

# Forsythiaside A Exhibits Anti-inflammatory Effects in LPS-Stimulated BV2 Microglia Cells Through Activation of Nrf2/HO-1 Signaling Pathway

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**Abstract** Inflammation and oxidative stress have been reported to play critical roles in the pathogenesis of neurodegenerative disease. Forsythiaside A, a phenylethano-side product isolated from air-dried fruits of *Forsythia suspensa*, has been reported to have anti-inflammatory and antioxidant effects. In this study, the anti-inflammatory effects of forsythiaside A on LPS-stimulated BV2 microglia cells and primary microglia cells were investigated. The production of inflammatory mediators TNF- $\alpha$ , IL-1 $\beta$ , NO and PGE<sub>2</sub> were detected in this study. NF- $\kappa$ B, nuclear factor-erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) expression were detected by western blot analysis. Our results showed that forsythiaside A significantly inhibited LPS-induced inflammatory mediators TNF- $\alpha$ , IL-1 $\beta$ , NO and PGE<sub>2</sub> production. LPS-induced NF- $\kappa$ B activation was suppressed by forsythiaside A. Furthermore, forsythiaside A was found to up-regulate the expression of Nrf2 and HO-1. In conclusion, this study demonstrates that

forsythiaside A inhibits LPS-induced inflammatory responses in BV2 microglia cells and primary microglia cells through inhibition of NF- $\kappa$ B activation and activation of Nrf2/HO-1 signaling pathway.

**Keywords** Forsythiaside A · LPS · Inflammatory mediators · NF- $\kappa$ B · Nrf2

## Introduction

It is well known that neuroinflammation and oxidative stress are implicated in the development of neurodegenerative disease, such as Parkinson's disease and Alzheimer's disease [1, 2]. Microglia, one of the most important cells in the central nervous system (CNS), plays a critical role in the progress of neuroinflammation [3]. Stimulating of microglia by LPS induces NF- $\kappa$ B activation and inflammatory mediators production [4]. These inflammatory mediators such as TNF- $\alpha$ , IL-1 $\beta$ , NO, and PGE<sub>2</sub> are known to be implicated in the pathogenesis of neurodegenerative diseases [5]. Recently studies showed that pharmacological or nutritional intervention to attenuate microglial activation could attenuate the severity of neurodegenerative diseases [6, 7].

Forsythiaside A, one of the main active ingredients isolated from air-dried fruits of *Forsythia suspensa*, has been reported to have anti-inflammatory and antioxidant effects [8]. Forsythiaside A was found to protect against transient cerebral global ischemia in gerbil [9]. Forsythiaside A was also found to inhibit hydrogen peroxide-induced oxidative stress in PC12 cell [10]. Previous studies showed that forsythiaside A inhibited LPS-induced injury on the bursa of Fabricius (BF) of chickens [11]. In addition, forsythiaside A has been reported to protect against LPS/D-

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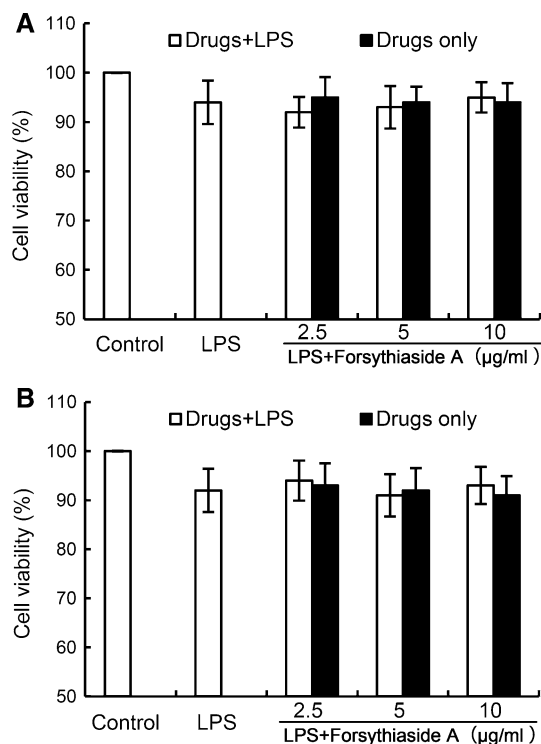
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**Fig. 1** Effects of forsythiaside A on the cell viability of BV-2 microglial cells (a) or primary microglia cells (b). Cells were cultured with different concentrations of PA (2.5, 5, 10 µg/ml) in the absence or presence of 0.5 µg/ml LPS for 24 h. The cell viability was determined by MTT assay. The values presented are the mean  $\pm$  SEM of three independent experiments

galactosamine-induced liver injury [12] and LPS-induced acute lung injury in mice [13]. However, the anti-inflammatory effects of forsythiaside A on LPS-stimulated BV2 microglia cells have not been reported. Therefore, this study aimed to investigate the anti-inflammatory effects and mechanisms of forsythiaside A on LPS-stimulated BV2 microglia cells.

