RESEARCH ARTICLE

Ginsenoside Rb1 protects dopaminergic neurons from inflammatory injury induced by intranigral lipopolysaccharide injection

Da-Wei Li[1, 2], Fa-Zhan Zhou[3], Xian-Chang Sun[4], Shu-Chen Li[2], Jin-Bin Yang[2], Huan-Huan Sun[2], Ai-Hua Wang[1, *]
1 Department of Neurology, Qianfoshan Hospital Affiliated to Shandong University, Jinan, Shandong Province, China
2 Department of Neurology, The People's Hospital of Xintai, Xintai, Shandong Province, China
3 Department of Cardiovascularology, Taian Central Hospital, Taian, Shandong Province, China
4 Department of Physiology, Taishan Medical University, Taian, Shandong Province, China

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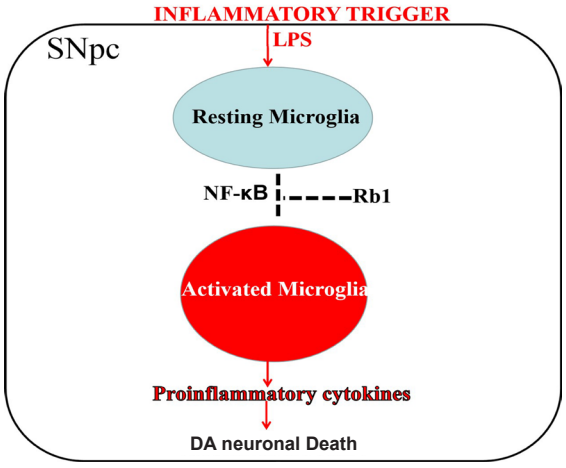
Graphical Abstract

Ginsenoside Rb1 alleviates lipopolysaccharide (LPS)-induced dopaminergic neuronal damage by inhibiting nuclear factor-κB (NF-κB) signaling pathway



Correspondence to:
Ai-Hua Wang, MD, doctorli1977@126.com.

orcid:
0000-0001-5771-2699 (Ai-Hua Wang)

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Abstract

Accumulating studies suggest that neuroinflammation characterized by microglial overactivation plays a pivotal role in the pathogenesis of Parkinson's disease. As such, inhibition of microglial overactivation might be a promising treatment strategy to delay the onset or slow the progression of Parkinson's disease. Ginsenoside Rb1, the most active ingredient of ginseng, reportedly exerts neuroprotective effects by suppressing inflammation *in vitro*. The present study aimed to evaluate the neuroprotective and anti-inflammatory effects of ginsenoside Rb1 in a lipopolysaccharide-induced rat Parkinson's disease model. Rats were divided into four groups. In the control group, sham-operated rats were intraperitoneally administered normal saline for 14 consecutive days. In the ginsenoside Rb1 group, ginsenoside Rb1 (20 mg/kg) was intraperitoneally injected for 14 consecutive days after sham surgery. In the lipopolysaccharide group, a single dose of lipopolysaccharide was unilaterally microinjected into the rat substantial nigra to establish the Parkinson's disease model. Lipopolysaccharide-injected rats were treated with normal saline for 14 consecutive days. In the ginsenoside Rb1 + lipopolysaccharide group, lipopolysaccharide was unilaterally microinjected into the rat substantial nigra. Subsequently, ginsenoside Rb1 was intraperitoneally injected for 14 consecutive days. To investigate the therapeutic effects of ginsenoside Rb1, behavioral tests were performed on day 15 after lipopolysaccharide injection. We found that ginsenoside Rb1 treatment remarkably reduced apomorphine-induced rotations in lipopolysaccharide-treated rats compared with the lipopolysaccharide group. To investigate the neurotoxicity of lipopolysaccharide and potential protective effect of ginsenoside Rb1, contents of dopamine and its metabolites in the striatum were measured by high-performance liquid chromatography. Compared with the lipopolysaccharide group, ginsenoside Rb1 obviously attenuated the lipopolysaccharide-induced depletion of dopamine and its metabolites in the striatum. To further explore the neuroprotective effect of ginsenoside Rb1 against lipopolysaccharide-induced neurotoxicity, immunohistochemistry and western blot assay of tyrosine hydroxylase were performed to evaluate dopaminergic neuron degeneration in the substantial nigra par compacta. The results showed that lipopolysaccharide injection caused a large loss of tyrosine hydroxylase-immunoreactive neurons in the substantia nigra and a significant decrease in overall tyrosine hydroxylase expression. However, ginsenoside Rb1 noticeably reversed these changes. To investigate whether the neuroprotective effect of ginsenoside Rb1 was associated with inhibition of lipopolysaccharide-induced microglial activation, we examined expression of the microglia marker Iba-1. Our results confirmed that lipopolysaccharide injection induced a significant increase in Iba-1 expression in the substantia nigra; however, ginsenoside Rb1 effectively

suppressed lipopolysaccharide-induced microglial overactivation. To elucidate the inhibitory mechanism of ginsenoside Rb1, we examined expression levels of inflammatory mediators (tumor necrosis factor-α, interleukin-1β, inducible nitric oxide synthase, and cyclooxygenase 2) and phosphorylation of nuclear factor kappa B signaling-related proteins (IκB, IKK) in the substantia nigra with enzyme-linked immunosorbent and western blot assays. Our results revealed that compared with the control group, phosphorylation and expression of inflammatory mediators IκB and IKK in the substantia nigra of lipopolysaccharide group rats were significantly increased; whereas, ginsenoside Rb1 obviously reduced lipopolysaccharide-induced changes on the lesioned side of the substantial nigra par compacta. These findings confirm that ginsenoside Rb1 can inhibit inflammation induced by lipopolysaccharide injection into the substantia nigra and protect dopaminergic neurons, which may be related to its inhibition of the nuclear factor kappa B signaling pathway. This study was approved by the Experimental Animal Ethics Committee of Shandong University of China in April 2016 (approval No. KYLL-2016-0148).

