Neuroprotective effects of ginsenoside Rg1 through the Wnt/β-catenin signaling pathway in both in vivo and in vitro models of Parkinson's disease

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Abstract

Ginsenoside Rg1 (Rg1) is a major bioactive ingredient in *Panax ginseng* that has low toxicity and has been shown to have neuroprotective effects. The objectives of the present study were to explore the potential of the application of Rg1 for the treatment of Parkinson’s disease (PD) and to determine whether its neuroprotective effects are exerted through the Wnt/β-catenin signaling pathway by using in vivo and in vitro models of PD. In the in vivo study, Rg1 treatment ameliorated the behavioral deficits of "Pole test", and reduced dopaminergic cell loss that were induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in a dose-dependent manner in an in vivo model of PD. In the in vitro study, cell viability was increased and cell apoptosis induced by 1-methyl-4-phenylpyridinium (MPP+) was decreased by Rg1 pretreatment. Rg1 induced protective effects on the protein and mRNA expression levels of markers of the Wnt/β-catenin signaling pathway in both the in vivo and the in vitro studies, and these neuroprotective effects were blocked by DKK1 in the in vitro study. Our results provide evidence that Rg1 has neuroprotective effects in both in vivo and in vitro PD models, and these effects act through the Wnt/β-catenin signaling pathway. Taken together, these results indicate that Rg1 may exert therapeutic effects on PD via the Wnt/β-catenin signaling pathway and may therefore provide a novel approach for the treatment of PD.

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1. Introduction

Parkinson's disease (PD) is a common progressive neurodegenerative disorder that is characterized by the gradual, progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and decreased dopamine levels in the striatum (caudate and putamen) of the basal ganglia (Lees et al., 2009; Badger et al., 2014). Despite decades of research, the further investigation of molecular mechanisms underlying the progressive loss of dopaminergic neurons in PD remain indispensable (Jenner and Olanow, 2006; Yang et al., 2009). Understanding these mechanisms is essential because it may provide the means for future therapeutic strategies. Current pharmacological treatments for PD provide only symptomatic treatment and do not prevent the progressive loss of dopaminergic neurons in PD patients (Schipera, 2009; Olanow and Schipera, 2013). Thus, further insights into the molecular mechanisms of PD and the discovery of new therapeutic agents for PD that have higher efficacy are needed.

While the underlying mechanisms of PD are not yet completely understood, accumulating evidence indicates that dysfunction in the Wnt/β-catenin signaling pathway may be an important aspect

Abbreviations: Rg1, ginsenoside Rg1; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP+, 1-methyl-4-phenylpyridinium; DKK1, Dickkopf-1; PFA, paraformaldehyde; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CKK-8, cell counting kit-8; RT-PCR, Reverse transcription polymerase chain reaction; CNS, central nervous system.

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The Wnt/β-catenin signaling pathway is also called the canonical Wnt signaling pathway, and it controls neuronal survival (Castelo-Branco et al., 2003), participates in the development of the central nervous system (CNS) and regulates the function of the adult nervous system (Inestrosa and Arenas, 2010). Evidence has indicated that dysfunction in the Wnt/β-catenin signaling pathway plays an important role in the pathophysiology of nigrostriatal dopaminergic neurons in PD models (L’Episcopo et al., 2014). The canonical Wnt signaling pathway can be blocked by the extracellular protein Dickkopf-1 (Dkk1), which binds to LRP5/6 and contributes to neurotoxicity in PC12 cells and in neurodegenerative disease (Dun et al., 2013; Scott and Brann, 2013). Because many of the components of this signaling pathway have been identified in the adult brain, Wnt/β-catenin signaling may be important for maintaining neuronal survival. Therefore, the activation of the Wnt/β-catenin signaling pathway in PD models may be crucial to modify disease progression (Parish and Thompson, 2014).