## Materials and Methods

### Materials

Forsythiaside (purity > 98 %) was purchased from the National Institutes for Food and Drug Control (China). LPS (*Escherichia coli* O55:B5) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). ELISA kits of PGE<sub>2</sub>, TNF- $\alpha$ , and IL-1 $\beta$  were purchased from R&D Systems (Minneapolis, MN, USA). Rabbit anti-human NF- $\kappa$ B p65, I $\kappa$ B $\alpha$ , Nrf2, HO-1, and  $\beta$ -actin monoclonal antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). All other reagents were of analytical grade.

### Cell Culture and Viability

BV2 microglia cells were purchased from the Institute of Basic Medical Sciences of the China Science Academy. The cells were cultured in DMEM supplemented with 10 % FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C. Primary rat microglia cells were cultured as reported elsewhere [14]. In brief, whole brains of 1-day-old neonatal Wistar rats were dissociated into individual cells that were cultured for 11 or 14 days as mixed glial cultures with 10 % fetal calf serum (FCS)-supplemented Dulbecco's Modified Eagle's Medium (DMEM; Wako, Osaka, Japan). For the analysis of cell viability, the cells ( $2 \times 10^5$  cells/ml) were treated with different concentrations of forsythiaside A in the presence or absence of LPS (0.5 µg/ml) for 24 h. Cell viability was detected by MTT assay.

### Cytokine Assays

The levels of TNF- $\alpha$ , IL-1 $\beta$ , and PGE<sub>2</sub> in the culture supernatant were determined using a commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The absorbance at 450 nm was measured using a microplate reader.

