Korean Red Ginseng protects dopaminergic neurons by suppressing the cleavage of p35 to p25 in a Parkinson’s disease mouse model

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Abstract

Background: Ginseng is known to have antiapoptotic, anti-inflammatory, and antioxidant effects. The present study investigated a possible role of Korean Red Ginseng (KRG) in suppressing dopaminergic neuronal cell death and the cleavage of p35 to p25 in the substantia nigra (SN) and striatum (ST) using a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson’s disease mouse model.

Methods: Ten-week-old male C57BL/6 mice were injected intraperitoneally with 30 mg/kg of MPTP at 24-h intervals for 5 d, and then administered KRG (1 mg/kg, 10 mg/kg, or 100 mg/kg) once a day for 12 consecutive days from the first injection. Pole tests were performed to assess the motor function of the mice, dopaminergic neuronal survival in the SN and ST was evaluated using tyrosine hydroxylase-immunohistochemistry, and the expressions of cyclin-dependent kinase 5 (Cdk5), p35, and p25 in the SN and ST were measured using Western blotting.

Results: MPTP administration caused behavioral impairment, dopaminergic neuronal death, increased Cdk5 and p25 expression, and decreased p35 expression in the nigrostriatal system of mice, whereas KRG dose-dependently alleviated these MPTP-induced changes.

Conclusion: These results indicate that KRG can inhibit MPTP-induced dopaminergic neuronal death and suppress the cleavage of p35 to p25 in the SN and the ST, suggesting a possible role for KRG in the treatment of Parkinson’s disease.

1. Introduction

Parkinson’s disease (PD) is a typical neurological disorder that causes dopaminergic neuronal death in the substantia nigra (SN) resulting in striatal dopamine depletion and leads to impaired motor functions such as tremor, bradykinesia, rigidity, postural instability, and akinesia [1]. Although various proteins and gene mutations have been related to PD, the exact mechanisms are still unknown. Therefore, many studies have focused on finding therapeutics that suppress neuronal death to prevent the progression of PD [2].

Cyclin-dependent kinase 5 (Cdk5), a serine/threonine cyclin-dependent kinase, plays an important role in neuronal functions including neuronal development, migration, synaptic activity, and cell survival [3]. In normal conditions, Cdk5/p35, by phosphorylating specific substrates, supports neuronal survival through activation of the antiapoptotic protein Bcl-2 [4] and inhibition of sustained extracellular signal-regulated kinase activation [5]. Whereas in insulated conditions, increased intracellular Ca2+ activates calpain, which cleaves p35 to p25. P25 induces hyperactivation of Cdk5, which causes abnormal hyperphosphorylation of cytoskeletal proteins in the cell body and consequently induces neuronal death [2]. Therefore inhibition of the cleavage p35 to p25 would be therapeutically beneficial in neurodegenerative diseases such as PD.

Panax ginseng (ginseng), a commonly used medical herb in Asian countries, has been used traditionally to increase vitality, promote mental stability, and prevent aging [6], and recent studies have reported that it has antiaging, antioxidant, anti-inflammatory and antiapoptotic effects [7,8]. Ginseng and its extracts also produce neuroprotective effects in PD models [9]. Water extract of ginseng suppressed 1-methyl-4-phenylpyridinium ion (MPP+)-induced...
apoptosis in SH-SY5Y cells by elevating the Bax/Bcl-2 ratio and suppressing the overproduction of reactive oxygen species [10], ginseng extract G115 alleviated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced behavioral impairment and dopaminergic neuronal death in C57BL/6 mice [11], and ginsenoside Rg1 showed neuroprotective effects by reducing iron levels in MPP+–treated MES23.5 cells [12] and in the SN of MPTP-treated mice [13]. These results indicate that ginseng has therapeutic efficacies in PD, but its mechanisms are not yet fully understood.

2. Materials and methods

2.1. Animals

This study was approved by the Pusan National University Institutional Animal Care and Use Committee (PNU-2013-0456) Yangsan, Korea. Male C57BL/6 mice (10 wk old, weighing 20–23 g; Orientbio Inc., Seongnam, Korea) were housed at room temperature (22 ± 3 °C) under a standard 12-h light/dark cycle (lights on at 07:00 h) with unlimited access to food and water. The animals were handled in accordance with the current guidelines established in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985), and all efforts were made to minimize animal suffering and reduce the number of animals used.