Key Words: *nerve regeneration; neurodegeneration; Parkinson's disease; ginsenoside Rb1; neuroinflammation; lipopolysaccharide; dopaminergic neuron; microglia; nuclear factor kappa B; dopamine; tyrosine hydroxylase; substantia nigra; neural regeneration*

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Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), a marked decrease of dopamine in the striatum, and the presence of Lewy bodies or Lewy neuritis in surviving neurons (Rizek et al., 2016; Zeng et al., 2018; Zhou et al., 2019). PD manifests clinically as motor disorders, such as bradykinesia, rigidity, postural instability and rest tremor, and non-motor features, such as olfactory deficits, sleep disturbance, and psychosis secondary to autonomic dysfunction. Although these clinical symptoms result from the gradual degeneration of dopaminergic neurons in SNpc, the precise mechanism of neuronal degeneration remains elusive. Several lines of evidence suggest that microglia-mediated neuroinflammation may contribute to nigrostriatal pathway degeneration and modulate the progression of PD (Hirsch and Hunot, 2009; Tansey and Goldberg, 2010; Wang et al., 2017; Gupta et al., 2018).

Microglia, the resident macrophages of the brain, have been recognized as primary actors in neuroinflammatory responses in the central nervous system. Overactivated microglia release various pro-inflammatory enzymes and/or cytotoxic cytokines that are deleterious to the surrounding neurons, resulting in a self-sustaining cycle of neuronal death (Wang et al., 2015; Le et al., 2016; Rai et al., 2017; Lee et al., 2019). Hence, anti-inflammatory treatment *via* inhibition of microglial release of these pro-inflammatory molecules may be a promising treatment strategy to delay the onset or slow the progression of PD.

Panax ginseng Meyer is a well-known medicinal herb that has been used as a tonic to improve well-being and alleviate fatigue in Asia, particularly China, for millennia. Numerous studies have indicated that the pharmacological actions of ginseng can be attributed to its bioactive ingredients, called ginsenosides, which have been shown to elicit beneficial therapeutic effects in several degenerative diseases (Song et al., 2017). Ginsenoside Rb1 (GRb1), the major component of ginseng, exhibits a wide range of neurotrophic and neuroprotective effects in the central nervous system. GRb1 can reportedly protect neurons from injuries induced by amyloid β-peptide (Chen et al., 2008) and α-synuclein (Ardah et al., 2015). Furthermore, Radad et al. (2004) and Hashimoto et al. (2012) reported that GRb1 could protect dopaminergic neurons from undergoing apoptosis after exposure to 1-methyl-4-phenylpyridinium-iodide. The results of these studies indicate that GRb1 may be a potent neuroprotectant for neurodegenerative disorders such as PD. Recently, several mechanistic studies demonstrated that the neuroprotective effects of GRb1 might be partly attributed to its anti-inflammatory effects. *In vitro* studies indicated that GRb1 alleviates lipopolysaccharide (LPS)-induced inflammatory reaction in N9 microglia (Ke et al., 2014), RAW267.4 macrophages (Smolinski and Pestka, 2003), EOC20 microglia (Beamer and Shepherd, 2012), and BV2 microglia (Lee et al., 2012). In an *in vivo* study, Lee et al. (2013) reported that GRb1 inhibited systemic LPS-induced microglial activation and expression of pro-inflammatory factors in both the cortex and hippocampus of mice. However, despite numerous studies, the modulatory effects of GRb1 on neuroinflammation still remain unclear (Yu et al., 2017), and no previous study has investigated the protective effect of GRb1 against inflammation in the nigrostriatal system *in vivo*.

Therefore, the present study was designed to investigate whether GRb1 attenuated dopaminergic neuronal damage *via* its anti-inflammatory actions in an LPS-induced neurotoxic rat model.

Materials and Methods

Animals

Forty-eight male Wistar rats aged 3 months and weighing 240–280 g were supplied by Shandong Experimental Animal Center, China (license number: SCXK (Lu) 2015002). Rats were housed under standard conditions of controlled temperature (22 ± 3°C) and light cycle (12-hour light and 12-hour dark) with free access to food and water. Rats were allowed to acclimate to their new surroundings for 1 week before experimental manipulations. All animal experimental procedures were approved by the Animal Ethics Committee of Shandong University, China in April 2016 (approval No. KYLL-2016-0148). All efforts were made to minimize the number of animals used and their suffering.

Stereotaxic surgery

Rats were intraperitoneally anesthetized with chloral hydrate (400 mg/kg) and placed on a stereotaxic frame (Standard Stereotaxic Frame, Stoelting, Wood Dale, IL, USA) to conform to the brain atlas of Paxinos and Watson (Paxinos

and Watson, 2007). As previously described (Sharma et al., 2017), LPS (5.0 μg dissolved in 2 μL of 0.9% saline) was injected into the right side of the SNpc at a rate of 0.5 μL/min using the following stereotaxic coordinates (Paxinos and Watson, 2007): anteroposterior: −5.2 mm, mediolateral: 2 mm; dorsoventral: 7.9 mm. After each injection, the needle remained in position for an additional 5 minutes to prevent reflux of the toxin along the injection tract. Sham-operated rats were subjected to the same surgical procedures, except that 2 μL of normal saline, instead of LPS, was injected into the right SNpc (Sun et al., 2016). Rats with more than 200 revolving cycles in 30 minutes were successfully modeled.