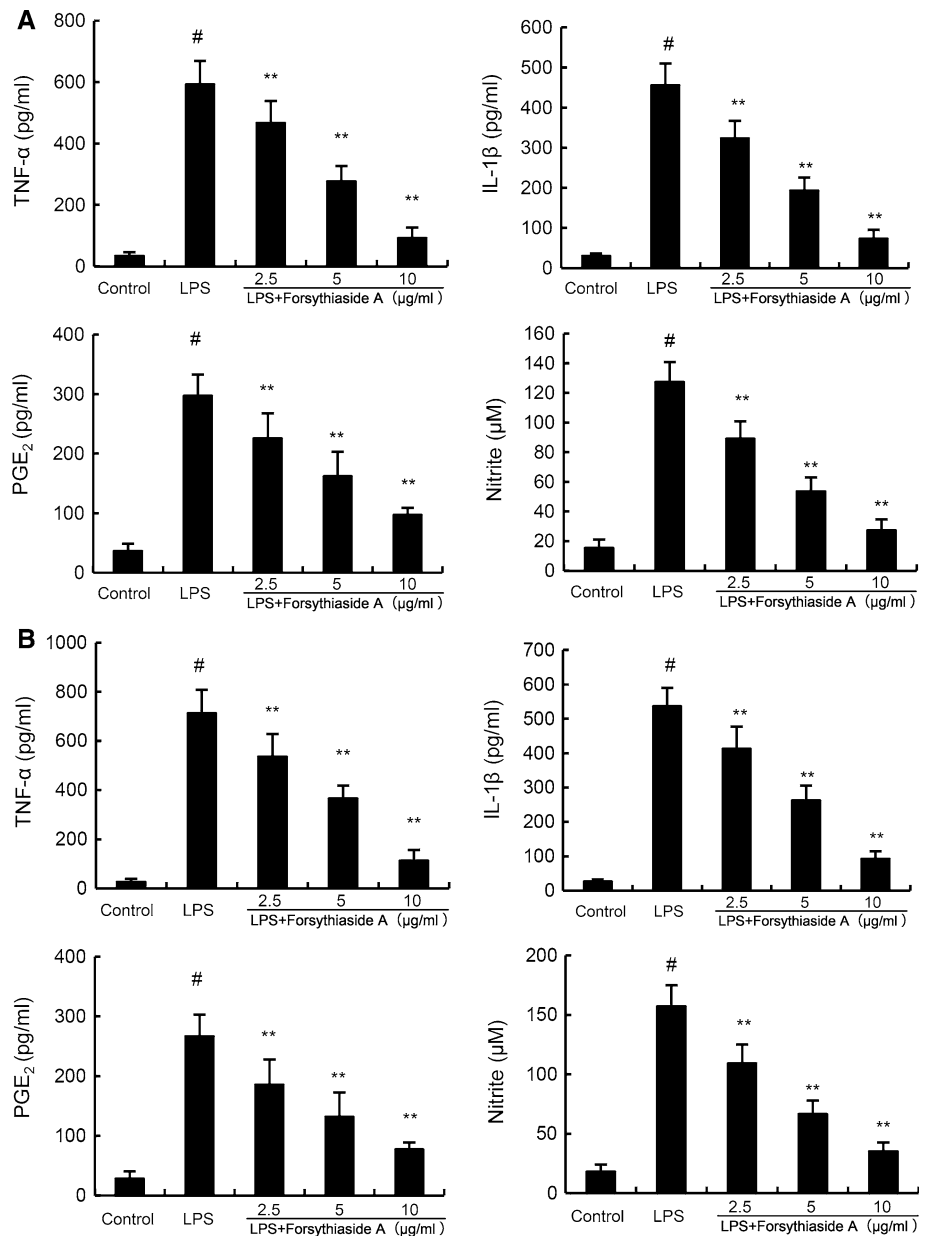
### Nitrite Quantification

The generation of NO was assessed by measuring the nitrite accumulation in the medium using the Griess reaction. Briefly, BV2 microglia cells or primary microglia cells were pretreated with forsythiaside A and stimulated with LPS for 24 h. 100 µl of the cell media was mixed with an equal amount of Griess reagent. Light absorbance was read at 540 nm.

### Western Blot Analysis

Nuclear and cytoplasmic proteins of cells were extracted using NE-PER protein extraction reagents (Pierce, Rockford, IL, USA). Protein concentration was determined by the Bradford method (Bio-rad, USA). Equal amounts of protein were separated on 10 % SDS-PAGE and then transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL). After blocking with 5 % skim milk for 1 h at room temperature, the membrane was incubated with primary antibodies against NF- $\kappa$ B, I $\kappa$ B $\alpha$ , Nrf2, and HO-1. After washing three times, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized using the ECL Prime Western blotting detection reagents (GE Healthcare, NJ, USA).

**Fig. 2** Effects of forsythiaside A on LPS-induced TNF- $\alpha$ , IL-1 $\beta$ , NO and PGE<sub>2</sub> production in BV-2 microglial cells (a) or primary microglia cells (b). The data presented are the mean  $\pm$  SEM of three independent experiments and differences between mean values were assessed by Student's *t* test. #*p* < 0.05 versus control group; \**p* < 0.05, \*\**p* < 0.01 versus LPS group



**Nrf2 siRNA Transfections**

BV2 microglia cells were transfected with Nrf2 siRNA (100 nM) or control siRNA (100 nM) using FuGENE HD transfection reagents (Promega, USA). 36 h later, the cells were treated with linalool and LPS. The production of TNF- $\alpha$ , IL-1 $\beta$ , NO and PGE<sub>2</sub> were detected 24 h after LPS treatment.

**Statistical Analysis**

The values are expressed as mean  $\pm$  SEM. Significance of the differences between groups were analyzed using one-