2.2. Group classification

Mice were randomly assigned to four groups: A saline-injected group (Saline), an MPTP injected group (MPTP), an MPTP-injected plus 1 mg/kg KRG-treated group (MPTP+KRG1), an MPTP-injected plus 10 mg/kg KRG-treated group (MPTP+KRG10) and an MPTP-injected plus 100 mg/kg KRG-treated group (MPTP+KRG100).

2.3. MPTP injection and medication

All mice except the Saline group received an intraperitoneal injection of MPTP-HCl (30 mg/kg of free base; Sigma, St. Louis, MO, USA) in normal saline at 24-h intervals for five consecutive days. The mice in the Saline group received an intraperitoneal injection of vehicle on the same schedule [15].

The KRG extract used in this experiment was offered by the Korea Ginseng Corporation (Daejeon, Korea). Briefly, KRG was obtained from 6-yr-old roots of *Panax ginseng Meyer*. First, the ginseng was steamed at 90 °C under no pressure for 3 h and was dried at 50–80 °C. The KRG was extracted three times with circulating hot water at 85–90 °C for 8 h. The water content of the pooled extract was 36% of the total weight, and the content of crude ginsenoside in the KRG was analyzed by high-performance liquid chromatography as follows: 6.92 mg/g of Rb1, 2.68 mg/g of Rb2, 3.24 mg/g of Rc, 0.94 mg/g of Rd, 1.40 mg/g of Re, 1.03 mg/g of Rf, 1.12 mg/g of Rg1, 1.23 mg/g of Rg2, 1.03 mg/g of Rg3r, 1.58 mg/g of Rg3s, 0.89 mg/g of Rb1, and other minor ginsenosides. The extract was diluted with sterilized mineral water to appropriate concentrations. Two hours after the MPTP or saline injection, mice in the KRG-treated groups received oral administration of the KRG extract (1 mg/kg, 10 mg/kg, or 100 mg/kg) once a day for 12 consecutive days, and mice in the Saline and the MPTP groups were also administered the same amount of vehicle on the same schedule.

2.4. Behavioral test (pole test)

The pole test was performed by modifying the method established by Abe et al. [16]. Mice (n = 6 in each group) were positioned head downwards near the top of a rough-surfaced wood pole (10 mm in diameter; 50 cm in height), and the time taken to arrive at the floor was recorded. The test was repeated three times at 30-s time intervals, and behavioral change was evaluated according to the average of the three descending times. The test was performed 1 d before MPTP administration (Day 0) and 2 h after feeding KRG on Day 5 and Day 12.

2.5. Tyrosine hydroxylase-positive immunohistochemistry

Immediately after the last pole test on Day 12, mice (n = 6 in each group) were anesthetized with isoflurane and perfused with 4% parafomaldehyde (PFA) in 0.1M phosphate buffer. The brain was quickly removed and postfixed in 4% PFA for 48 h, washed in 0.1M phosphate buffer, and immersed in 30% sucrose solution for storage at 4 °C prior to sectioning. Frozen sections (40 μm) were cut using a Leica CM3050S cryostat (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry was performed as described previously [17,18] on free-floating cryotome-cut sections that encompassed the entire SN (level between AP −3.08 ~ −3.28 mm from the bregma) and the ST (level between AP +0.38 ~ +0.98 mm from the bregma). After incubation with 1% H2O2 in 0.05M phosphate-buffered saline (PBS) for 15 min, followed by 0.3% Triton X-100 and 3% normal blocking serum in 0.05M PBS at room temperature for 1 h, the sections were stained overnight at room temperature using an antityrosine hydroxylase (TH) (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody for dopaminergic neurons. The next day, the sections were washed with 0.05M PBS, incubated with Vectastain Elite ABC reagents (Vector Laboratories Inc., Burlingame, CA, USA) at room temperature for 1 h, washed with 0.05M PBS, and then incubated with a diaminobenzidine (DAB) substrate kit (Vector Laboratories Inc.) for 5 min. After the DAB reaction, the tissues were rinsed with 0.05M PBS, mounted on gelatin-coated slides, air-dried, dehydrated, and coverslipped. Histological pictures were taken using an Axio Scope.A1 microscope (ZEISS, Oberkochen, Germany) and an AxioCam ICc3 camera (ZEISS). The survival of dopaminergic neurons was evaluated by the number of TH-positive neuronal cells in the SN. An independent observer, without knowing the expected results, manually counted TH-positive neurons bilaterally in five continuous SN sections, and the cell counting was confirmed three times to validate the data. For dopaminergic fiber analysis, the mean value of optical density (OD) in the ST was determined using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