Experimental groups and drug administration

GRb1 (purity > 99%) was purchased from Jilin University, Changchun, China. The chemical formula of Rb1 is $C_{54}H_{92}O_{23}$ and its molecular weight is 1109.29. Purity of GRb1 powder was 99% as determined by reverse phase high-performance liquid chromatography (HPLC). GRb1 was dissolved in physiological saline (10 mg/mL) and administered intraperitoneally.

Forty-eight male Wistar rats were divided into the following four groups (12 rats per group): (1) Control group: sham-operated rats were intraperitoneally administered with normal saline (2 mL/kg per day) for 14 consecutive days; (2) GRb1 group: Same as control group except GRb1 (20 mg/kg, 10 mg/mL) was injected intraperitoneally; (3) LPS group followed by vehicle treatment: LPS-injected rats were treated as described in the control group; (4) GRb1 + LPS group: Same as LPS group except that GRb1 (20 mg/kg) was injected intraperitoneally. GRb1 dosage was chosen in accordance with previous reports (Zhu et al., 2012; Chen et al., 2015; Wang et al., 2018; Zhao et al., 2018) and preliminary experiments. After behavioral testing on day 15, rats were sacrificed and used for HPLC analysis, immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and western blot assay.

Rotational behavior analysis

Ten rats in each group were tested for rotational behavior after 14 days of treatment. The rotational behavior test was carried out as previously described (Hritcu and Ciobica, 2013; Bjorklund and Dunnett, 2019). Briefly, rats were placed in stainless steel bowls and allowed to adapt for 10 minutes to the testing environment on day 15. The rat chest was surrounded by a harness that connected to an automatic four-channel rotameter (Taishan Medica University, China). Next, rats were intraperitoneally injected with apomorphine (0.5 mg/kg) dissolved in normal saline. The rotational behavior test began at 5 minutes after injection and lasted for 30 minutes under minimal external stimuli. Rats were placed in a rotating container, and the number of rotations for each group of rats was counted for 30 minutes for statistical comparison.

HPLC

The brain was quickly obtained from 32 rats after behavioral testing. The striatum was stored at −80°C until subsequent HPLC detection. Contents of dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid in the rat striatum were determined by HPLC, as previously described (Liu et al., 2008). Briefly, the striatum was weighed and homogenized in 0.3 mL liquid A (0.4 M perchloric acid) and centrifuged at 12,000 r/min for 20 minutes at 4°C. In total, 240 μL of supernatant was collected and mixed with 120 μL liquid B, which contained 20 mM citromalic acid-potassium, 300 mM dipotassium phosphate, and 2 mM EDTA-2Na. Subsequently, the supernatant was evaluated with an HPLC system (Waters e2695, Milford, MA, USA) equipped with an Electrochemical Detector (Waters 2465).

Immunohistochemistry

After behavioral testing, 16 rats were anesthetized with chloral hydrate and transcardially perfused with 150 mL of normal saline and 350 mL of 4% paraformaldehyde. Brains were fixed and processed for immunostaining as previously described (Sun et al., 2016). Serial coronal sections (25 μm) were cut through the SNpc (from anteroposterior: −4.8 mm to anteroposterior: −6.2 mm) on a cryostat (Leica, Germany). Free-floating brain sections were pre-incubated in 5% bovine serum albumin containing 0.2% Triton X-100 for 30 minutes at 37°C. These sections were incubated overnight at 4°C with a rabbit polyclonal anti-tyrosine hydroxylase (TH) (1:2000) or mouse monoclonal ionized calcium-binding adaptor molecule-1 (1:200) antibody, both purchased from Millipore (Bedford, MA, USA). Afterwards, sections were incubated with goat anti-rabbit and goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA) for 2 hours at room temperature, separately. Stained samples were visualized by diaminobenzidine, and six sections of each animal were counted by two individuals in a blind fashion using a microscope (Olympus, Tokyo, Japan). The survival rate of TH neurons was calculated as the number of TH neurons on the lesioned side relative to the number of TH neurons on the non-lesioned side.

ELISA

After behavioral testing, the brain was quickly obtained from 16 rats, and the substantia nigra was stored at −80°C until subsequent ELISA. Amounts of TNF-α and IL-1β in the rat substantia nigra were determined with ELISA Kits (R&D Systems, Minneapolis, MN, USA). Tissues were weighed and homogenized in ice-cold lysis buffer containing 1 mM phenylmethylsulfonyl fluoride according to the manufacturer's instructions. The supernatant was collected and protein concentrations were measured using a PierceMT BCA protein assay (Thermo Scientific, Waltham, MA, USA). Sandwich ELISA was then performed in accordance with the manufacturer's instructions. Sensitivity of the ELISA assay was 5 pg/mL for both TNF-α and IL-1β. The protein concentration was determined using a detergent-compatible protein assay with a bovine serum albumin standard.

Western blot assay

After behavioral testing, the brain was quickly obtained from

16 rats, and the substantia nigra was stored at −80°C until subsequent western blot assay.