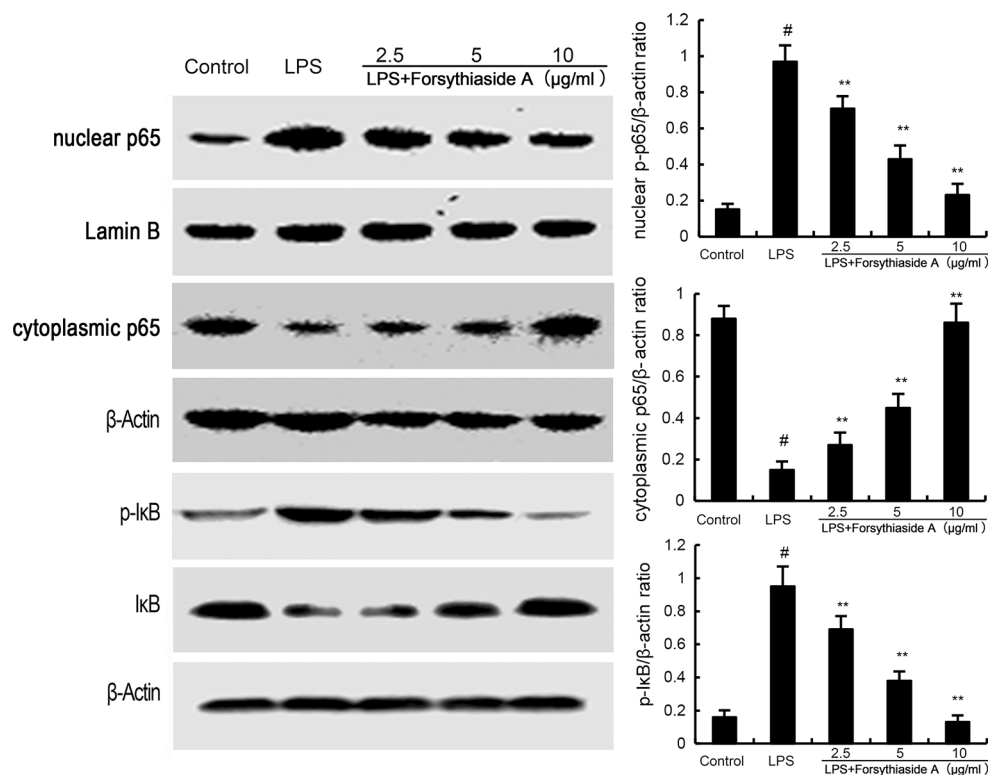
way ANOVA followed by the Bonferroni test. Statistical significance was accepted *p* < 0.05 or *p* < 0.01.

**Results**

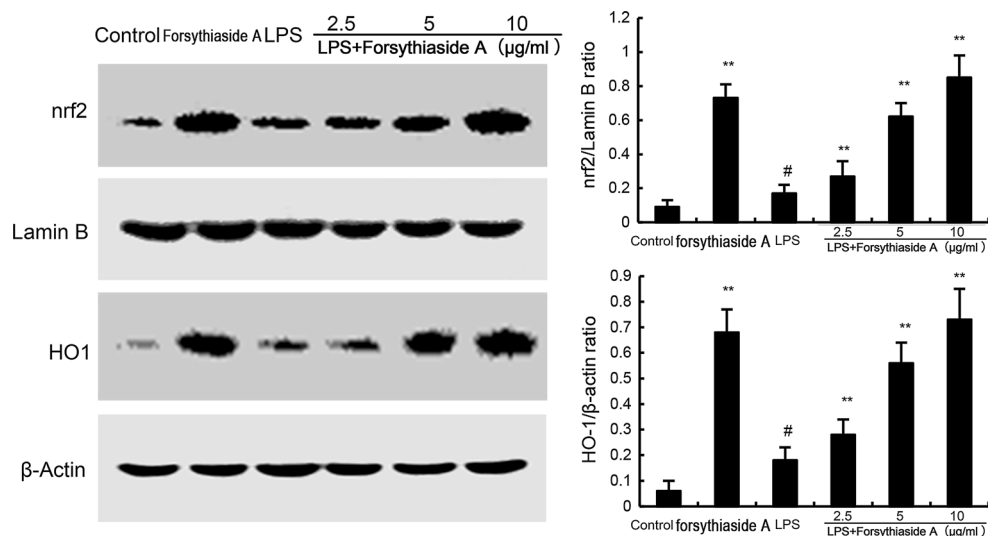
**Effects of Forsythiaside A on Cell Viability**

The effects of forsythiaside A on the viability of BV2 microglia cells or primary microglia cells were detected by MTT assay. As shown in Fig. 1, the results showed that forsythiaside A at the concentration of 2.5, 5, 10  $\mu$ g/ml exerted no significant cytotoxicity on BV-2 microglial cells (Fig. 1a) or primary microglia cells (Fig. 1b).