Ginsenoside Rg1 (Rg1), one of the most active ingredients in ginseng, has been suggested to have potential therapeutic effects for neurodegenerative diseases. Its chemical structure is shown in Fig. 1. Some studies have investigated the beneficial effects of Rg1 on the CNS in animal models and cultured neuronal cells (Radad et al., 2004; Shen and Zhang, 2007). In recent years, some studies have shown that ginsenosides such as Rg1 have beneficial effects in PD models that are associated with antiapoptotic, antioxidant and other molecular mechanisms (Chen et al., 2005, 2003; Xu et al., 2009). However, the mechanisms underlying the neuroprotective effects of Rg1 on dopaminergic neurons remain to be elucidated. To date, no report has investigated whether the neuroprotective effects of Rg1 are exerted through the Wnt/β-catenin signaling pathway in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced and 1-methyl-4-phenylpyridinium (MPP+) ‐induced PD models.

In this study, we established an in vivo PD model by using MPTP, a type of neurotoxin that can cause the loss of dopaminergic neurons in the SNpc of animals, leading to a parkinsonian-like syndrome (Przedborski et al., 2000). In an in vitro PD model, MPP+ was chosen. MPP+ is the active metabolite of MPTP, and it induces cell death in a rat adrenal gland pheochromocytoma cell line (PC12 cells) (Itano et al., 1994).

In the present study, we wished to investigate whether Rg1 treatment exerts neuroprotective effects in PD models and whether these effects were associated with the activation of the Wnt/β-catenin signaling pathway. Our results indicated that the neuroprotective effects of Rg1 in both in vivo and in vitro PD models might be mediated through the activation of the Wnt/β-catenin pathway.

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies complied with the ARRIVE guidelines. The animal protocols were approved by the Committee on the Ethics of Animal Experiments of the Dalian Medical University. All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to in vivo techniques, if available.

2.2. Animals and treatment

C57BL/6J mice (6–8 weeks old, male, weighing 16–25 g), purchased from the Experimental Animal Center of Dalian Medical University (SPF level), were used for this study. The mice were maintained in a constant environment with a temperature of 20–22 °C and 50–60% humidity under a 12-h light/dark cycle of artificial light (lights on at 08:00) with free access to food and water. After adaptive feeding for one week, the mice were randomly divided into six groups: (i) control group; (ii) Rg1 20 mg/kg group; (iii) MPTP 30 mg/kg group; (iv) MPTP 30 mg/kg + Rg1 5 mg/kg group; (v) MPTP 30 mg/kg + Rg1 10 mg/kg group; and (vi) MPTP 30 mg/kg + Rg1 20 mg/kg group. Each experimental group consisted of 10 mice, and a total of 60 mice were used.

The subchronic method of an MPTP-induced PD model was established. Groups receiving MPTP injections (groups iii, iv, v, and vi) were administered an intraperitoneal injection (i.p.) with MPTP-HCl (Sigma—Aldrich, USA) in saline at a dosage of 30 mg/kg/day for each mouse for 5 consecutive days (Jackson-Lewis and Przedborski, 2007). Groups with Rg1 administration (groups iv, v, and vi) were treated i.p. with Rg1 12 h before the MPTP injection and then treated with Rg1 for another 10 days post-treatment. The group with only Rg1 (group ii) was treated with Rg1 for 15 consecutive days. The control group (i) was administered i.p. with the same volume of saline for 15 consecutive days.

2.3. Drugs

Rg1 (HPLC > 98%) was purchased from Dalian Melonepharma Biological Technology Co., Ltd. (Dalian, China). MPTP and MPP+ were purchased from Sigma—Aldrich (Sigma, USA). Monoclonal anti-mouse TH was purchased from ImmunoStar, Inc. (ImmunoStar, USA). Polyclonal anti-mouse/rat Wnt1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology, USA). Polyclonal anti-mouse/rat β-catenin, GSK-3β and p-GSK-3β were purchased from Cell Signaling Technology, Inc. (Beverly, USA). The Wnt/β-catenin signaling pathway inhibitor DKK1 was purchased from Sigma—Aldrich, Inc. (St. Louis, MO, USA). The secondary antibodies for immunofluorescence were purchased from Jackson ImmunoResearch Laboratories, Inc. (Jackson ImmunoResearch Laboratories, USA). The reagents for reverse transcription polymerase chain reaction (RT-PCR) were purchased from Life Technologies Inc. (Carlsbad, CA, USA). TUNEL staining kits were purchased from Boehringer Mannheim (detection kit, BM, Germany). The cell counting kit-8 (CCK-8) kit was purchased from Biosynthesis Biotechnology Co. Ltd. (Beijing, China). The BCA kit and Enhanced Chemiluminescence (ECL) were purchased from Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Gibco BRL (Gaithersburg, MD, USA).

