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Farrerol protects dopaminergic neurons in a rat model of lipopolysaccharide-induced Parkinson's disease by suppressing the activation of the AKT and NF-κB signaling pathways

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ABSTRACT

Neuroinflammation, characterized by the activation of microglia, is one of the major pathologic processes of Parkinson's disease (PD). Overactivated microglia can release many pro-inflammatory cytokines, which cause an excessive inflammatory response and eventually damage dopaminergic neurons. Therefore, the inhibition of neuroinflammation that results from the overactivation of microglia may be an method for the treatment of PD. Farrerol is a 2,3-dihydro-flavonoid obtained from Rhododendron, and it possesses various biological functions, including anti-inflammatory, antibacterial and antioxidant activities. However, the effect of farrerol on neuroinflammation has not been investigated. The present study uncovered a neuroprotective role for farrerol. In vitro, farrerol markedly decreased the production of inflammatory mediators, including interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), cyclooxygenase 2 (COX-2) and induced nitric oxide synthase (iNOS), induced by lipopolysaccharide (LPS) in BV-2 cells. This anti-inflammatory effect was regulated via inhibiting NF-κB p65 and AKT phosphorylation. Furthermore, we found that farrerol alleviated microglial activation and dopaminergic neuronal death in rats with LPS-induced PD. Pretreatment with farrerol markedly improved motor deficits in rats with LPS-induced PD. Taken together, our results indicate that the neuroprotective effect of the farrerol, which prevents microglial overactivation in rats with LPS-induced PD, may provide a potential therapy for patients suffering from PD.

1. Introduction

The main feature of Parkinson's disease (PD), the second most common neurodegenerative disease in the world, is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain [1]. The main clinical symptoms of PD are resting tremor, stiffness and motor delay as well as progressive nonmotor symptoms [2]. Although the pathogenesis of PD is unclear, neurodegeneration has been demonstrated to be an important pathological process in PD [3,4]. In the brain, microglia are the major immune cells, and they play a crucial role in maintaining central nervous system (CNS) homeostasis [5,6]. Microglia activated by environmental stressors can release a large number of proinflammatory mediators, including interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), cyclooxygenase 2 (COX-2), and induced nitric oxide synthase (iNOS), which lead to neuroinflammation and damage neuronal cells [7,8]. It has been reported that overactivated microglia are accompanied by the pathogenesis of brain disease, including Alzheimer's disease, Huntington chorea and PD [9]. Therefore, the inhibition of proinflammatory mediator production induced by farrerol may represent a potential therapeutic strategy for PD.

Lipopolysaccharide (LPS), a major component of the cell wall of gram-negative bacteria, can activate many types of cells (macrophages and epithelial cells) to secrete pro-inflammatory cytokines [10–12]. LPS has been widely used to establish inflammatory models in vivo and in vitro [13–15]. Furthermore, LPS-induced neuroinflammatory models have been widely used to screen antineuroinflammatory drugs [16–18].

It has been demonstrated that farrerol has many biological effects, such as antibacterial, anti-inflammatory and antioxidant activities

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[19–21]. Additionally, our study showed that farrerol provides a protective effect in trinitro-benzene-sulfonic acid (TNBS)-induced colitis [22]. However, the effects of farrerol on neurodegenerative disease are still unclear. In the present study, the effects of farrerol in rats with LPS-induced PD and in LPS-treated BV-2 cells were investigated. This study revealed that farrerol treatment suppressed the loss of dopaminergic neurons in rats with LPS-induced PD. In addition to the in vivo study, farrerol alleviated the production of pro-inflammatory mediators and the phosphorylation of NF-κB p65 and AKT in LPS-treated BV-2 cells.

2. Material and methods

2.1. Animals and treatments

Experimental Wistar rats weighing approximately 280 g were provided by the Laboratory Animal Center, Bethune Medical College of Jilin University (Changchun, China). Farrerol (> 98% purity; Yuan ye Biotech, Shanghai, China) was dissolved in DMSO. The rats were provided with adequate drinking water and food and were raised under specific pathogen-free (SPF) conditions on a 12-h light-dark cycle. The rats were randomly divided into the following 5 groups with 10 rats in each group: the sham group, in which PBS was injected into the right SNpc; the LPS-injected group, in which the rats were anesthetized with sodium pentobarbital (45 mg/kg) and then LPS was injected into the right SNpc at a concentration of 5 μg/μL; and the farrerol-treated (10, 20 and 40 mg/kg) groups, in which farrerol was administered intraperitoneally three days before the injection of LPS and once a day for 28 days. We complied with animal experimental programs and guidelines approved by the animal protection and use commission of Jilin University (approved on 27 February 2015, Permit Number: 2015047).

2.2. Rotational behavior assay

To assess the severity of the lesion, we injected amphetamine into the rat abdominal cavity and performed rotational behavior tests in the second and fourth weeks, as previously described [16,26]. Briefly, each rat was first placed in a rotating test environment for a period of time for adaptation and was then injected with the dopamine receptor agonist amphetamine. The trial begins 5 min after injection to record the number of turns in rats in 30 min.

2.3. Immunohistological analysis

Midbrain tissue was washed with PBS, fixed in a 4% formaldehyde solution, embedded in paraffin and sectioned (3 μm). The sections the immersed in xylene and dewaxes twice for 15 min each. The sections were dehydrated in different concentrations of alcohol. Antigen retrieval was performed by incubating the sections for 5 min in citric acid salt at 95 °C, and then the sections were washed with PBS. Immunohistochemistry was performed with an Ultra-SensitiveTM S-P kit (containing endogenous peroxidase blocking solution, sheep serum, a biotin-labeled goat anti-rat secondary antibody, and streptavidin-peroxidase) (MBX Biotechnologies, Fuzhou, China). The sections were incubated with the endogenous peroxidase blocking agent for 10 min and washed with PBS 3 times. After being washed, the sections were incubated with sheep serum for 1 h and then treated with an anti-TH (1:1000; Abcam, Cambridge, CA, USA) and Iba-1 (1:1000, Proteintech, Chicago, IL, USA) antibody overnight at 4 °C. The sections were washed three times with PBS for 5 min each. The sections were then incubated in a biotin-labeled secondary antibody for 10 min. The anti-TH antibody labeled dopaminergic neurons and the anti-Iba-1 antibody labeled activated microglia. To quantify the Iba-1- and TH-positive cells, the cells were counted by three researchers blinded to the experimental treatments.

