Breviscapine alleviates MPP+-induced damage and apoptosis of SH-SY5Y cells by activating Nrf2 pathway

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Abstract

Purpose: To investigate the role and mechanism of action of breviscapine (Brp) in 1-methyl-4-phenylpyridinium ion (MPP+)-induced cell injury in human neuroblastoma cell line, SH-SY5Y.

Methods: The injury on SH-SY5Y cells was induced using MPP+. Cell viability and apoptotic ability were determined by CCK8 assay and Annexin V/PI staining, respectively. Protein expressions of nuclear factor E2-related factor 2 (Nrf2) and its related downstream proteins - hemeoxygenase 1 (HO-1) and NAD(P)H-quinone oxidoreductase 1 (NQO1), were determined using Western blotting.

Results: Brp dose-dependently attenuated MPP+ induced reduction in the viability of SH SY5Y cells, but alleviated MPP+-induced oxidative stress (OS) and cell injury, as evidenced by the levels of reactive oxygen species (ROS), tyrosine hydroxylase (TH), lactic dehydrogenase (LDH), and dopamine transporter (DAT) (p < 0.05). Brp decreased the amount of apoptotic cells induced by MPP+, as well as the protein levels of Bax and cleaved-caspase 3, and also induced the activation of Nrf2 signaling pathway (p < 0.05).

Conclusion: Brp alleviates MPP+-induced cellular damage and cell apoptosis in SH-SY5Y cells by activating Nrf2 pathway. Thus, Brp is a potential therapeutic candidate for the treatment of PD.

Keywords: Breviscapine, Parkinson's disease, Oxidative stress, Nrf2 pathway, SH-SY5Y cell

INTRODUCTION

Parkinson’s disease (PD), a degenerative neurological disorder, displays motor symptoms including bradykinesia, resting tremor, and difficulties with gait and balance. The typical feature of PD is the prominent death of dopaminergic neurons in the Substantia nigra compact (SNpc) area, and the development of neuronal Lewy bodies [1]. Inflammation and oxidative stress (OS) are critically involved in the pathophysiological process of PD. Potential therapeutic targets for PD treatment include agents that can work against neuroinflammation, mitochondrial dysfunction and OS [2]. Higher levels of basal ganglia were found in SNpc neurons which are related to elevated intracellular calcium loads. Nuclear factor-erythroid 2-related factor 2 (gene: Egr2) is an important activator of the Nrf2 signaling pathway, which plays a critical role in cellular antioxidant defenses and resistance to oxidative stress.
**EXPERIMENTAL**

**Culture and treatment of SH-SY5Y**

Human neuroblastoma cell line SH-SY5Y (ATCC, Manassas, VA, USA) was cultured in DMEM/F12 medium with the supplementary of FBS (10%). When 80 % confluence was attained, the cells were collected and seeded onto a microtiter plate (2×10^5 cells/mL) for further treatment. To induce cellular injury, molecule 1-methyl-4-phenylpyridinium ion (MPP+) was administered into SH-SY5Y cell line at a dose of 500 μM for 24 h. For the Brp group, the cells were exposed to Brp (20, 50 and 80 μM) for 2 h, and then administered with MPP+ (500 μM) for further 24 h. After treatment, cell viability, apoptosis and protein expression were investigated.

**Evaluation of cell viability**

After treatment, CCK-8 solution was added to each test well and incubated (1 h). The absorbance (at 450 nm) of each test well was read using microplate reader.

**Flow cytometry**

A commercial kit (Annexin V-FITC/PI kit, ThermoFisher, V13242, Waltham, MA, USA) was applied to determine the cell apoptotic rate. The cells were briefly cultured in six-well plates, collected and stained (Annexin-V/PI). Flow cytometry (Becton Dickinson Medical Devices Co. Ltd. Sparks, MD, USA) was applied to determine apoptotic cells, and then quantified. The normal (Q4), necrotic (Q1), early apoptotic (Q3) and late apoptotic cells (Q2) were distinguished in a scatter plot.

**Lactic dehydrogenase (LDH) leakage analysis**

LDH leakage was performed using a commercial kit (Beyotime Biotechnology, Shanghai, China). Briefly after treatment, the cell supernatant (100 μL) was centrifuged and collected in a 96-well plate, and the reaction solution (100 μL) was added and incubated (30 min). The absorbance (at 490 nm) was detected.