![Fig. 1. Chemical structure of ginsenoside Rg1.](image-url)
2.4. Behavior test

All mice were submitted to the “Pole test” to measure motor coordination 1 day prior to MPTP injection and then on the 5th, 10th, and 15th day after the MPTP injection, and they were trained for the test 3 days before the MPTP injection (Sedelis et al., 2001). The “Pole test” consists of a gauze-taped pole (50 cm high, 1 cm in diameter) with a small cork ball at the top. The mice were placed with their head upward right below the ball. Two times were recorded: the time it took for the mouse to turn completely downward (T-turn) and the time it took the mouse to descend to the floor (T-total), with a cut-off limit of 60 s. The tests were performed 3 times with 10-min intervals and the average time was calculated.

2.5. Brain tissue preparation

The mice in each group were sacrificed when the treatment was completed, and the brains were isolated for further experiments. Half of the mice in each group were prepared for immunofluorescence. These mice were anesthetized and rapidly perfused through the aorta with saline for 15 min and then with precooled (4 °C) 4% paraformaldehyde (PFA) for 10 min. Then, the brains were quickly removed and immersion postfixed in 4% PFA at 4 °C for 12 h. The brain was then dehydrated sequentially in 20% and 30% sucrose in 0.1 M PBS for immunofluorescence. The other half of the mice in each group were prepared for western blot analysis and RT-PCR. The mice were sedated under general anesthesia and sacrificed by cervical dislocation. Then, the brains were quickly removed and stored at −80 °C for further analysis.

2.6. Cell culture

Rat pheochromocytoma PC12 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) with 100 U/ml penicillin and 100 μg/ml streptomycin and placed in a water-saturated atmosphere of 95% air and 5% CO2 at 37 °C. The culture medium was changed every 3 days, and the cells were subcultured every 5 days. The cells were seeded at a density of 30,000 cells/cm2. The medium was changed to serum-deprived medium or medium supplemented with 1% FBS 24 h before experiments, and PC12 cells were seeded in 96- or 6-well plates. The cells were pre-treated or not with 20 μM Rg1 for 24 h and then treated with 500 μM MPP+ for 24 h. The Wnt/β-catenin signaling pathway inhibitor DKK1 was added to PC12 cells 1 h prior, when used.

2.7. Immunofluorescence

Brain sections (20 μm thick) from each mouse were cut and mounted on glass slides. Hydrated slides were washed in 0.01 M PBS for 10 min. Antigen retrieval was performed with sodium citrate (pH 6.0) for 6 min, 2 times, in a microwave and then cooled at room temperature for 30 min. The slides were then rinsed 3 times and incubated in 0.3% Triton X-100 for 30 min. After washing in PBS, the sections were incubated for 30 min at 37 °C with 10% normal goat serum. The sections were then incubated with mouse anti-tyrosine hydroxylase (TH; 1:4000; Immunostar, USA) at 4 °C for 16–24 h. After overnight incubation, sections for TH were rinsed and incubated in darkness for 2 h with TRITC-conjugated goat anti-mouse antibody (1:500; Jackson ImmunoResearch Laboratories, USA). After washing, the slides were coverslipped with glycerol-based mounting medium. Positive cells were measured using image analysis software (ImageJ, 1.46, Scion Corporation). For cell immunofluorescence, PC12 cells were seeded in glass-bottom 6-well plates and fixed with 4% PFA for 20 min. Then, the protocols were the same as for brain sections, except that the cells were incubated with rabbit anti-β-catenin (1:200; Cell Signaling Technology, USA) and rabbit anti-GSK-3β (1:400; Cell Signaling Technology, USA) antibodies and Alexa Fluor 488-conjugated goat anti-rabbit antibodies (1:200; Jackson ImmunoResearch Laboratories, USA), followed by DAPI-staining of nuclei. Images were acquired with a fluorescence microscope (LEIDA DM4000B, LEICA, Germany).