2.4. Cell culture and treatments

BV-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA), with 10% fetal bovine serum (FBS) (Clark, Claymont, DE, USA) and incubated at 37 °C in a humidified atmosphere of 5% CO2. Before each experiment, the cells were incubated for 4 h in serum-free DMEM, which was used to reduce the mitotic effect. The cells were pretreated with farrerol for 1 h and then treated with a certain concentration of LPS (Sigma-Aldrich, St. Louis, MO, USA) for different times.

2.5. MTT assay

An MTT assay was used to detect the effect of farrerol on cell survival. In brief, BV-2 cells were seeded in 96-well plates. The cells were cultured in a humidified incubator at 37 °C and 5% CO2. Different concentrations of farrerol were then added to the cultures for 24 h. After being incubated with MTT (5 mg/ml), the cells were cultured at 37 °C for 4 h. DMSO (150 μl/well) was transferred to each well to dissolve the crystals, and then the absorbance was determined at 570 nm. There were five replicates per farrerol concentration.

2.6. Quantitative real-time PCR

Total RNA was extracted from BV-2 cells using TRIzol (Sigma-Aldrich, St. Louis, MO, USA) following the supplier's instructions. A SYBR Green quantitative RT-PCR kit was used to obtain cDNA templates. Quantitative real-time PCR (qPCR) was used to determine mRNA transcription via the SYBR Green quantitative RT-PCR kit. The mRNA levels of different genes were evaluated using specific primers, and there were three replicates for each sample. These primer sequences are shown in Table 1.

2.7. ELISA

First, BV-2 cells were pretreated for 1 h with various concentrations of farrerol, and the cells were stimulated with LPS (1 μg/mL) for 12 h. The upper culture medium was collected and centrifuged at 4 °C separate the cells from the supernatant. Finally, the levels of IL-1β, PGE2, IL-6 and TNF-α were evaluated with a BioLegend ELISA kit (Biolegend, San Diego, CA, USA) in accordance with the manufacturer's instructions.

2.8. Western blotting analysis

BV-2 cells were split into a 6-cm diameter dish at 80% confluence, and they were treated with various concentrations of farrerol for 1 h and stimulated with LPS for different times. After behavioral tests were performed, the midbrains of the rats were quickly removed and cryopreserved until the beginning of the Western blotting analysis. BV-2 cells and the midbrains of the rats were lysed for 20 min in lysis buffer

Table 1
The primers sequences of β-actin, iNOS, COX-2, TNF-α, IL-1β and IL-6.

Gene	Sequences	Length (bp)
β-actin	(F) 5′-GTCAGGTCATCACTATCGGCAAT-3′ (R) 5′-AGAGGTCTTTACGGATGTCAACGT-3′	147
iNOS	(F) 5′-GAACTGTAGCACAGCACAGGAAAT-3′ (R) 5′-CGTACCGGATGAGCTGTGAAT-3′	158
COX-2	(F) 5′-CAGTTTATGTTGTCTGTCCAGAGTTTC-3′ (R) 5′-CCAGCACTTCACCCATCAGTT-3′	127
TNF-α	(F) 5′-CCCCAAAGGGATGAGAAGTTC-3′ (R) 5′-CCTCCACTTGGTGGTTTGCT-3′	136
IL-1β	(F) 5′-GTTCCCATTAGACAACTGCACTACAG-3′ (R) 5′-GTCGTTGCTTGGTTCTCCTTGTA-3′	139
IL-6	(F) 5′-CCAGAAACCGCTATGAAGTTCC-3′ (R) 5′-GTTGGGAGTGGTATCCTCTGTGA-3′	138

(Beyotime Inst. Biotech, Beijing, China). Then, the samples were centrifuged at 4 °C for 15 min at 12000 rpm. The protein in the supernatant was used for Western blotting analysis. SDS-polyacrylamide gel electrophoresis (12%) was used to separate 50 μg of protein, and the proteins were transfer to PVDF membranes (Millipore, ON, Canada; Bedford, MA, USA). After 5% of skim milk was used to block the membranes, the PVDF membranes were incubated with primary antibodies against TH (1:800), OX-42 (1:800), iNOS (1:1000), COX-2 (1:2000), AKT (1:2000), phospho-AKT (1:2000), NF-κB p65 (1:1000), phospho-NF-κB p65 (1:1000), phospho-JNK1/2 (1:2000), JNK1/2 (1:2000), phospho-ERK1/2 (1:2000), ERK1/2 (1:2000), phospho-p38 (1:2000), p38 (1:2000) (Cell Signaling Technology, Danvers, MA, USA), and β-actin (1:5000) (Santa Cruz, CA, USA). Then, the PVDF membranes were incubated for 1 h with a goat anti-rabbit (1:2000) or goat anti-mouse (1:2000) secondary antibody. The proteins were evaluated with ECL Western blot detection reagents (Amersham Pharmacia Biotech, Tokyo, Japan).

2.9. Statistical analysis

All results are shown as the mean ± SD and were analyzed by the SPSS 12.0 statistical software package (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to compare differences between two groups. Differences between groups were assessed by one-way analysis of variance (ANOVA) , post hoc comparisons using Tukey's method of adjustment were conducted to determine the location of significant pairwise differences. $p < 0.05$ was considered significant, and $p < 0.01$ was considered highly significant.

3. Results

3.1. Farrerol protects rats from the motor dysfunction

The unilateral injection of LPS into the SNpc causes microglial activation and dopaminergic neuron damage, consequently leading to behavioral disorders in PD rats. Apomorphine, an indirect agonist of the dopamine receptor, can be used to induce rotational behavior in PD rats [16]. To evaluate the role of farrerol in motor dysfunction in PD rats, the number of rotations performed by the PD rats was investigated. After the rats were injected with LPS for two weeks (Fig. 1A) and four weeks (Fig. 1B), the number of rotations induced by apomorphine was markedly increased. However, treatment with farrerol (10, 20 or 40 mg/kg/d) obviously reduced the rotation number (Fig. 1). Our results reveal that farrerol treatment improves motor dysfunction in rats with LPS-induced PD.