2.6. Western blot

Immediately after the pole test on Day 5 or Day 12, mice (n = 6 in each group) were sacrificed with CO2 gas; tissues of SN and ST were then dissected rapidly and kept at −80 °C until use. The tissues were homogenized with protease inhibitor and RIPA buffer, centrifuged for 20 min at 4 °C at 12,000 rpm, and the supernatants were separated. Equal amounts of protein (30 μg) from each sample were separated on 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membrane was blocked with 5%...
bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature and incubated overnight with rabbit polyclonal anti-p35 or anti-Cdk5 antibodies (Santa Cruz Biotechnology) that were diluted 1:1,000 in blocking solution antibody at 4°C. Then the membrane was washed and incubated with horseradish peroxidase-conjugated secondary antirabbit antibody (1:2,000, Santa Cruz Biotechnology) at room temperature for 1 h. After washing the membrane, bands were detected using the enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL, USA). Then, these blots were reprobed with rabbit monoclonal anti-β-actin antibody (1:1,000; Santa Cruz Biotechnology) as a loading control for all experiments. Quantification of immunoreactivity corresponding to the total bands was performed by densitometric analysis using an Image Quant LAS 4000 (Fujifilm, Tokyo, Japan).

2.7. Statistical analysis

All data are expressed as the mean ± standard deviation and analyzed by one-way analysis of variance (ANOVA) with the Newman-Keuls post hoc test. All statistical testing was performed using Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA) and statistical significance was set at \( p < 0.05 \).

3. Results

3.1. Effect of Korean Red Ginseng on MPTP-induced behavioral impairment

To confirm the effect of KRG on behavioral impairment by MPTP, the pole test was performed. Before MPTP injection, there was no significant difference in the descending time of all mice. On Day 5, the descending time of mice in the MPTP group was significantly prolonged (39.13 ± 12.89 s; \( p < 0.05 \)) compared with the time of those in the Saline group (11.35 ± 4.11 s). All doses of KRG reduced the descending time dose dependently (32.45 ± 13.68 s, 22.87 ± 11.91 s, and 14.08 ± 8.56 s for the MPTP+KRG1,
MPTP + KRG10, and MPTP + KRG100 groups, respectively). However, only mice in the MPTP + KRG100 group showed a significant difference \((p < 0.05)\) compared with those in the MPTP group. On Day 12, mice in the MPTP group still descended significantly slower \((18.00 \pm 5.86 \text{ s})\) compared with the Saline-injected mice \((8.57 \pm 1.63 \text{ s}, p < 0.05)\). By contrast, KRG-treated mice showed dose-dependently decreased descending times \((13.57 \pm 3.56 \text{ s}, 10.43 \pm 0.81 \text{ s}, 7.54 \pm 1.67 \text{ s} \text{ for the MPTP + KRG1, MPTP + KRG10 and MPTP + KRG100 groups, respectively})\) compared with mice in the MPTP group, and 10 mg/kg and 100 mg/kg KRG-treated mice showed a significant difference \((p < 0.05 \text{ and } p < 0.01, \text{ respectively; Fig. 1})\).

3.2. Effect of Korean Red Ginseng on MPTP-induced neuronal loss in the SN and the ST

To evaluate the neuroprotective effect of KRG on MPTP-induced neuronal death, the number of TH-positive neurons in the SN was counted. Compared with the number in mice of the Saline group \((100.00 \pm 31.09\%\), those in mice of the MPTP \((40.66 \pm 24.54\%, p < 0.001)\), MPTP + KRG1 \((46.76 \pm 24.82\%, p < 0.01)\), and MPTP + KRG10 \((59.54 \pm 24.91\%, p < 0.05)\) groups were significantly reduced in the SN. However, the number in mice of the MPTP + KRG100 group \((79.84 \pm 28.77\%)\) was significantly increased compared with that in the MPTP group \((p < 0.05)\).