2.8. TUNEL assay

Brain sections were incubated with 0.3% Triton X-100 in 0.1 M PBS for 30 min at room temperature and then with FITC-conjugated anti-fluorescein antibody (TUNEL detection kit, BM, Germany) for 2 h at room temperature. The slides were then rinsed 3 times and coverslipped with glycerol-based mounting medium. For cell immunofluorescence, PC12 cells were seeded in 6 glass-bottom well plates and fixed with 4% PFA for 20 min. Then, the protocols were the same as for brain sections. Images were acquired with a fluorescence microscope (LEICA DM4000B, LEICA, Germany). The numbers of TUNEL-positive cells in the brain sections were counted.

2.9. Western blotting analysis

Brain tissue was stored at −80 °C until homogenization in lysis buffer for 15 min. The homogenate was centrifuged, and the supernatants were collected. The total protein of PC12 cells was isolated in the same way as described for in vivo experiments. Total protein was estimated using a Bicinchoninic Acid Kit for Protein Determination (Biosynthesis Biotechnology, China). Then, the samples were mixed with SDS sample buffer and boiled for 10 min. Equal amounts of protein samples (40 μg) were separated by 10% SDS/PAGE gel electrophoresis and then transferred to PVDF membranes (Amersham Biosciences, Uppsala, Sweden). The membranes were incubated in 5% fat-free milk in wash buffer (0.01 M Tris, 0.05% Tween-20) for 30 min to block non-specific binding. Then, the membranes were incubated with primary rabbit polyclonal antibodies for Wnt1 (1:400; Santa Cruz Biotechnology, USA), β-catenin (1:200, Cell Signaling Technology, USA), GSK-3β (1:400; Cell Signaling Technology, USA); p-GSK-3β (1:400; Cell Signaling Technology, USA); cleaved caspase-3 (1:200; Cell Signaling Technology, USA) or Bcl-xL (1:400; Cell Signaling Technology, USA) overnight at 4 °C. The next day, PVDF membranes were incubated with goat anti-rabbit secondary antibodies (1:2000; Santa Cruz) and visualized via chemiluminescence. The bands were imaged by a Molecular Imager Chemic Doc XR system (Bio-Rad, Richmond, USA). The optical density of the bands was determined using Quantity One (4.62, Bio-Rad).

2.10. Reverse transcription polymerase chain reaction (RT-PCR)

The bilateral SNpc regions were rapidly dissected on ice under a microscope, and total RNA was isolated from the dissected tissue with TRIzol reagent (Invitrogen, San Diego, CA, USA). The total RNA of PC12 cells was isolated in the same way as described for in vivo experiments. Then, first-strand cDNA was synthesized from total RNA using the reverse transcription kit from Life Technologies Inc. (Carlsbad, CA, USA) according to the manufacturer’s recommendations. For cDNA synthesis, total RNA samples (2 μg) were subjected to reverse transcription with 50 uM Oligo(dT)18 and 1ul (15U/ul) ThermoScript™ RT enzyme in 20ul of reaction mixture at 50 °C for 30 min. Terminate the reaction by incubating at 85 °C for 5 min. Then, amplification reactions were performed accordingly. The
2.11. Cell viability

PC12 cells were plated in 96-well plates at 2 × 10^3 per well in a final volume of 100 µL following treatment using the CCK-8 kit according to the manufacturer's recommendations. In brief, cells were incubated in 10% CCK-8 that was diluted in DMEM at 37 °C for 4 h (37 °C, 5% CO2 atmosphere). The absorbance of the samples was measured at 450 nm with a microplate reader (Epoch; BioTek, Winooski, VT, USA).

2.12. Statistical analyses

All quantitative data were analyzed using SPSS 17.0. The results are expressed as the mean ± standard error of the mean (mean ± SEM). The data were analyzed using a one-way ANOVA followed by Tukey's multiple comparison test. Differences with P < 0.05 were considered significant. All statistical analyses in this study were performed using Prism 5 for Windows, version 5.01 (GraphPad Software, Inc.).