3.2. Farrerol ameliorates dopaminergic degeneration and α-synuclein increase in rats with LPS-induced PD

One of the histopathological features of PD is the damage and death of dopaminergic neurons. Tyrosine hydroxylase (TH) is a rate-limiting enzyme for dopamine synthesis and plays an important role in the synthesis of dopamine. The number of TH-positive cells and the protein expression level of TH can be used to evaluate the morphology of dopaminergic neurons. To investigate the effect of farrerol on dopaminergic neuronal damage, the number of TH-positive cells was evaluated by IHC staining. Our results showed that LPS treatment significantly decreased the number of TH-positive cells compared to that in the sham group, while farrerol treatment significantly inhibited the decrease in the number of TH-positive cells in PD rats [23] (Fig. 2A, B). To further confirm this observation, the expression level of TH in the SNpc was detected by Western blotting. The Western blot results showed that farrerol treatment inhibited the reduction in the expression of the TH protein in the rat model of LPS-induced PD (Fig. 2C, E). To detect whether LPS-induced dopaminergic neuronal degeneration is associated with an increase in α-synuclein, we examined the expression of α-synuclein by Western blotting. The Western blot results showed that farrerol treatment suppressed the increase in α-synuclein in the rat model of LPS-induced PD (Fig. 2C, D). Overall, our data reveal that farrerol has a neuroprotective effect on dopaminergic neurons and can attenuate the increase in α-synuclein in a rat model of LPS-induced PD.

3.3. Farrerol suppresses the overactivation of microglia induced by LPS in the SNpc of rats

Previous studies have shown that microglial overactivation contributes to the reduction of dopaminergic neurons in rats with LPS-induced PD [24,25]. Ionized calcium binding adaptor molecule-1 (Iba-1) is a marker of microglial activation and is present in activated microglia [26]. Our IHC staining results indicated that LPS injection dramatically increased the number of Iba-1-positive cells, while farrerol treatment inhibited the effect induced by LPS; this change was characterized by a reduction in the number of Iba-1-positive cells (Fig. 3A, B). When microglia are activated, the protein expression of OX-42 in microglia is increased. To further verify the results, the protein expression of OX-42 in the SNpc was detected by Western blotting. Similar observations were found. The protein level of OX-42 in LPS-injected rats was markedly increased, while this effect was rescued by farrerol treatment (Fig. 3C). These results suggest that the neuroprotective effect of farrerol may be attributed to the inhibition of microglial activation.

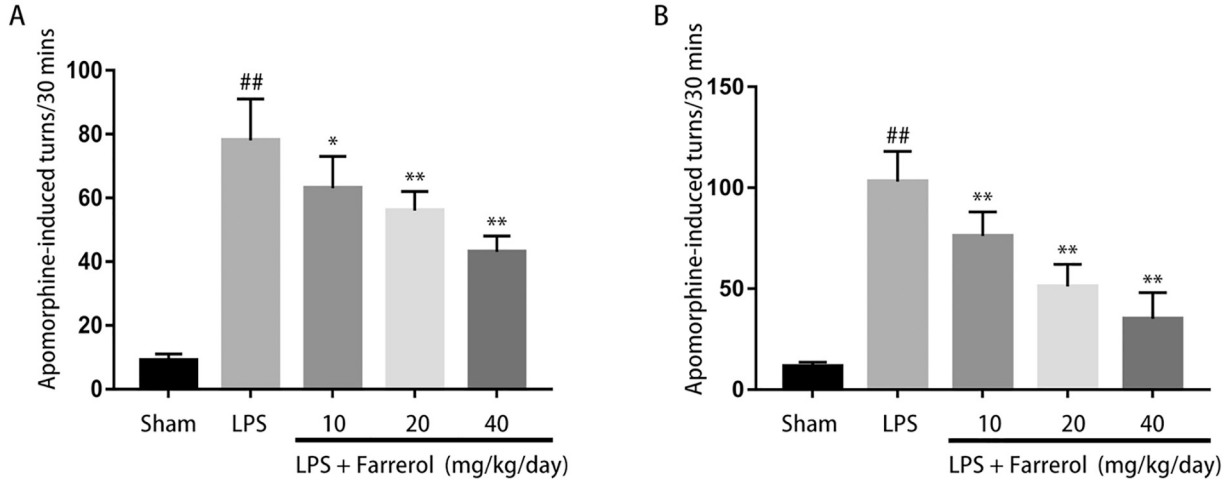

Fig. 1. Farrerol relieves motor dysfunction in rats with LPS-induced PD. After being pretreated with farrerol (10, 20 or 40 mg/kg/d) for three days, the rats were unilaterally injected with LPS. After being injected for two weeks (A) and four weeks (B), the number of rotations induced by apomorphine in the PD rats was counted. $^{\#\#}$ $p < 0.01$ compared to the sham group, * $p < 0.05$ and ** $p < 0.01$ compared to the LPS groups.