**Fig. 3** Forsythiaside A inhibits LPS-induced NF- $\kappa$ B activation. The values presented are the mean  $\pm$  SEM of three independent experiments and differences between mean values were assessed by Student's *t* test. #*p* < 0.05 versus control group; \**p* < 0.05, \*\**p* < 0.01 versus LPS group



**Fig. 4** Forsythiaside A induces Nrf2 nuclear translocation and HO-1 expression. The values presented are the mean  $\pm$  SEM of three independent experiments and differences between mean values were assessed by Student's *t* test. #*p* < 0.05 versus control group; \**p* < 0.05, \*\**p* < 0.01 versus LPS group



### Forsythiaside A Inhibits LPS-Induced TNF- $\alpha$ , IL-1 $\beta$ , NO, and PGE<sub>2</sub> Production

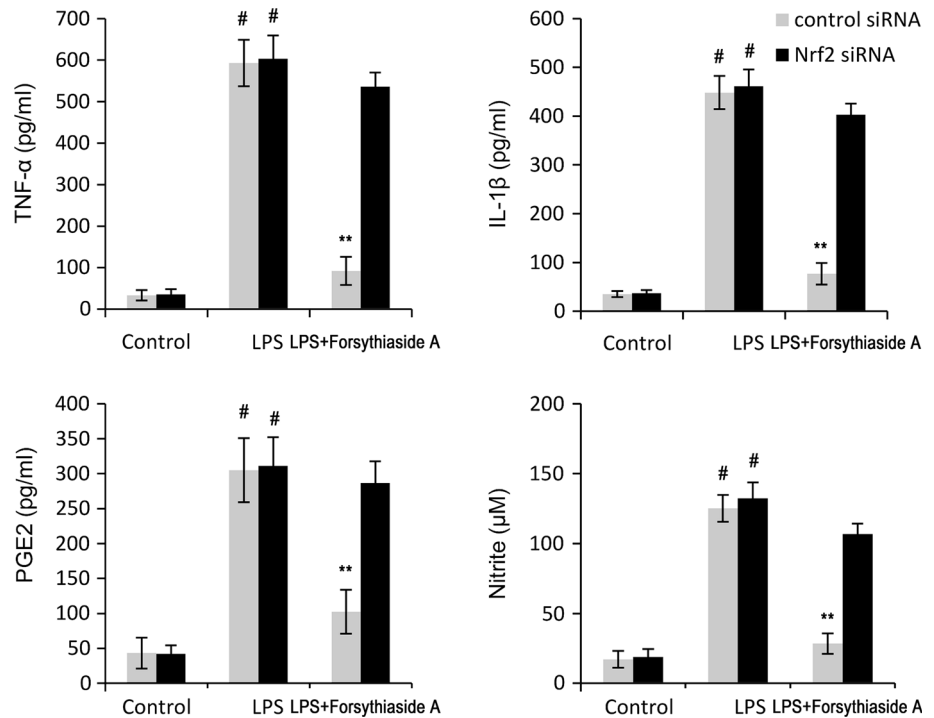
To evaluate the anti-inflammatory effects of forsythiaside A, the inhibitory effects of forsythiaside A on inflammatory mediators production were detected. Our results showed that LPS challenge significantly increased the production of inflammatory mediators TNF- $\alpha$ , IL-1 $\beta$ , NO and PGE<sub>2</sub> production in BV2 microglia cells or primary microglia cells. However, treatment of forsythiaside A markedly

attenuated LPS-induced TNF- $\alpha$ , IL-1 $\beta$ , NO and PGE<sub>2</sub> production in BV2 microglia cells (Fig. 2a) or primary microglia cells (Fig. 2b).

### Forsythiaside A Inhibits LPS-Induced NF- $\kappa$ B Activation

NF- $\kappa$ B is the main regulator of inflammatory mediators production. Therefore, the effects of forsythiaside A on LPS-induced NF- $\kappa$ B activation were detected in this study.

**Fig. 5** Nrf2 knockdown reversed the anti-inflammatory effects of forsythiaside A. The values presented are the mean  $\pm$  SEM of three independent experiments and differences between mean values were assessed by Student's *t* test. #*p* < 0.05 versus control group; \**p* < 0.05, \*\**p* < 0.01 versus LPS group



As shown in Fig. 3, LPS challenge significantly increased NF- $\kappa$ B p65 translocation from cytoplasm to nuclear and I $\kappa$ B $\alpha$  degradation. However, LPS-induced NF- $\kappa$ B activation was significantly inhibited by treatment of forsythiaside A.