3. Results

3.1. Effects of Rg1 on mouse behavior in the “Pole test”

The behavioral deficits in the MPTP-induced mouse model of PD are useful for investigating the relationship between dopaminergic neuron degeneration and recovery processes, particularly in motor activity. A “Pole test” was applied to assess whether the MPTP-induced mouse model successfully induced bradykinesia and to analyze the therapeutic effects of Rg1. As shown in Fig. 2, the effects of Rg1 on mouse behavior was assessed compared with the no Rg1-treated groups. The “T-turn” and “T-total” times significantly increased following injection of MPTP (30 mg/kg) (P < 0.01, compared with the control group) and were significantly reduced following administration of Rg1 (P < 0.01, compared with the MPTP group). The data revealed that Rg1 (5, 10, or 20 mg/kg, i.p.) treatment produced a dose-dependent reduction in the “T-turn” and “T-total” times. Motor immobility times were significantly reduced with higher doses of Rg1 (n = 10, P < 0.01 compared with control group).

3.2. Effects of Rg1 on SNpc region cells in vivo

The number of TH-positive neurons in the SNpc region (TH is a diagnostic marker of dopaminergic neurons) was used to evaluate the neuronal protective effects of Rg1 in the MPTP-induced mouse model. The quantification of TH-positive cells was performed by counting the number of TH-positive cells in the SNpc. As shown in (Fig. 3A, B), MPTP-treated mice exhibited a significant reduction in the number of TH-positive neurons compared with the control group (P < 0.01). However, when MPTP was combined with Rg1 treatment, the number of TH-positive neurons significantly increased compared with the MPTP-treated group (P < 0.01). The number of TH-positive neurons in the Rg1 group was similar to that in the control group. The results showed a dose-dependent reduction following Rg1 (5, 10, 20 mg/kg, i.p.) treatment in the number of TH-positive cells. The number of TH-positive cells was significantly increased with higher doses of Rg1 (n = 5, P < 0.01 compared with control group). TUNEL staining of the SNpc after MPTP injection showed a significant increase in apoptotic bodies, and this effect was attenuated by Rg1 treatment (Fig. 3C, D, P < 0.01). The data showed a dose-dependent reduction in apoptotic cell bodies following Rg1 (5, 10, or 20 mg/kg, i.p.) treatment. These results show that Rg1 provides neuroprotective effects against MPTP-induced injury.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primer sequences used in reverse transcription polymerase chain reactions.</th>
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<tr>
<td>mRNA</td>
<td>Forward primers</td>
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<tr>
<td>Mouse</td>
<td>Wnt1</td>
</tr>
<tr>
<td></td>
<td>β-Catenin</td>
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<td></td>
<td>GSK-3β</td>
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<tr>
<td></td>
<td>β-actin</td>
</tr>
<tr>
<td>Rat (PC12)</td>
<td>Wnt1</td>
</tr>
<tr>
<td></td>
<td>β-Catenin</td>
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<tr>
<td></td>
<td>GSK-3β</td>
</tr>
<tr>
<td></td>
<td>β-actin</td>
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Fig. 2. Effects of Rg1 on the “T-turn” and “T-total” times. The “Pole test” was conducted on mice on days 0, 5, 10, and 15 following treatment with MPTP (30 mg/kg/d, i.p.) and/or Rg1, and the average time was recorded. (A) T-turn. (B) T-total. Statistical evaluations were conducted using a one-way ANOVA ( *P < 0.01 and **P < 0.05 compared with the control group; #P < 0.01 compared with the MPTP group). The data represent the mean ± SEM (n = 10).
3.3. Effects of Rg1 on the Wnt/β-catenin signaling pathway in vivo