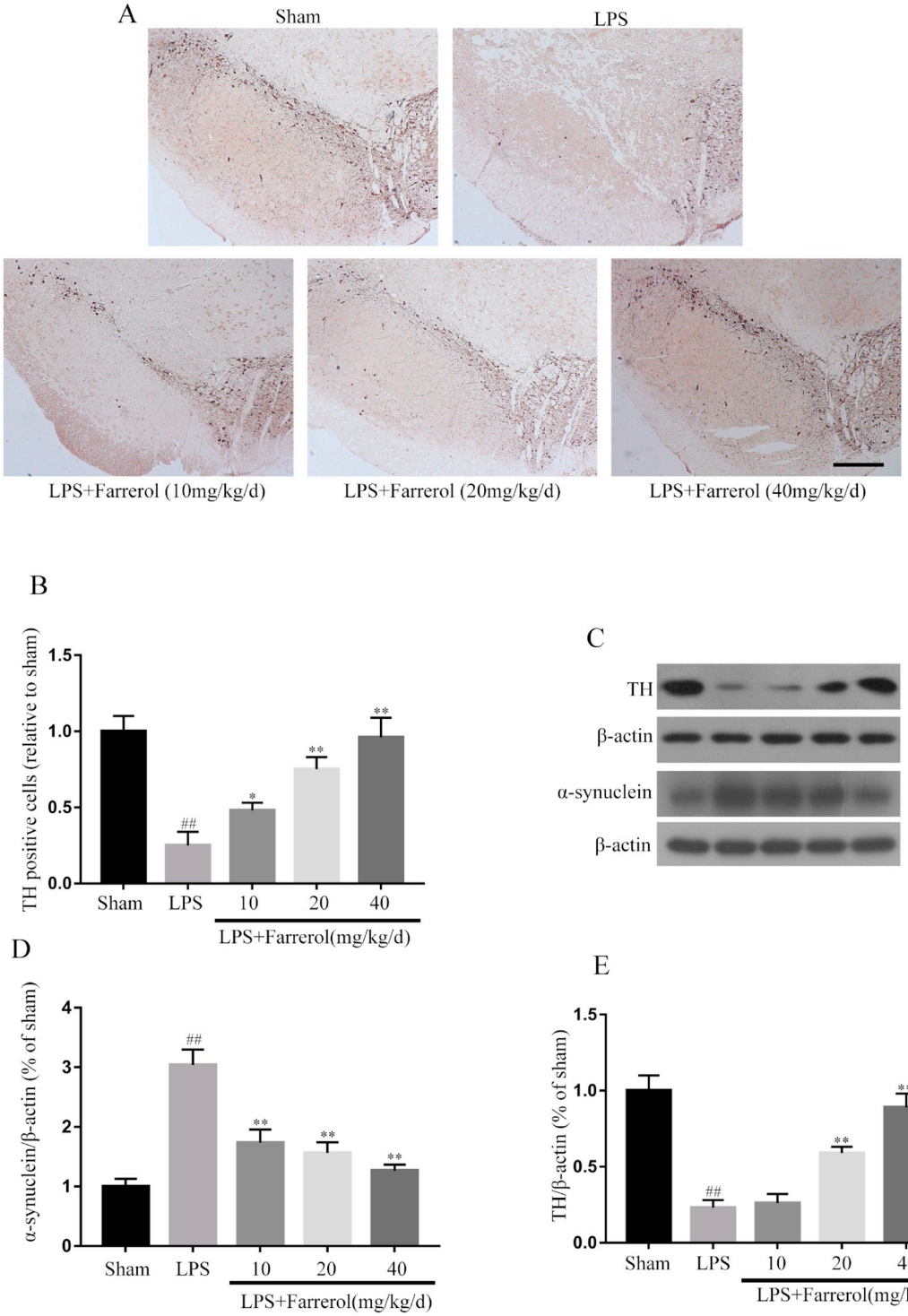

Fig. 2. Farrerol inhibits the reduction of dopaminergic neurons in rats with LPS-induced PD. The midbrains of PD model rats were removed under anesthesia on the 28th day. (A) The evaluation of TH-positive cells stained by IHC; the scale bar represents 100 μm (n = 5). (B) The survival ratio of the TH-positive neurons in the SNpc (n = 5). (C–E) The expression level of TH and α-synuclein (n = 5). $^{\#\#}$ $p < 0.01$ compared to the sham group, * $p < 0.05$ and ** $p < 0.01$ compared to the LPS groups.

3.4. Farrerol inhibits the production of pro-inflammatory cytokines in LPS-treated BV-2 cells

Our results reveal that farrerol treatment relieves behavioral disorders, dopaminergic neuronal loss, and microglial activation in a rat model of LPS-induced PD. To further confirm the neuroprotective mechanisms of farrerol, the effects of farrerol on the activation of the microglial cell line BV-2 were measured. First, the effect of farrerol on BV-2 cell viability was evaluated by the MTT assay (Fig. 4B). We found that BV-2 cells treated with farrerol at a concentration lower than 90 μM did not exhibit changes in viability. However, farrerol treatment at a concentration of 120 μM or above reduced the viability of BV-2 cells. Hence, we chose a dose of < 90 μM to investigate the effect of farrerol on BV-2 cells. Our data showed that LPS injection into the SNpc caused the activation of microglia, which can release a large number of proinflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-