**Intracellular reactive oxygen species (ROS) production analysis**

Cells were incubated with 2’,7’-dichlorofluorescin diacetate (DCFDA, 15 min). Fluorescence was captured using a fluorescence microscope equipped with a filter capable of measuring an excitation filter (485-nm) and an emission filter (535-nm).

**Dopamine (DA) analysis**

Evaluation of DA was performed using Dopamine ELISA kit (Novus Biologicals, KA1887, Littleton, Colorado, USA).

**Western blotting**

SH-SY5Y cells were lysed using a RIPA buffer. The BCA method was used to obtain the protein concentration. Proteins in equal amounts were separated using electrophoresis on SDS-PAGE gel (10 %v/v) and transferred to PVDF membranes. The membranes were blocked with milk (5 %, at room temperature for 1 h), and with the incubation (overnight at 4 °C) of anti-Bax (1:1500, CST #2772), anti-cleaved caspase 3 (1:1000, CST #9661, Cell Signaling Technology, Danvers, MA, USA), anti-TH (1:1000, CST #2332) and anti-NRF2 (1:1000, CST #3215).
anti-DAT (1:1500, Sigma #AB1591P, St. Louis, MO, USA), and then with the incubation of anti-Rabbit-IgG (1:10000, CST #7233, 2 h). Protein expression was visualized and quantified using an ECL system and ImageJ software.

Statistical analysis

The data are expressed as mean ± SD, and were analyzed by SPSS software. One-way ANOVA and Tukey’s test were applied for statistical analysis. \( P < 0.05 \) was considered statistically significant.

RESULTS

Brp attenuated the decreased in vitro cell viability induced by MPP+

In MPP+-treated SH-SY5Y cells, the functional role of Brp on in vitro viability was analyzed using CCK-8 assay. Compared to control group, MPP+ treatment decreased the in vitro viability by about 50 % (Figure 1, \( p < 0.01 \)). However, in MPP+-treated SH-SY5Y cells, Brp dose-dependently attenuated the reduction in in vitro viability (Figure 1, \( p < 0.05 \) for 20 \( \mu M \), \( p < 0.01 \) for 50 and 80 \( \mu M \) vs. MPP+ group). In particular, Brp at a dose of 80-\( \mu M \) restored the in vitro viability of MPP+-treated cells to about 90 % of that in the control group.

Brp alleviated OS and injury in MPP+-treated cells

MPP+ induced OS and led to injury in SH-SY5Y cells, evidenced by ROS production (Figure 2 A) and release of LDH (Figure 2 B), respectively. However, Brp strongly inhibited the MPP+ induced levels of ROS and LDH. The accumulation of dopamine (DA) in the cytosol led to the formation of ROS in the culture medium. The results showed that DA level in the culture medium markedly decreased to about 60 % after MPP+ treatment when compared to the control group, which was then reversed using Brp (Figure 2 C). In addition, MPP+ significantly decreased the level of tyrosine hydroxylase (TH, for DA biosynthesis) and dopamine transporter (DAT, for DA reuptake), indicating that MPP+ causes cytotoxicity in SH-SY5Y cells. Brp treatment reduced MPP+-induced cytotoxicity, as evidenced by the decreased expression levels of TH and DAT (Figure 2 D). Therefore, Brp protected SH-SY5Y cells against OS and cellular injury caused by MPP+.

Brp inhibited cell apoptosis caused by MPP+

Cells that emerged in Q2 (late apoptotic cell) and Q3 (early apoptotic cell) were considered as part of the apoptotic cell numbers. In the MPP+ group, the apoptotic level of SH-SY5Y cells was markedly increased to 42.20 %, showing higher percent apoptotic than the control group (2.28 %, Figure 3 A, \( p < 0.01 \)). After treatment with Brp, the amount of apoptotic cells dose-dependently decreased, especially in the group that was treated with MPP+ and Brp, the amount of apoptotic cells decreased to 10.95 % (Figure 3 B). The activity of caspase 3 and the levels of Bax and cleaved-caspase 3 were further determined. As shown in Figure 3C, MPP+ significantly increased the activity of caspase 3, indicating the activation of apoptosis-related pathway. Brp dose-dependently decreased the MPP+-induced activity of caspase 3. The anti-apoptotic effects of Brp were further confirmed by determining the expression levels of Bax and cleaved-caspase 3. Western blot results (Figure 3D) indicated that MPP+ induced these protein
expressions, which was then inhibited by Brp (Figure 3 E and F).