Dysfunctional Wnt/β-catenin signaling has been shown to play an important role in the pathophysiology of SNpc dopaminergic neurons and an MPTP model of PD (L’Episcopo et al., 2011). We examined the protein levels of Wnt-1, β-catenin, GSK-3β and p-GSK-3β in the SNpc region of mice. Dose-response studies were also performed. Our results revealed that the protein levels (Fig. 4A) of Wnt-1 and β-catenin were significantly decreased and that GSK-3β and p-GSK-3β were significantly increased in the MPTP group (P < 0.01, compared with the control group). With Rg1 administration, the protein levels of Wnt-1 and β-catenin...
were significantly increased, and GSK-3β and p-GSK-3β were significantly decreased ($P < 0.01$, compared with MPTP group). The changes in mRNA (Fig. 4B) levels of Wnt-1, β-catenin, GSK-3β were consistent with changes in protein levels. Our results also revealed that Rg1 (5, 10, or 20 mg/kg, i.p.) treatment had a dose-dependent effect on protein and mRNA levels. These results indicate that the neuroprotective effects of Rg1 (5, 10, or 20 mg/kg) appear to be mediated through the activation of the Wnt/β-catenin signaling pathway in vivo.

3.4. Effects of Rg1 on MPP+-induced cell viability loss in vitro

The ability of Rg1 to reverse the cytotoxicity of MPP+ in PC12 cells was investigated using CKK-8. The measurements revealed a significant decrease in the viability of PC12 cells following exposure to 500 μM MPP+ for 24 h, while the cells treated with 20 μM Rg1 alone did not show a decrease in cell viability. Pre-treatment with 5 μM, 10 μM, or 20 μM Rg1 for 24 h significantly decreased MPP+-induced cytotoxicity. The results also revealed that Rg1 (5, 10, or 20 μM) treatment exhibited concentration-dependent effects (Fig. 5A). Pre-treatment with 20 μM Rg1 for 6, 12, 24 and 48 h showed time-dependent effects (Fig. 5B). The present results demonstrate that MPP+-induced apoptosis in PC12 cells could be alleviated by pre-treatment with Rg1. According to these results, pre-treatment with 20 μM Rg1 for 24 h was chosen as the condition for the following experiments because it most effectively protected PC12 cells from apoptosis.

3.5. Effect of Rg1 against in vitro MPP+-induced apoptosis, and Dkk1 blocks the effects of Rg1

TUNEL staining of PC12 cells after MPP+ administration revealed a significant increase in apoptotic bodies. This effect was attenuated by Rg1 (20 μM) treatment. Groups with DKK1 treatment also showed many cells undergoing apoptosis, and Rg1 treatment had no effect on this apoptosis (Fig. 6A, B). In addition, our results show that cleaved caspase-3 expression was significantly increased in the MPP+ group and that pretreatment with Rg1 reduced its expression. Bcl-xl expression showed a pattern opposite to that of cleaved caspase-3. The DKK1 treatment groups showed the same protein expression tendencies as in previous results of apoptosis (Fig. 6C). From these results, it can be concluded that the Wnt/β-catenin signaling pathway plays an important role in MPP+-induced PC12 cell apoptosis, and Rg1 (20 μM) could protect PC12 cells from MPP+-induced apoptosis.

3.6. Effects of Rg1 on the Wnt/β-catenin signaling pathway in vitro, and Dkk1 blocks the effects of Rg1

To further investigate whether the Wnt/β-catenin signaling pathway was implicated in Rg1-induced neuroprotective effects in vitro, we examined the expression of Wnt-1, β-catenin, GSK-3β and p-GSK-3β in PC12 cells. Our results indicated that the protein (Fig. 7A) levels of Wnt-1 and β-catenin were significantly increased, and GSK-3β and p-GSK-3β were significantly increased in MPP+-treated cells ($P < 0.01$, compared with control group). In contrast, when treated with 20 μM Rg1, the protein levels of Wnt-1 and β-catenin were significantly increased, and GSK-3β and p-GSK-3β were significantly decreased ($P < 0.01$, compared with the MPP+ group). The mRNA (Fig. 7B) levels of Wnt-1, β-catenin, and GSK-3β were consistent with the protein levels. The expression levels of protein and mRNA after the blockade of Wnt/β-catenin signaling with DKK1 were similar to those observed in the MPP+-only group. The results of immunofluorescence for β-catenin and GSK-3β in PC12 cells were consistent with the protein and mRNA levels (Fig. 7C). These results indicated that the neuroprotective effects of Rg1 (20 μM) are mediated through the activation of the Wnt/β-catenin signaling pathway in vitro.