#### Forsythiaside A Induces Nrf2 Nuclear Translocation and HO-1 Expression

Nrf2/HO-1 signaling pathway has been reported to play a critical role in regulation of NF- $\kappa$ B activation. In the present study, the effects of forsythiaside A on Nrf2 nuclear translocation and HO-1 expression were detected in this study. The results showed that forsythiaside A could induce the translocation of Nrf2 from cytoplasm to nuclear and up-regulated the expression of HO-1 (Fig. 4).

#### Forsythiaside A Inhibits LPS-Induced Inflammatory Mediators Through Activating Nrf2

To investigate whether activation of Nrf2 is responsible for the anti-inflammatory effects of forsythiaside A, Nrf2 was knockdown by siRNA. Our results showed that Nrf2 knockdown significantly reversed the inhibition of NF- $\alpha$ , IL-1 $\beta$ , NO and PGE<sub>2</sub> production by forsythiaside A (Fig. 5).

#### Discussion

Forsythiaside A, a phenylethanoid product isolated from air-dried fruits of *Forsythia suspensa*, has been reported to have anti-inflammatory effects. In the present study, the anti-inflammatory effects of forsythiaside A on LPS-stimulated BV2 microglia cells and primary microglia cells were detected. Our results showed that forsythiaside A significantly inhibited LPS-induced inflammatory mediators production. Forsythiaside A may be a therapeutic agent for neurodegenerative diseases.

Microglia are resident immune cells that play important roles in the progress of neuroinflammation [15]. During bacterial infection, the uncontrolled activation of microglia can cause neuronal injury [16]. LPS, a constituent of the cell wall of *Gram-negative* bacteria, has been recognized as the primary factor that induces microglia activation [17, 18]. LPS significantly induced the production of inflammatory mediators including TNF- $\alpha$ , IL-1 $\beta$ , PGE<sub>2</sub> and NO [19]. It has been reported the inflammatory mediators TNF- $\alpha$ , IL-1 $\beta$ , PGE<sub>2</sub> and NO were increased in CNS-related diseases [20]. Overproduction of these inflammatory mediators could induce neuronal cell death [21]. In addition, TNF- $\alpha$  and IL-1 $\beta$  could induce the production of other inflammatory mediators such as PGE<sub>2</sub> and NO to amplify the inflammatory responses [22]. In this study, the results showed that forsythiaside A inhibited LPS-induced TNF- $\alpha$ , IL-1 $\beta$ , PGE<sub>2</sub> and NO production in a dose-dependent

manner, suggesting forsythiaside A had the ability to attenuate neuroinflammation.

It is well known that NF- $\kappa$ B plays an important role in inflammatory mediators production [23]. During neuroinflammation, NF- $\kappa$ B regulates the production of TNF- $\alpha$ , IL-1 $\beta$ , PGE<sub>2</sub> and NO [7]. Thus, in this study, the effects of forsythiaside A on LPS-induced NF- $\kappa$ B activation were detected. The results showed that treatment of forsythiaside A significantly inhibited NF- $\kappa$ B activation and I $\kappa$ B $\alpha$  degradation. Previous studies showed that HO-1 played important anti-inflammatory roles in LPS-stimulated BV2 microglia cells [24]. Activating HO-1 attenuated LPS-induced inflammatory mediators production [25]. Nrf2, an important transcription factor, has been shown to be involved in inflammatory and antioxidant responses [26]. Activation of Nrf2 could regulate the transcription of antioxidant enzymes such as HO-1 [27]. In this study, our results showed that forsythiaside A dose-dependently up-regulated the expression of Nrf2 and HO-1. Furthermore, our results showed that Nrf2 knockdown significantly reversed the anti-inflammatory effects of forsythiaside A. These results suggested that forsythiaside A inhibited LPS-induced inflammatory response by inhibiting Nrf2 signaling pathway.

In conclusion, the present study demonstrates that forsythiaside A inhibits TNF- $\alpha$ , IL-1 $\beta$ , PGE<sub>2</sub> and NO production in LPS-stimulated BV2 microglia cells. The promising anti-inflammatory mechanism of forsythiaside A occurs by inhibiting NF- $\kappa$ B activation and activating of Nrf2/HO-1 signaling pathway. These findings suggest that forsythiaside A may be a possible agent in the treatment of neurodegenerative diseases.

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#### Compliance with Ethical Standards

**Conflict of interest** All authors declare that they have no conflict of interest.

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