Fig. 3. Validation of Cdk5, p35, and p25 expressions in the substantia nigra. (A) Changes in the protein expressions on Day 5 (left) and Day 12 (right). (B) Relative values of Cdk5, p35, and p25 expressions. Data are shown as means \(\pm\) standard deviation \((n = 6 \text{ in each group})\). * \(p < 0.05\) compared with the saline group. ** \(p < 0.05\) and *** \(p < 0.01\) compared with the MPTP group. Cdk5, cyclin-dependent kinase; KRG, Korean Red Ginseng; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPTP, MPTP-injected group; MPTP + KRG, MPTP-injected and KRG-treated group; Saline, saline-injected group.
In addition, the OD of TH-positive fibers in the ST was evaluated. After MPTP administration, the OD in the MPTP (73.27 ± 16.69%) group was significantly reduced versus that in the Saline group (100.00 ± 27.58%, p < 0.05). The OD in KRG-treated mice was dose-dependent increased versus that in mice of the MPTP group (86.24 ± 16.66%, 92.73 ± 16.73%, 109.20 ± 19.04% in the MPTP+KRG1, MPTP+KRG10, MPTP+KRG100 groups, respectively) compared with mice in the MPTP group, however, only 100 mg/kg KRG-treated mice showed a significant difference (p < 0.05; Fig. 2).

3.3. Changes of Cdk5, p35, and p25 expressions in the substantia nigra

Cdk5, p35, and p25 expressions in the SN and the ST were evaluated using Western blot analysis. On Day 5, Cdk5 expression in the MPTP group was significantly upregulated versus that in the Saline group (p < 0.05), and this was significantly suppressed by 10 mg/kg and 100 mg/kg KRG administration (p < 0.05 and p < 0.01, respectively). MPTP administration significantly suppressed p35 expression (p < 0.05) and enhanced p25 overexpression (p < 0.05),

Fig. 4. Validation of Cdk5, p35, and p25 expressions in the striatum. (A) Changes in the protein expressions on Day 5 (left) and Day 12 (right). (B) Relative values of Cdk5, p35, and p25 expressions. Data are shown as means ± standard deviation (n = 6 in each group). *p < 0.05 compared with the saline group. **p < 0.05 and ***p < 0.01 compared with the MPTP group. Cdk5, cyclin-dependent kinase 5; KRG, Korean Red Ginseng; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPTP, MPTP-injected group; MPTP+KRG, MPTP-injected and KRG-treated group; Saline, saline-injected group.
whereas 100 mg/kg KRG administration significantly normalized these changes \( p < 0.05 \). On Day 12, there were no significant differences in Cdk5 and p25 expressions among the groups. However, p35 expression in the MPTP group was significantly suppressed compared with that in the Saline group \( p < 0.05 \), and this was significantly alleviated by 100 mg/kg KRG administration \( p < 0.05 \); Fig. 3).

3.4. Changes of Cdk5, p35, and p25 expressions in the ST

On Day 5, Cdk5 expression in the MPTP group was significantly increased versus that in the Saline group \( p < 0.05 \), and this was significantly suppressed by 10 mg/kg and 100 mg/kg KRG administration \( p < 0.05 \) and \( p < 0.01 \), respectively. MPTP administration also significantly suppressed p35 expression \( p < 0.05 \) and enhanced p25 overexpression \( p < 0.05 \), whereas 100 mg/kg KRG administration in the MPTP group significantly normalized these changes \( p < 0.05 \). On Day 12, there was no significant difference in Cdk5 expression among the groups. However, significant suppression of p35 \( p < 0.05 \) and overexpression of p25 \( p < 0.05 \) were observed in the MPTP group compared with those in the Saline group, and these were significantly normalized by 10 mg/kg \( p < 0.05 \) and 100 \( p < 0.01 \) mg/kg KRG administration (Fig. 4).

4. Discussion

Despite reports concerning the neuroprotective effects of ginseng, there is little information in studies relating to the action of KRG in PD. Our study demonstrated that KRG can improve the behavioral impairment, protect dopaminergic neurons from MPTP-induced neuronal death, and suppress the MPTP-induced over-expression of Cdk5 and cleavage of p35 to p25 in the SN and the ST.

To evaluate the effect of KRG on MPTP-induced behavioral impairment, the pole test was performed because it is a precise expression of Cdk5 and cleavage of p35 to p25 in the SN and the ST.

In conclusion, this study demonstrated that KRG alleviates MPTP-induced behavioral impairment and dopaminergic neuronal death in the SN and ST, suggesting that the effects may be mediated by suppressing MPTP-induced inappropriate hyperactivation and increase of Cdk5 in the nigrostriatal system, and that the suppression of the cleavage of p35 to p25 by KRG may play an important role in suppressing Cdk5 hyperactivation. However, the possibility remains that KRG may mitigate MPTP-induced behavioral impairment via other mechanisms. Despite this limitation, our findings suggest important clues for understanding the mechanism of KRG in PD intervention.

Conflicts of interest

The authors declare that no competing financial interests or conflicts of interest exist. The funders had no role in the study design, data collection and analysis, and the decision to publish the manuscript.

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