4. Discussion

Ginseng has been considered an important herbal drug that exerts beneficial effects on the CNS and in patients with neurodegenerative diseases (González-Burgos et al., 2015; Radad et al., 2011). Ginsenosides, which are the principal active ingredients in ginseng, have been considered to be responsible for most of ginseng’s effects (Nah et al., 2007; Ardah et al., 2015). Many studies have indicated the protective effects of the Rg1 in in vivo or in vitro PD models (Chen et al., 2002; Radad et al., 2006). The investigation of the therapeutic effects and molecular mechanisms involving Rg1 in animal models of PD will provide further evidence for clinical trials. The results of our study demonstrate that Rg1 exerts neuroprotective effects in both in vivo and in vitro models of PD, and these neuroprotective effects appear to be mediated by the Wnt/β-catenin signaling pathway.

Studies have indicated that MPTP toxicity causes dopaminergic neuronal loss in the SNpc region and reduces striatal dopamine levels, which leads to motor deficits in mice (Goldberg et al., 2011; Scoce et al., 2015). The “Pole test” was used to evaluate the degeneration of dopaminergic cells by observing behavioral recovery in mice (Matsura et al., 1997). In the present study, the “Pole test” of mice showed that the times required for both “T-turn"
and “T-total” significantly increased in the MPTP-treated group compared with the control group and were reduced by Rg1 treatment. Our results also revealed that Rg1 produced a dose-dependent effect on the results of the “Pole test”. These results demonstrate that Rg1 exerts neuroprotective effects that manifest as motor improvements in mice. We further examined the number of TH-positive dopaminergic neurons and the number of apoptotic cells in the SNpc region to assess the neuroprotective effects of Rg1. Our results showed that the MPTP-treated group exhibited a significant reduction in number of TH-positive neurons and an increase in the number of apoptotic cells in the SNpc region. Co-treatment of Rg1 with MPTP increased the survival of TH-positive neurons and reduced the apoptotic rate in a dose-dependent manner. These results indicate that Rg1 protects dopaminergic neurons from MPTP-induced toxicity.

Apoptosis in the MPTP/MPP+ models of PD has been investigated for decades. The JNK pathway, Bax signaling, oxidative stress and nitric oxide are considered to contribute to cell death, and an anti-apoptotic therapy that is also a neuroprotective therapy would be a promising strategy (Eberhardt and Schulz, 2003; Nicotra and Parvez, 2002; Wang et al., 2004). However, few studies have investigated whether the Wnt/β-catenin signaling pathway is associated with the apoptosis observed in PD models (Dun et al., 2013). In the present study, the protective effects of Rg1 on MPP+ -induced apoptosis in PC12 cells were investigated by CKK-8 assay measurements, TUNEL assays and the expression of cleaved caspase-3 and Bcl-xL. The pro-apoptotic protein cleaved caspase-3 indicates early apoptosis while Bcl-xL, which belongs to the Bcl-2 family, inhibits apoptosis (Akhtar et al., 2004; Jiang et al., 2012). The results of this study indicate that PC12 cells treated with MPP+ showed a loss of cell viability and increased apoptosis, while Rg1...
reduced MPP\(^+\)-induced loss of viability and decreased apoptosis in PC12 cells. However, the anti-apoptotic effects of Rg1 were abrogated by co-treatment with DKK1, a specific blocker of the Wnt/β-catenin signaling pathway. These results demonstrate that Rg1 has anti-apoptotic effects in an in vitro model of PD induced by MPP\(^+\) and that these effects may be exerted through the activation of the Wnt/β-catenin signaling pathway.