Substantia nigra tissues were lysed in RIPA buffer (Beyotime, Haimen, China) supplemented with 1% phenylmethyl sulfonylfluoride. After centrifugation at 12,000 r/min for 15 minutes at 4°C, supernatants were collected and protein concentrations in the supernatants were tested using a Pierce BCA protein assay kit. In total, 30 μg of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transblotted onto polyvinylidene fluoride membranes (Immobilin-P, Millipore, Bedford, MA, USA). Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween for 1 hour at room temperature, washed with Tris-buffered saline with 0.1% Tween three times for 10 minutes, then incubated separately with primary antibodies for TH (1:2000; rabbit polyclonal antibody), Iba-1 (1:500; mouse monoclonal antibody), COX-2 (1:1000; rabbit polyclonal antibody), inducible nitric oxide synthase (iNOS) (1:2000; rabbit polyclonal antibody), IκB (1:1000; rabbit polyclonal antibody), phospho-IκB (1:1000; rabbit polyclonal antibody), IKK and phospho-IKK (1:1000; rabbit polyclonal antibody), and β-actin (1:5000) overnight at 4°C. Primary antibodies against phospho-IκB, IκB, phospho-IKK, IKK, COX-2, iNOS, β-actin (Hertfordshire, England), TH and Iba-1 (Bedford) were supplied by Cell Signaling Biotechnology, Beverly, MA, USA. Membranes were washed with Tris-buffered saline containing 0.1% Tween three times for 15 minutes each, and incubated with rabbit or goat secondary horseradish peroxidase-linked antibodies (Santa Cruz Biotechnology) for 2 hours at room temperature. Blots were detected with enhanced chemiluminescence reagent (Millipore) and visualized with an imaging system (UVP Biospectrum 810, USA). Density of the target protein and β-actin was measured using ImageJ software (NIH, Bethesda, MD, USA), and their ratio was used as the relative optical density.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis was carried out using one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

Results

Quantitative analysis of experimental animals

A total of 48 rats were selected for this experiment. Three died of infection during the experiment, and were supplemented with new ones. A total of 48 animals entered final analysis. Behavioral testing was performed on all animals, and 10 out of each group ($n = 12$) were randomly selected for analysis ($n = 10$). After behavioral testing, 8 rats from each group were selected to isolate the striatum and substantia nigra. The striatum was used for HPLC analysis ($n = 8$); half of the substantia nigra was used for ELISA ($n = 4$), and the other half was used for immunoblot detection ($n = 4$). In addition, four rats from each group were perfused with paraformaldehyde and fixed for immunohistochemistry ($n = 4$).

GRb1 administration ameliorates apomorphine-induced behavioral impairments

Apomorphine-induced rotation to the lesioned side is the most commonly used quantitative index of dopaminergic neuronal damage in animal models of PD (Alzoubi et al., 2018; Miyanishi et al., 2019). To investigate the therapeutic effects of GRb1, behavioral tests were performed on day 15 after LPS injection. The results showed that treatment with apomorphine, an indirect dopamine receptor agonist, elicited rotational behavior towards the lesioned side in LPS-induced PD model rats. Compared with rats lesioned with LPS alone, GRb1 treatment remarkably attenuated apomorphine-induced rotation (**Figure 1**). These data suggest that GRb1 had beneficial effects on motor dysfunction in LPS-induced PD model rats.

GRb1 administration attenuates depletion of dopamine and its metabolites DOPAC and homovanillic acid in the striatum induced by intranigral injection of LPS

Reduction of dopamine and its metabolites DOPAC and homovanillic acid in the striatum is believed to be a neurochemistry hallmark of LPS neurotoxicity. Therefore, contents of dopamine and its metabolites in the striatum were measured using HPLC. As shown in **Figure 2**, contents of dopamine, DOPAC, and homovanillic acid on the LPS-injected side were noticeably decreased compared with the uninjected side or control animals. After treatment with GRb1 (20 mg/kg per day) for 14 days, LPS-induced depletion of dopamine and its metabolites in the striatum was obviously attenuated. No changes in the contents of dopamine or its metabolites were found on the unlesioned side of the striatum in all groups. These results indicated that GRb1 effectively increased levels of dopamine neurotransmitter in PD rats induced by LPS through intranigral injection.

Protective effect of GRb1 on LPS-induced dopaminergic neuronal deficits

To further explore the neuroprotective effect of GRb1 against LPS-induced neurotoxicity, immunohistochemistry and western blot assay of TH were performed to evaluate dopaminergic degeneration in the substantia nigra. As shown in **Figure 3**, severe nigrastriatal lesions with a marked loss of TH-immunoreactive neurons and their dendrites were observed in rats receiving vehicle treatment after intranigral LPS injection. Only 33.6% of TH-immunoreactive neurons in the SNpc on the LPS-injected side survived compared with the non-injected side. In contrast, GRb1 treatment remarkably increased the survival of TH neurons, such that the survival ratio was 57.8% on the LPS-injected side. Western blot assay results revealed that LPS injection markedly decreased TH protein levels on the lesioned side compared with the control group. In the GRb1 group, expression of TH was remarkably increased compared with the PD group. The above results show that GRb1 can alleviate damage to dopa-

minergic neurons induced by intranigral LPS injection.