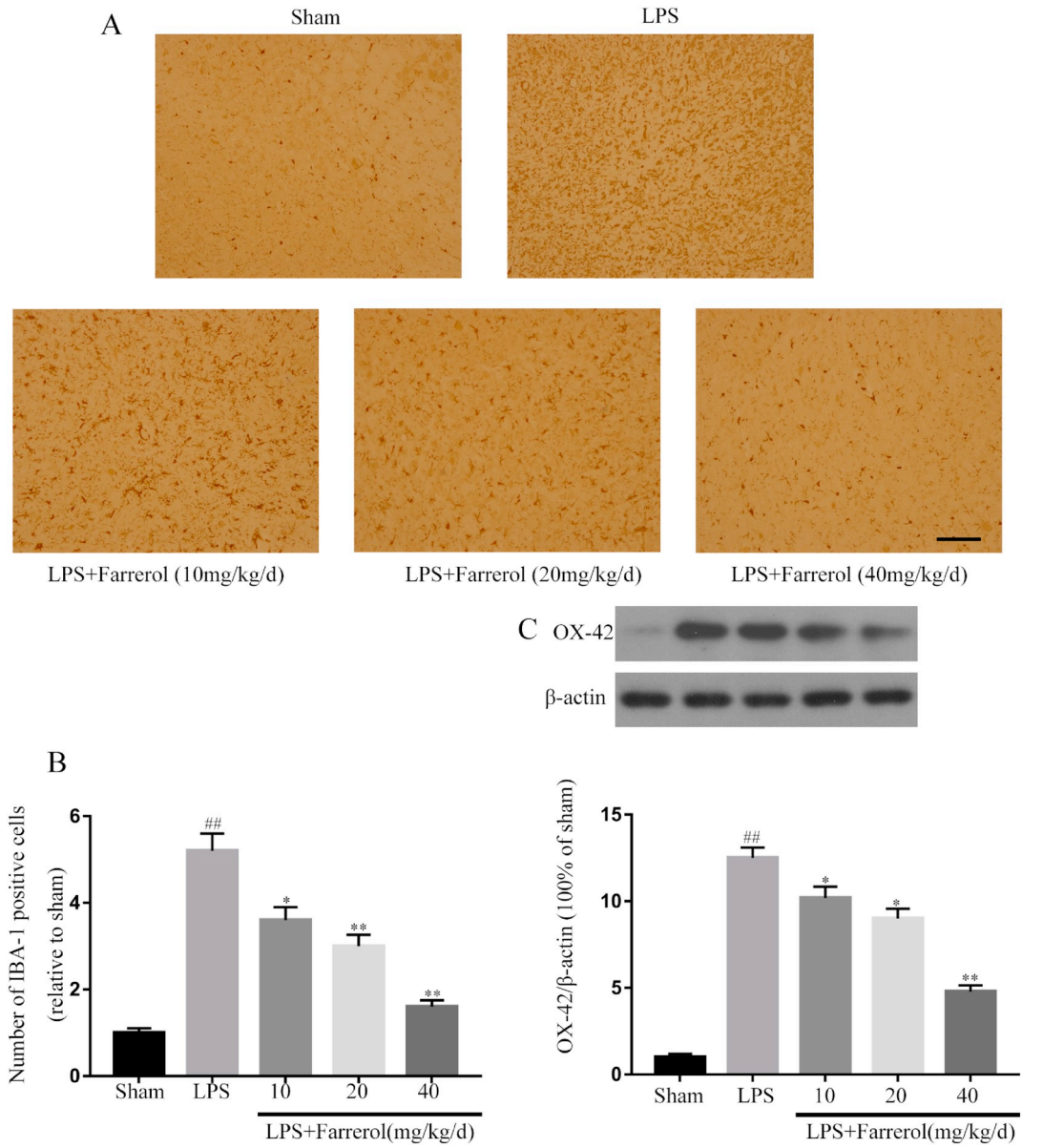

Fig. 3. Farrerol inhibits microglial activation induced by LPS. The midbrains of PD model rats were removed under anesthesia on the 28th day. (A) The morphology of the activated microglia in the SNpc was revealed via Iba-1 IHC staining (n = 5). A representative photomicrograph of the SNpc is shown. The scale bar represents 100 μm (n = 5). (B) The number of Iba-1-positive cells (n = 5). (C) The protein expression levels of OX-42 (n = 5). $^{\#\#}$ $p < 0.01$ compared with the sham group, * $p < 0.05$ and ** $p < 0.01$ compared with LPS groups.

α). To explore the effects of farrerol on neuroinflammation produced by activated microglia, BV-2 cells were pretreated with 30, 60 and 90 μM of farrerol and then exposed to 1 μg/mL of LPS for 4 h or 12 h. The gene and protein levels of these neurotoxic factors were measured by RT-PCR and ELISA, respectively. The results suggested that farrerol significantly suppressed proinflammatory cytokine production, including that of IL-1β, IL-6 and TNF-α (at the mRNA and protein level), in a dose-dependent manner in BV-2 cells treated with LPS (Fig. 4C–H).

3.5. Farrerol suppresses the production of pro-inflammatory enzymes and the release of its end-product in LPS-stimulated BV-2 cells

Activated microglia cause neuroinflammation accompanied by an increased production of NO, PGE2, iNOS and COX-2 [27]. To further explore the effect of farrerol on these inflammatory mediators, BV-2 cells were pretreated with different concentrations of farrerol and then exposed to LPS for 24 h. In addition to the effect on inflammatory cytokine production, LPS stimulation increased NO and PGE2 production in BV-2 cells, while farrerol treatment rescued this effect (Fig. 5F, G). NO and PGE2 are products regulated by iNOS and COX-2, respectively [28]. Subsequently, iNOS and COX-2 production in BV-2 cells was investigated. We found that the mRNA and protein expression of inflammatory enzymes (iNOS and COX-2) were increased in LPS-activated BV-2 cells (Fig. 5A–E). The results showed that farrerol alleviated the effect induced by LPS in BV-2 cells, reducing inflammatory enzyme production at the mRNA and protein levels in a dose-dependent manner (Fig. 5A–E).

3.6. Farrerol inhibits the activation of the AKT and NF-κB p65 signaling pathways in LPS-treated BV-2 cells

Accumulating evidence has revealed that the mitogen-activated protein kinase (MAPK), AKT and NF-κB signaling pathways play a significant role in regulating inflammatory responses [29–31]. In this study, our data showed that farrerol treatment suppressed LPS-induced neuroinflammation in vivo