**Figure 3:** Brp inhibited cell apoptosis caused by MPP+.
A. Cell apoptosis was analyzed by Annexin V/PI staining. B. The quantification in the rate of apoptotic cells. C. Caspase 3 activity. D. Western blotting and then quantified (E - F). **p < 0.01 vs. control, & p < 0.05, && p < 0.01 vs. MPP+**

**Brp induced activation of Nrf2 signaling pathway in MPP+-treated cells**

When cells are under OS, Nrf2 translocates to the nucleus and induces the transcriptional activation of detoxifying or antioxidant enzymes such as HO-1 and NQO1. Thus, their protein expression levels were assessed further. The results (Figure 4) showed that, compared with control group, MPP+ decreased Nrf2, HO-1 and NQO1 protein expression levels (p < 0.01), which were then reversed by Brp treatment. These results indicate that Brp activated the Nrf2 pathway.

**Figure 4:** Brp induced the activation of Nrf2 signaling pathway in MPP+-treated cells. Western blotting and then quantified. **P < 0.01 vs. Control, &p < 0.05, &&p < 0.01 vs. MPP+**

**DISCUSSION**

Research on the pathogenesis of PD has advanced substantially. Inflammation, OS, excitotoxicity, and loss of neurotrophic support are important factors that contribute to this disease [2]. PD is still an incurable progressive disease, and therapies for PD are mainly symptomatic treatments and measures to improve quality of life. Dopamine replacement therapy is one of the effective methods which can be utilized to reduce motor handicap and PD associated depression, pain [10].

Earlier research suggested that SNpc neurons are particularly prone to higher levels of mitochondrial OS [11]. SH-SY5Y is a commonly used cell line for the construction of cell models of neurodegenerative diseases like PD, due to their specific neuroproteins, functional axonal vesicle, as well as transport and synaptic structures [12]. MPP+, the active toxic metabolite of neurotoxin MPTP, lead to impaired mitochondrial energy metabolism, thus enhancing the production of ROS [13]. Besides, MPP+ decreases mitochondrial DNA, aggravates OS and leads to subsequent apoptotic cell death [14]. In this research, MPP+ (final concentration of 500 μM) induced apoptosis and OS in SH-SY5Y cells. However, the effects of MPP+ were inhibited by Brp.

A recent study indicated that Brp showed obvious neuroprotective effects [9]. Jiang et al. have reported that Brp promoted neurobehavioral function following neurotrauma through the inhibition of the GSK3β signaling pathway in traumatic brain-injured rats [15]. In AD transgenic mouse model, Brp treatment for three months rescue learning deficits, relieved memory retention, decreased Aβ burden, and attenuated the function of hippocampal neurons [8]. In addition, some researchers have reported that the neuroprotective effects of Brp were partly dependent on its anti-oxidant properties. Li et al found that Brp alleviated traumatic brain injury (TBI)-induced neuronal cell apoptosis and further improved neurobehavioral functions through the upregulation of Nrf2 [9]. The present results are in accordance with previous reports. This research demonstrated that Brp attenuated the MPP+-induced ROS production and apoptosis of SH-SH5Y cells by increasing the levels Nrf2, HO-1 and NQO1. Nrf2 is an essential TF that encodes antioxidant and phase II enzymes including HO-1 and NQO1, which are closely associated with protection against OS [16].

Progressive decline in the DA of PD patients is responsible for both motor and non-motor...
symptoms [17]. DA initiates OS and induce neuronal cell death in neurodegenerative disorders. Also, others reported that OS partly contributes to the cascade DA deficiency in PD. Although it remains to be studied which occurs first, it is certain that OS and DA deficiency are closely related processes [18]. The present results showed that MPP+ induced ROS production, accompanied by DA reduction. In SH-SY5Y cells, the effects of MPP+ were reversed by Brp. The efficacy of Brp on PD in vivo experiments needs further investigation.

CONCLUSION

This research suggests that in MPP+ -treated SH-SY5Y cells, Brp alleviates damage and apoptosis by activating Nrf2 pathway. Thus, Brp is a probable novel therapeutic candidate for PD.

DECLARATIONS

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. Dongzhu Li and Xianpei Tan designed the study and supervised the data collection; Qiang Tu and Mingqing Xiang analyzed and interpreted the data; and Yamei Wang, Ming Yu and Tao Tan prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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REFERENCES