GRb1 treatment inhibits LPS-induced microglial activation in the substantia nigra

Compelling evidence indicates that microglial overactivation is a key factor in the loss of nigral dopaminergic neurons in PD. To determine whether GRb1 has a neuroprotective effect *via* regulation on microglial inflammation, Iba-1 expression (a marker of microglial activation) in the substantia nigra was examined by immunohistochemistry and immunoblotting. As shown in **Figure 4**, Iba-1-immunoreactive cells were ramified resting microglia with two or three fine processes in the SNpc of sham-operated rats. After LPS injection, activated amoeboid microglia were readily identifiable throughout the SNpc. However, treatment with GRb1 for 14 days markedly reduced activated microglia/macrophage cells and LPS-induced elevations in Iba-1 expression levels. These results confirmed that GRb1 was capable of suppressing neuroinflammation in the LPS-infused substantia nigra.

GRb1 inhibits LPS-induced release of inflammatory mediators in the substantia nigra

To further analyze whether the neuroprotective and anti-inflammatory effects of GRb1 are associated with inhibited release of inflammatory mediators, expression levels of TNF-α, IL-1β, COX-2, and iNOS were examined using ELISA and western blot assay, respectively. As shown in **Figure 5**, intranigral LPS injection surprisingly upregulated TNF-α, IL-1β, iNOS, and COX-2 expression levels. Compared with vehicle-treated rats, systemic administration of GRb1 markedly inhibited the upregulation of pro-inflammatory mediators induced by intranigral LPS injection.

GRb1 downregulates LPS-induced activation of the NF-κB signaling pathway

The NF-κB pathway is known to be a major mediator of inflammation *via* the control of pro-inflammatory cytokine synthesis in microglial overactivation. To explicate the anti-inflammatory mechanism of GRb1 in LPS-lesioned substantia nigra, expression of IκB and IKK and their phosphorylation levels in the substantia nigra were examined by western blot assay. Our data clearly showed that GRb1 treatment significant reduced IκB and IKK phosphorylation compared with LPS-injected rats (**Figure 6**), indicating that GRb1 suppresses LPS-induced microglial activation *via* NF-κB signaling.

Discussion

Accumulating studies have confirmed the neuroprotective effects of GRb1 on neurodegenerative diseases in the central nervous system *in vivo* and *in vitro* (Liu et al., 2013; Ahmed et al., 2016). However, the precise mechanisms underlying neuroprotection at the molecular and cellular levels remain elusive. In the present study, our results clearly demonstrated that GRb1 exerted protective effects on dopaminergic neurons by suppressing neuroinflammation characterized by microglial overactivation in an *in vivo* rat model induced by single intranigral LPS injection. Further investigation showed that the inhibitory effect of GRb1 on neurogliocytes was mediated by the downregulation of pro-inflammatory mediators corresponding to suppression of the NF-κB sig-

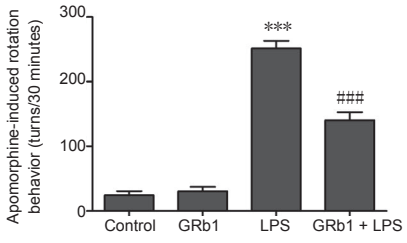

Figure 1 GRb1 treatment improves apomorphine-induced rotation behavior.

Rats were pretreated with GRb1 (20 mg/kg per day) or vehicle 3 days before LPS (5.0 μg) injection and subsequently for 14 days after LPS injection. Apomorphine-induced rotational behavior was tested and total turns were counted for 30 minutes after intraperitoneal injection with apomorphine (0.5 mg/kg). ***P < 0.001, *vs*. control group; ###P < 0.001, *vs*. LPS group. Data are expressed as the mean ± SEM (n = 10; one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test). LPS: Lipopolysaccharide; GRb1: ginsenoside Rb1.

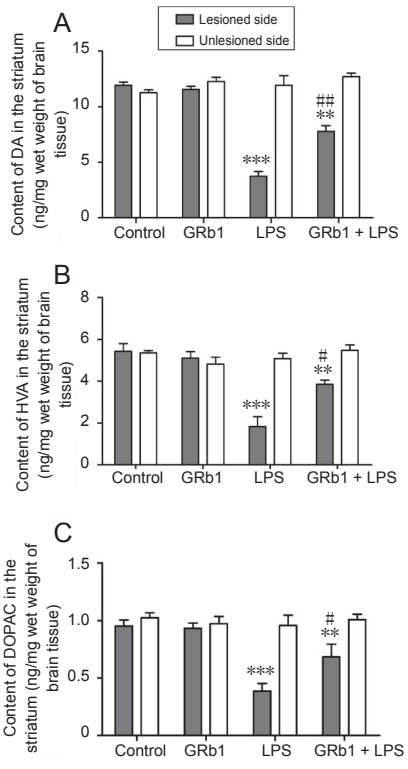

Figure 2 Effect of GRb1 on contents of DA and its metabolites on the lesioned side of the striatum.

After LPS (5.0 μg) injection, rats were treated with GRb1 (20 mg/kg) or vehicle for 2 weeks. (A–C) Contents of (A) DA, (B) HVA and (C) DOPAC in the striatum as detected by high-performance liquid chromatography (ng/mg wet weight of brain tissue). **P < 0.01, ***P < 0.001, *vs*. control group; #P < 0.05, ##P < 0.01, *vs*. LPS group (lesioned side). Data are expressed as the mean ± SEM (n = 8; one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test). DA: Dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; GRb1: ginsenoside Rb1; LPS: lipopolysaccharide.