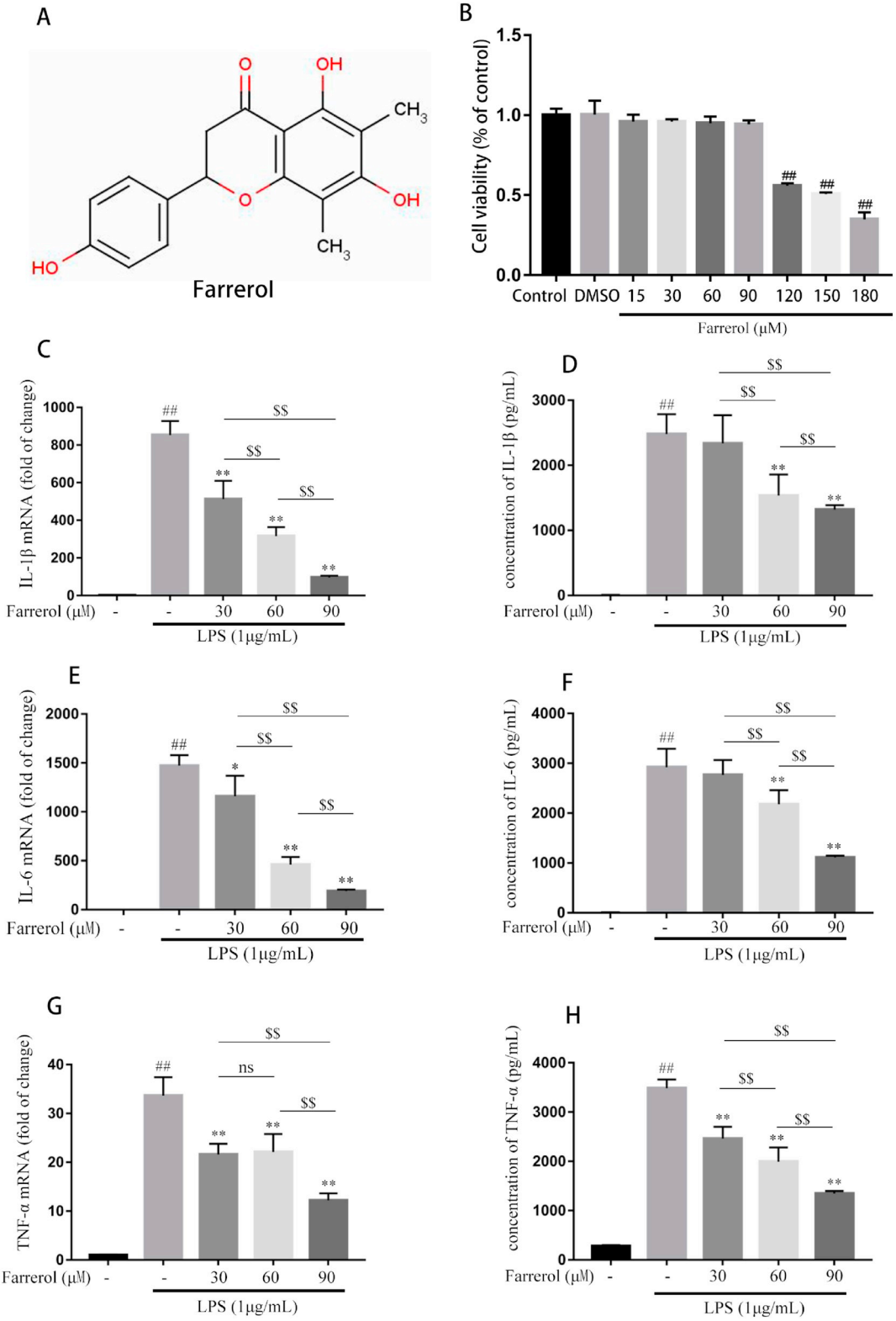

Fig. 4. Farrerol inhibits inflammatory cytokine production in BV-2 cells. (A) The chemical structure of farrerol. (B) The viability of BV-2 cells treated with farrerol (n = 5). (C, E, G) The transcriptional levels of cytokines were detected by qRT-PCR (n = 3). (D, F and H) The protein levels of cytokines were analyzed by ELISA (n = 3). $^{\#\#}$ $p < 0.01$ compared to the untreated group, * $p < 0.05$ and ** $p < 0.01$ compared to the LPS groups, $^{\$\$}$ $p < 0.01$ represented the statistical analysis of the two groups, ns means no significant difference.

and in vitro. To explore the effect of farrerol on neuroinflammation, the activation of inflammatory signaling pathways, including the MAPK, AKT, and NF-κB signaling pathways, in LPS-stimulated BV-2 cells was examined (Fig. 6A). We found that LPS significantly increased the phosphorylation levels of ERK1/2, p38 and JNK1/2, AKT and NF-κB p65 in BV-2 cells, while farrerol treatment dramatically reduced the activation of the AKT and NF-κB p65 signaling pathways (Fig. 6B, C) but showed no effect on ERK1/2, p38 or JNK1/2 phosphorylation (Fig. 6D–F).

