS activated Keap1/Nrf2/HO-1 pathway in PD model.

DISCUSSION

Parkinson's disease, a type of neuro-degenerative disorder, affects approximately 2 % of aged persons. However, its pathogenesis is still not fully understood. Therefore, development of efficacious drugs for Parkinson's disease management is an imperative and pressing necessity [2]. RUS has a significant protective effect against brain injury. It can alleviate the blood-brain barrier dysfunction [1]. This study focused on determining whether RUS has significant activity against PD. First, MPP+ was selected to treat SH-SY5Y cells for *in vitro* model establishment. MPP+ is transported into dopaminergic neurons and segregated into synaptic vesicles or enriched in mitochondria [6]. Due to the sensitivity of dopaminergic neurons to MPP+, MPP+ is widely used to establish various *in vitro* and *in vivo* PD models [7].

Ruscogenin is involved in various pharmaco-logical activities, including anti-thrombotic, anti-inflammatory, and antioxidant stress. One of the mechanisms through which RUS exerts its antioxidant effects is by inhibiting lipid peroxidation. In lipid peroxidation, the free radicals oxidize poly-unsaturated fatty acids, leading to cellular damage and dysfunction. Ruscogenin has been shown to inhibit this process by scavenging free radicals and stabilizing cell membranes [11].

Mitochondrial dysfunction refers to a state where the energy-producing organelles in cells are not functioning properly. When the mitochondria fail to function properly, they do not produce enough energy for the cell to work effectively [14]. In mitochondrial dysfunction, increased oxidative stress further exacerbates mitochondrial damage and dysfunction, thereby creating a vicious cycle. This can result in a variety of health issues, including neurodegenerative diseases, cardiovascular disease, diabetes, and cancer. Reducing oxidative stress is an important part of treating mitochondrial dysfunction [15]. In this study, the results show that 10 μM RUS significantly enhanced ATP production and the membrane potential of mitochondria, indicating that RUS has an inhibitory effect on oxidant stress. Therefore, RUS plays a role in relieving mitochondrial dysfunction.

The Keap1/Nrf2/HO-1 pathway protects cells from oxidative stress and inflammation. The pathway involves three main proteins: Keap1, Nrf2, and HO-1. While Keap1 is a cytoplasmic

protein involved in Nrf2 activity, Nrf2 is a transcription factor which regulates the genes involved in cellular defense against oxidative stress [16]. When exposed to oxidative stress or other triggers, Nrf2 is liberated from Keap1 and translocated to the nucleus, where Nrf2 binds to antioxidant response elements [17]. This binding leads to the transcriptional activation of these genes, including HO-1. HO-1 is an enzyme that catalyzes heme into biliverdin, carbon monoxide (CO), and iron. HO-1 is induced by Nrf2 and is a critical antioxidant and anti-inflammatory protein. It has been shown to protect against a range of pathological conditions, including cardiovascular disease, neurodegenerative diseases, and cancer. Activation of Keap1/Nrf2/HO-1 pathway has been proposed as a potential therapeutic target for a variety of diseases associated with oxidative stress and inflammation [13]. Strategies which activate this pathway include dietary interventions such as consumption of flavonoids and other plant-derived compounds, as well as pharmacological interventions targeting Nrf2 and related proteins. This study demonstrates that RUS inhibits Keap1 expression, but increases Nrf1 and HO-1, thus activating Keap1/Nrf2/HO-1 signaling pathways. Thus, RUG is a therapeutic option for the inhibition of dopamine neuron death and reducing the rate of PD.

CONCLUSION

RUS promotes SH-SY5Y cell viability by activating Keap1/Nrf2/HO-1 signaling pathway, alleviates MPP+-induced dopaminergic neuronal cytotoxicity, and reduces lipid peroxidation levels and mitochondrial dysfunction. Thus, RUS can potentially be developed for the treatment of PD.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Kai Shi, Bing Wang designed the study and carried them out, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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