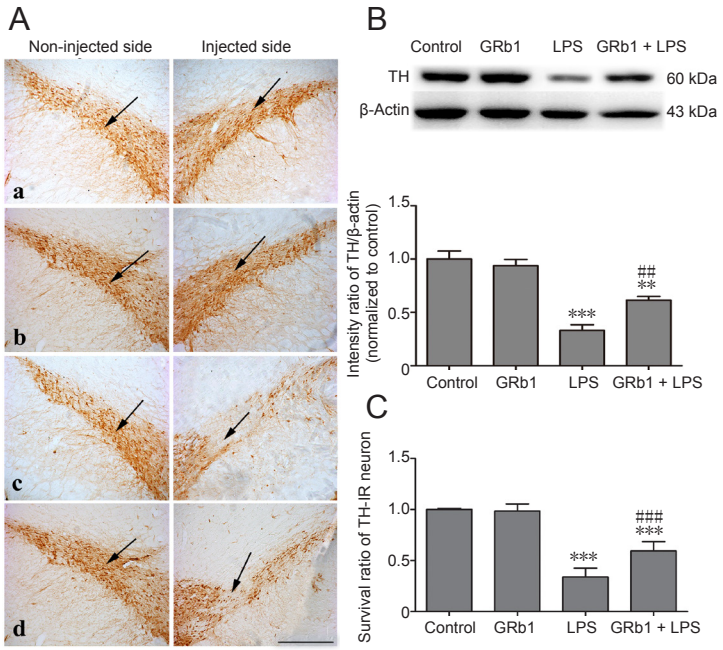

Figure 3 Effect of GRb1 on TH-IR neurons and TH expression in the substantia nigra pars compacta of LPS-induced Parkinson's disease model rats.

Normal saline or 5 μg LPS was unilaterally injected into the right substantia nigra pars compacta of rats. After 2 weeks of treatment with vehicle or GRb1 (20 mg/kg), rats were sacrificed and then coronal sections passing through the substantia nigra were processed for TH immunostaining. Sections were photographed using an Olympus BX51 microscope. (A) Representative microphotographs of TH-positive neurons on unlesioned and lesioned sides of the substantia nigra in (a) control, (b) GRb1, (c) LPS, and (d) GRb1 + LPS groups (immunohistochemical staining, original magnification, 100×). Scale bar: 200 μm. (B) Immunoblotting analysis of TH expression on the lesioned side of substantia nigra. (C) Quantitative analysis of TH-positive neuron survival in the substantia nigra pars compacta (lesioned side *vs.* unlesioned side). $^{**}P < 0.01$, $^{***}P < 0.001$, *vs.* control group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, *vs.* LPS group. Data are expressed as the mean ± SEM ($n = 4$; one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test). The arrow point represents dopaminergic neurons in the compact part of the substantia nigra. TH: Tyrosine hydroxylase; TH-IR: TH-immunoreactive; LPS: lipopolysaccharide; GRb1: ginsenoside Rb1.

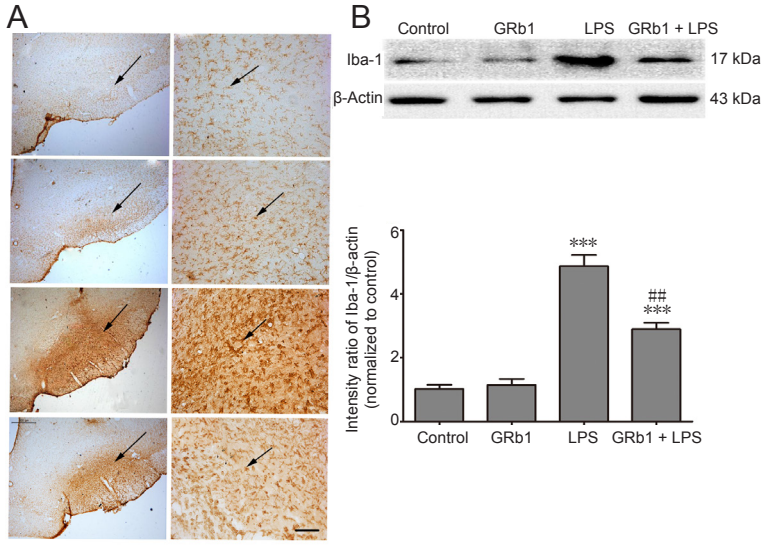

Figure 4 GRb1 treatment suppresses microglial activation and downregulates Iba-1 expression in the substantia nigra.

(A) Representative microphotographs of Iba-1 immunoreactive microglial cells (arrows) on the lesioned side of SNpc in (a) control, (b) GRb1 (20 mg/kg), (c) LPS (5.0 μg), and (d) GRb1 (20 mg/kg) + LPS (5.0 μg) groups (immunohistochemical staining). Original magnification, 100× in the left column and 200× in the right column. Scale bar: 30 μm. (B) Immunoblotting analysis of Iba-1 expression on the lesioned side of substantia nigra. $^{***}P < 0.001$, *vs.* control group; $^{\#\#}P < 0.01$, *vs.* LPS group. Data are expressed as the mean ± SEM ($n = 4$; one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test). The arrow represents microglia in the compact part of the substantia nigra. Iba-1: Ionized calcium binding adapter molecule 1; LPS: lipopolysaccharide; GRb1: ginsenoside Rb1.

naling pathway. These data further suggest the potential of GRb1 as a prospective therapy for PD.