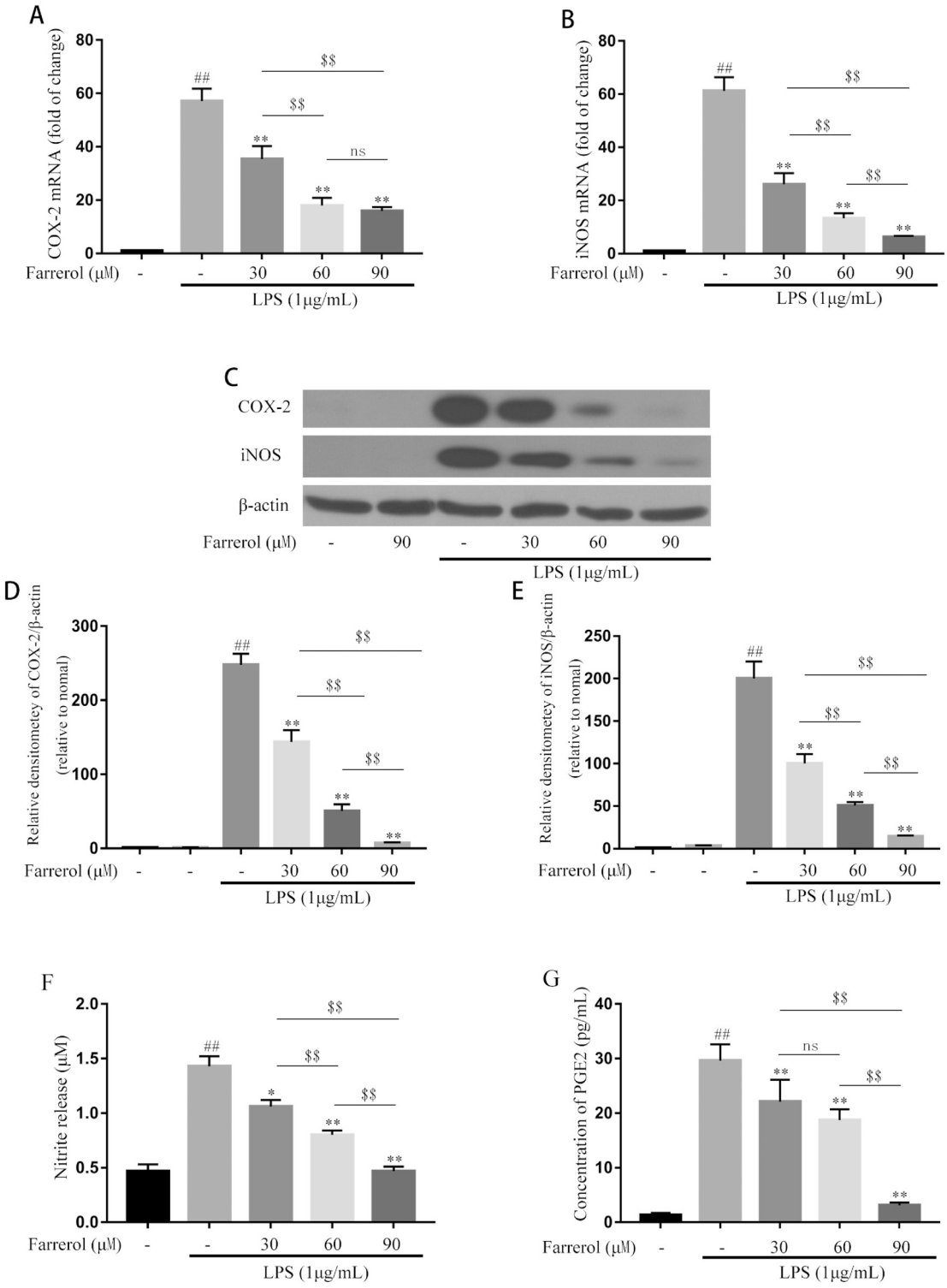

Fig. 5. Farrerol suppresses the production and release of proinflammatory enzymes in LPS-treated BV-2 cells. (A, B) The gene levels of COX-2 and iNOS were detected by RT-PCR (n = 3). (C–E) The protein levels of iNOS and COX-2 were determined by Western blotting (n = 3). (F) The release of NO was detected by Griess reagents; (G) The release of PGE2 was measured by ELISA (n = 3). $^{\#\#}$ $p < 0.01$ compared to the untreated group, * $p < 0.05$ and ** $p < 0.01$ compared to the LPS groups, $^{\$\$}$ $p < 0.01$ represented the statistical analysis of the two groups, ns means no significant difference.

4. Discussion

In this study, our results indicated that farrerol exerted neuroprotective effects in PD rats. The behavioral tests showed that farrerol treatment improved motor dysfunction in LPS-injected rats. Additionally, farrerol treatment rescued the loss of dopaminergic neurons and downregulated the activation of microglia. To further investigate the neuroprotective mechanisms of farrerol in PD rats, we investigated the important signaling pathways in LPS-treated BV-2 cells. We found that farrerol treatment inhibited microglia-mediated neuroinflammation by suppressing activation of the NF-κB and AKT signaling pathways.

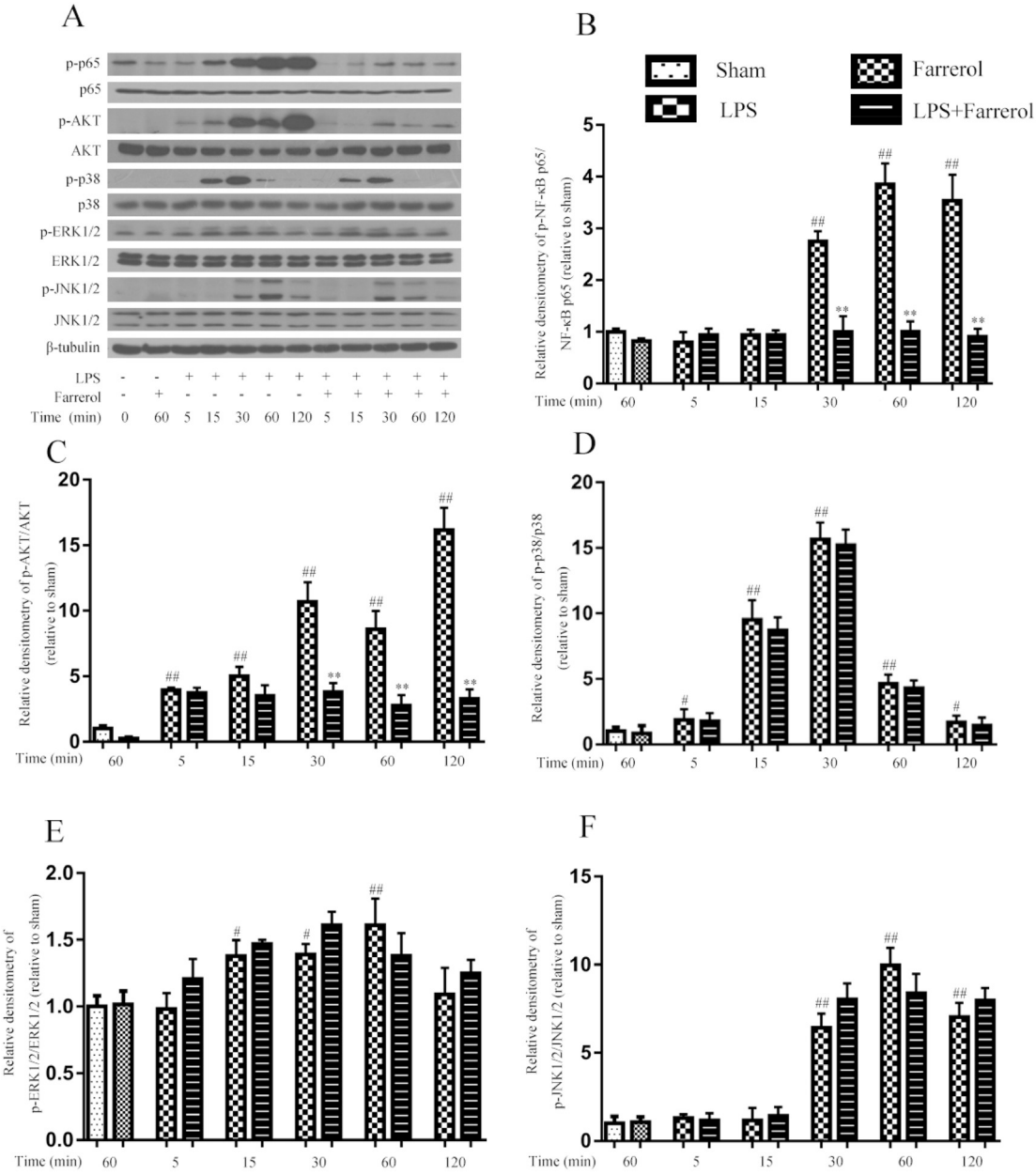

Fig. 6. Effect of farrerol on inflammatory signaling activation in LPS-treated BV-2 cells. BV-2 cells were treated with farrerol (90 μM) for 1 h and then stimulated by LPS (1 μg/ml) for 5, 15, 30, 60 or 120 min. (A) Inflammatory signaling activation was detected by Western blotting. (B–F) The phosphorylation of AKT, NF-κB p65, p38, ERK1/2 and JNK1/2 was analyzed under relative control conditions. $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ compared to the sham group, * $p < 0.05$ and ** $p < 0.01$ compared to the LPS groups at same time point.