Wnt signaling pathways play vital roles in many cellular processes, including survival, proliferation, cell fate and movement (Moon et al., 2004; Veeman et al., 2003). The Wnt/β-catenin pathway is the best studied of the Wnt signaling pathways and has been highly conserved throughout evolution (Clevers, 2006; Clevers and Nusse, 2012). In PD, the Wnt/β-catenin signaling pathway plays a vital role in the survival and protection of dopaminergic neurons (Harvey and Marchetti, 2014; Marchetti et al., 2013) and regulates the development, proliferation and differentiation of ventral midbrain precursors, and overexpression of β-catenin increases the number of dopaminergic neurons (Castelo-Branco et al., 2004; Chen et al., 2013; Arenas, 2014). Increasing evidence shows disturbances in Wnt/β-catenin signaling are implicated in the pathogenesis of PD (MacDonald et al., 2009; Andersson et al., 2013). In the present study, the results of

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**Fig. 7.** Effects of Rg1 on Wnt/β-catenin signaling following MPP\(^+\), Rg1 and DKK1 treatment in vitro. The Wnt antagonist Dkk1 (100 ng/ml) was applied either alone or in combination with Rg1 and/or MPP\(^+\). (A) Representative western blot showing levels of Wnt-1, β-catenin, GSK-3β and p-GSK-3β. (B) Representative mRNA levels of Wnt-1, β-catenin, and GSK-3β. (C) Dual immunofluorescence staining for β-catenin (red) and DAPI (blue) or GSK-3β (red) and DAPI (blue) in PC12 cells. a. Control; b. 500 μM MPP\(^+\) alone; c. 500 μM MPP\(^+\)+ 20 μM Rg1; d. 500 μM MPP\(^+\)+ 20 μM Rg1 + 100 ng/ml Dkk1; e. 500 μM MPP\(^+\)+ 100 ng/ml Dkk1. The differences are significant at \(P < 0.01\) compared with the control group and \#\(P < 0.01\) compared with the MPP\(^+\) group. The data represent the mean ± SEM. The data are representative of three experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
protein/mRNA expression analysis of Wnt-1, β-catenin, GSK-3β and p-GSK-3β in the MPTP or MPP+ treated groups indicate that Wnt/β-catenin signaling is impaired in models of PD, which is consistent with a previous study (L’Episcopo et al., 2012). Groups receiving Rg1 showed the opposite changes in expressions of Wnt-1, β-catenin, GSK-3β and p-GSK-3β, supporting the protective effects of Rg1. In addition, the Wnt/β-catenin signaling antagonist DKK1 suppressed all of the Rg1-mediated protective effects against MPP+–induced neurotoxicity. The above results once again confirm that Rg1-mediated activation of Wnt/β-catenin signaling transduction mechanisms may be responsible for alleviating deficits in both in vivo and in vitro PD models.

Here, we reported that the neuroprotective effects of Rg1 against MPTP/MPP+ toxicity may mediated through the activation of the Wnt/β-catenin signaling pathway. The discovery that the Wnt/β-catenin signaling pathway is implicated in the neuroprotective effects of the ginsenoside Rg1 against MPTP/MPP+–induced injuries to dopaminergic neurons and PC12 cells provides new insight into our understanding of the pharmacological actions of Rg1.

5. Conclusions

In summary, we assessed the neuroprotective effects of Rg1 through the Wnt/β-catenin signaling pathway in both in vivo and in vitro models of PD. The results indicated that the Wnt/β-catenin signaling pathway is indeed involved in mediating the action of Rg1 in PD models. We therefore hope that modulation of the Wnt/β-catenin signaling pathway by Rg1 will lead to new PD therapies with higher efficacy than current treatments that will slow PD progression and provide a theoretical basis for future clinical applications.

Authors’ contributions

Conceived and designed the experiments: ZHL, JZ, TTZ and GZ. Performed the experiments: TTZ and GZ. Analyzed the data: TTZ, GZ, ZHL and JZ. Contributed reagents/materials/analysis tools: TTZ, ZHL and JZ. Contributed reagents/materials/analysis tools: TTZ, ZHL and JZ. Contributed reagents/materials/analysis tools: TTZ, ZHL and JZ. Contributed reagents/materials/analysis tools: TTZ, ZHL and JZ. Contributed reagents/materials/analysis tools: TTZ, ZHL and JZ. Wrote the paper: TTZ, ZHL and JZ.

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