Recent studies have demonstrated that neuroinflammation induced by microglial activation plays a pivotal role in the pathogenesis of PD (Nolan et al., 2013; Roussakis and Piccini, 2018; Tansey and Romero-Ramos, 2019). Microglial overactivation contributes to progressive dopaminergic neuron death through the production of pro-inflammatory cytokines. As such, inhibition of overactivated microglia might be beneficial to retard or reverse the development of neuroinflammatory diseases. Therefore, anti-inflammatory strategies have attracted increasing attention for their potential to inhibit microglial activation and subsequently prevent further deterioration of dopaminergic neurons in the substantia nigra (Tonges et al., 2018; Zhang et al., 2018a). A growing body of evidence indicates that PD models induced by LPS may offer a good *in vivo* model for studying the selective effects of inflammatory reactions on the dopaminergic system (Castano et al., 1998; Herrera et al., 2000; Machado et al., 2011). As the major component of the cell wall of gram-negative bacteria, LPS can act on Toll-like receptor 4 on the surface of microglia to activate a series of inflammatory signaling pathway elements, as well as the synthesis and release of downstream inflammatory mediators (Woller et al., 2016). Simultaneously, LPS had no direct effect on neurons because of a lack of Toll-like receptor 4, which makes it suitable to study inflammation-mediated neuronal loss (Erny et al., 2015). Therefore, in the current study, a single dose of LPS was microinjected into the rat substantia nigra to establish an inflammation-induced PD model with the aim of identifying potential therapeutic agents.

Panax ginseng has been extensively used in Traditional

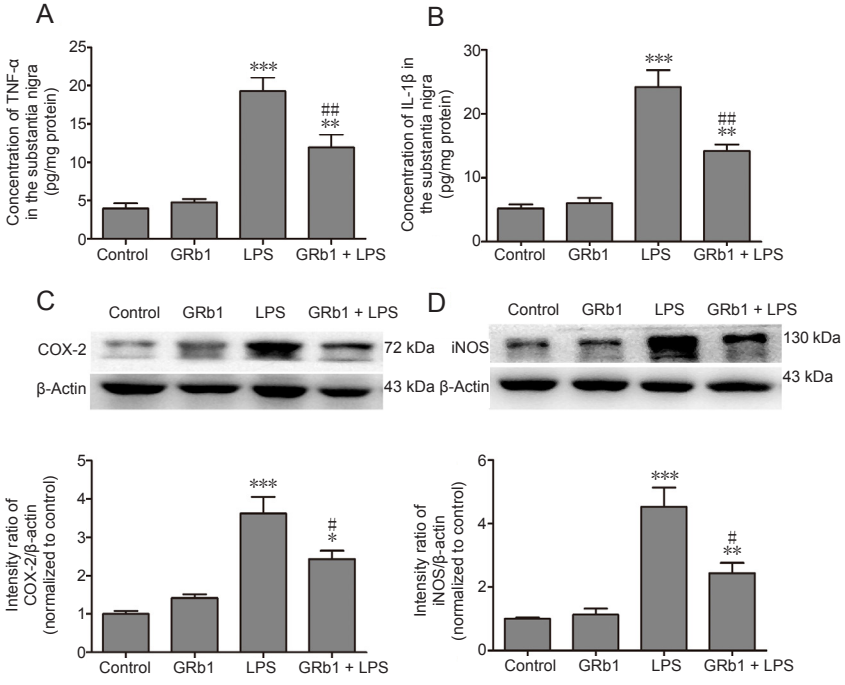

Figure 5 Inhibitory effect of GRb1 on LPS-induced release of TNF-α, IL-1β, COX-2, and iNOS in the substantia nigra on the lesioned side.

After LPS injection, rats were treated with GRb1 or normal saline for 14 days. Contents of TNF-α (A) and IL-1β (B) in lesioned substantia nigra were detected using ELISA kits. Expression of COX-2 (C) and iNOS (D) were detected by western blot assay. Band intensities were measured with ImageJ software. Ratios of intensities of COX-2 and iNOS to β-actin were calculated. Data are expressed as the mean ± SEM (n = 4; one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, *vs.* control group; $\#P < 0.05$, $\#\#P < 0.01$, *vs.* LPS group (lesioned side). ELISA: Enzyme-linked immunosorbent assay; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase 2; LPS: lipopolysaccharide; GRb1: ginsenoside Rb1.

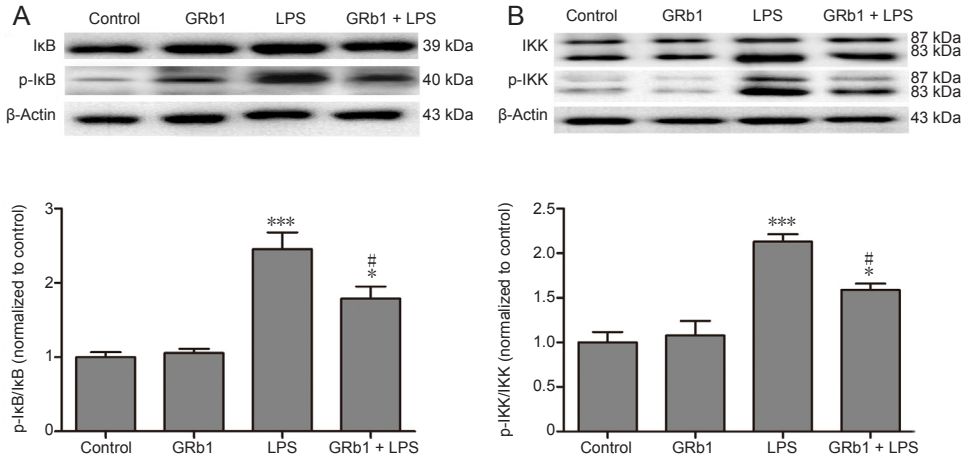

Figure 6 Inhibitory effect of GRb1 on LPS-induced activation of NF-κB signaling in the rat substantia nigra.