Microglia are innate immune cells in the SN and play an important role in the pathogenesis of neurological diseases [32,33]. Accumulating evidence has demonstrated that a large number of overactivated microglia exist in the SN of PD patients [17,18,34]. Cytotoxic factors produced by activated microglia contribute to neuronal damage and neurodegenerative diseases [35]. Thus, inhibiting microglial activation has become an underlying method for preventing and treating PD. Increasing evidence has indicated that neuroinflammation induced by microglia plays a key role in the development of dopaminergic neurodegeneration [36–38]. In PD patients, there is a vicious cycle between the overaction of microglia and neuronal damage, which is the driving force of the progressive development of PD [39,40]. Therefore, in this study, we aimed to find a drug to inhibit the activation of microglia.

Farrerol has been reported to protect many animal models of disease [22,41,42]. Previous studies have also demonstrated that farrerol functions as an antibacterial, anti-inflammatory, antiallergy and antioxidant molecule [41,43,44] and suppresses the production and release of proinflammatory cytokines in LPS-treated human gingival fibroblasts by inhibiting the PI3K/AKT/NF-κB signaling pathway [45]. Many natural products have been demonstrated to exert neuroprotection in rats with LPS-induced PD [18,46,47]. To detect whether farrerol can prevent dopaminergic neurodegeneration resulting from neuroinflammation, we measured the effect of farrerol in a rat model of LPS-induced PD. LPS is a natural activator of microglia and is widely used to induce a number of diseases [48]. It has been reported that LPS injection into the SNpc of rats causes the excessive activation of microglia and sustained dopaminergic neurodegeneration [49]. In addition, rats with LPS-induced PD are commonly used to investigate the connection between neuroinflammation and dopaminergic neurodegeneration and to explore new anti-inflammatory drugs for PD. In the present study, our data showed that LPS injection into the SNpc of rats induces behavioral disorders, microglial activation and dopaminergic

neurodegeneration. Apomorphine is an indirect agonist of dopamine receptors and can cause rotational behavior in LPS-injected PD rats. After rats were injected with apomorphine, the rotational behavior of the rats was measured. Surprisingly, farrerol treatment inhibited the number of rotations in rats with LPS-induced PD, suggesting that farrerol improves behavioral dysfunction in PD rats. Dopaminergic neuronal damage and loss and reduced TH expression reflect the pathological processes of PD. The results showed that farrerol treatment significantly alleviated the reduction of TH expression as well as the number of TH-positive cells in PD rats, suggesting that farrerol prevents dopaminergic neurodegeneration. Accumulating evidence has revealed that microglia-mediated neuroinflammation plays a vital role in the pathological process of PD [50–52]. The large number of neurotoxic factors released by overactivated microglia can damage dopaminergic neurons and result in neuronal death. Our IHC staining and Western blotting results showed that LPS injection led to an increase in Iba-1 and OX-42 expression. Our data revealed that farrerol treatment significantly reduced the production of Iba-1 and OX-42 in rats with LPS-induced PD. These results s that Farrerol protected dopaminergic neurons partly through inhibiting activated microglia in PD rats.

Overactivated microglia release a large number of proinflammatory mediators, including iNOS and COX-2, IL-1β, IL-6, TNF-α, NO, and PGE2. PGE2 plays an important role in immune monitoring, cell proliferation, apoptosis and inflammation [53]. The overproduction of NO can cause cell death and lead to functional disorders. A previous study showed that the inhibition of NO production provides neuroprotective effects [54]. iNOS and COX-2 regulate the synthesis of NO and PGE2, respectively. Previous studies have demonstrated that the inhibition of iNOS and COX-2 suppresses microglial activation and exerts neuroprotective effects [27,55]. In our study, we found that farrerol suppressed the production of proinflammatory mediators (iNOS, COX-2, IL-1β, IL-6, TNF-α, NO and PGE2) in LPS-treated BV-2 cells. In this study, we evaluated the MAPK, AKT and NF-κB signaling pathways, which play crucial roles in the inflammatory response, such as regulating the production of IL-1β, IL-6, TNF-α, COX-2 and iNOS [16,29,54,56]. Our results demonstrated that farrerol significantly inhibited the phosphorylation level of AKT and NF-κB p65 in LPS-induced microglia but had no significant effect on the phosphorylation of MAPKs. There is much evidence that farrerol has therapeutic and protective effects in many disease models [57–59]. In our study, we found that farrerol reduced the neurotoxicity induced by activated microglia. The study provided new insights into the mechanism by which farrerol exerts its protective anti-inflammatory mechanisms. Farrerol is a flavonoid, which guarantees the safety of farrerol [60]. Therefore, farrerol is likely become a safe and effective natural alternative for traditional medicines to prevent or improve the progression of neurodegenerative diseases. In conclusion, our research showed that farrerol protects dopaminergic neurons by suppressing the release of proinflammatory mediators via inhibiting the activation of AKT and the NF-κB signaling pathway, suggesting that farrerol may be developed as a new drug for the prevention or intervention of PD.

Author contributions

Y.L., Y.Z., T.M. and X.G. completed most of the experiments, analyzed the results and wrote the manuscript. G.H. conceived and designed this experiment. B.H., D.H., X.R., J.D., Y.Z., S.F., performed the qRT-PCR experiments and the western blot experiments. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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