(A, B) The lesioned side of the substantia nigra was removed from sacrificed rats to detect phosphorylated or total IκB (A) and phosphorylated IKK or total IKK (B). Expression of phosphorylated and total protein was monitored on the same membrane after stripping and reprobing with specific antibodies. Band intensities were measured with ImageJ software. Ratios of intensities of phosphorylated IκB and IKK to total IκB and IKK were calculated. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, *vs.* control group; $\#P < 0.05$, $\#\#P < 0.01$, *vs.* LPS group (lesioned side). Data are expressed as the mean ± SEM (n = 4; one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test). GRb1: Ginsenoside Rb1; LPS, lipopolysaccharide; NF-κB: nuclear factor κB; IκB: inhibitor protein of κB; IKK: IκB-kinase complex.

Chinese Medicine or as a dietary supplement. Many studies have shown that GRb1, the major bioactive component of ginseng, possesses a variety of biological activities such as anti-inflammatory and neuroprotective effects (Beamer and Shepherd, 2012; Xu et al., 2019). It has been shown to alleviate many central nervous system disorders both *in vitro* and *in vivo* (Ahmed et al., 2016; Zhu et al., 2018). In the present study, GRb1 (20 mg/kg) treatment remarkably ameliorated apomorphine-induced turning behavior compared with the LPS group. Further experiments showed that GRb1 attenuated LPS-induced deficits of dopamine content on the lesioned side of the striatum and reversed LPS-induced loss of TH-immunoreactive neurons in the SNpc. Western blot assay results also demonstrated that LPS-induced decreases in TH protein expression on the lesioned side of the substantia nigra could be restored by GRb1 treatment. No significant changes were observed on the unlesioned side of the substantia nigra or striatum. Our results are in agreement with previous reports, further suggesting that GRb1 conferred the protective effect on dopamine neurons *in vivo* (Ardah et al., 2015; Zhang et al., 2018b). However, little is known about potential mechanisms and anti-inflammatory roles of GRb1 in the nigrostriatal system. Several lines of evidence indicate that the neuroprotective activity of GRb1 involves its an-

ti-inflammatory function (Ke et al., 2014). Zhu et al. (2012) showed that suppression of local inflammation contributed to the neuroprotection elicited by GRb1 in cerebral ischemia rats. A recent study demonstrated that GRb1 alleviated cognitive impairment by inhibiting neuroinflammation in the hippocampus (Wang et al., 2011) and cerebral cortex (Miao et al., 2017). The present study further suggested that GRb1 inhibits microglial overactivation and the release of pro-inflammatory factors that are paramount in the generation of an inflammatory response, and may be responsible for the degeneration of dopaminergic neurons in the substantia nigra following LPS infusion. These data are the first to suggest that GRb1 exerts protective effects on mesencephalic dopaminergic neurons by inhibiting microglial inflammation in an LPS-induced PD model.

NF-κB is located in the cytoplasm as an inactive complex bound to its inhibitory factor IκB. Once activated, NF-κB dissociates from IκB and translocates into the nucleus to induce target gene transcription. It has been well documented that the NF-κB signaling pathway, the most important modulator of pro-inflammatory gene expression, is involved in LPS-induced microglial activation (Sun et al., 2016). GRb1 attenuated pro-inflammatory mediator expression by inactivating NF-κB in LPS-stimulated RAW264.7 cells (Park et al., 2005; Rhule et al., 2006). In the present study, the anti-inflammatory effect of GRb1 on LPS-induced NF-κB activation was determined by western blot assay. Our results showed that IκB phosphorylation levels were markedly increased by LPS stimulation in the substantia nigra, but were effectively reversed by GRb1 treatment. Expression of IKK, a central component of the signaling cascade that controls NF-κB-dependent gene transcription, was also inhibited by GRb1. These results clearly suggest that NF-κB signaling is involved in the anti-inflammatory effect of GRb1 against LPS-induced microglial activation in the SNpc. In addition, some studies have reported that the anti-neuroinflammatory effects of GRb1 may be related to protection of blood-brain barrier and estrogen receptor activation (Cho et al., 2004; Chen et al., 2015). Therefore, the detailed mechanism of the anti-inflammatory effect of GRb1 still needs to be verified *in vivo* and *in vitro*.

Collectively, the above results indicate that GRb1 can prevent LPS-induced neuroinflammation and dopaminergic neuron loss in the nigrostriatal system. Regulation of local inflammation, as demonstrated by inhibition of microglial overactivation and a reduction of pro-inflammatory factors *via* inactivation of NF-κB phosphorylation, may be potential mechanisms by which GRb1 elicits neuroprotection. Thus, the very low toxicity and multifactorial pharmacological activities of GRb1 make it a potential therapeutic to prevent or delay the neuroinflammatory progression of PD.

Taken together, previous studies on GRb1 in PD mainly focused on neuroprotective aspects, such as antioxidant properties (Liu et al., 2018) and scavenging of oxygen free radicals (Liu et al., 2013; Fernandez-Moriano et al., 2017); whereas, our research focused on inhibiting microglial inflammation. This study only observed the effect of GRb1 on the NF-kB inflammatory pathway in PD models rats, but did not observe changes of other inflammatory pathways. There are many inflammatory-related factors involved in PD. As such, in subsequent experiments, we will continue to observe the effects of GRb1 on various inflammatory pathways, as well as interacting effects occurring between different pathways.

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