Original article

Anti-neuroinflammatory effect of Sophoraflavanone G from Sophora alopecuroides in LPS-activated BV2 microglia by MAPK, JAK/STAT and Nrf2/HO-1 signaling pathways

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A B S T R A C T

Background: Neuroinflammation plays a vital role in Alzheimer’s disease (AD) and other neurodegenerative conditions. Sophora alopecuroides is widely used in traditional Uighur’s medicine for the treatment of inflammation. Sophoraflavanone G (SG), a major flavonoid found in the S. alopecuroides, has also been reported to exhibit anti-inflammatory activity both in vitro and in vivo. However, the effect of S. alopecuroides and SG on microglia-mediated neuroinflammation has not been investigated.

Purpose: The present study was designed to evaluate the anti-neuroinflammatory effect of S. alopecuroides and SG against lipopolysaccharide (LPS)-activated BV2 microglial cells and to explore the underlying mechanisms.

Methods: We measured the production of pro-inflammatory mediators and cytokines, and analyzed relevant mRNA and protein expressions by qRT-PCR and Western Blot.

Results: S. alopecuroides extract (SAE) and SG inhibited the LPS-induced release of nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β). Additionally, SG reduced gene expressions of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-6 and IL-1β, and further decreased the protein expressions of iNOS and COX-2. Mechanism studies found that SG down-regulated phosphorylated mitogen-activated protein kinases (MAPKs), phosphoinositide-3-kinase (PI3K)/AKT and Janus kinase/signal transducer and activator of transcription (JAK/STAT), and up-regulated heme oxygenase-1 (HO-1) expression via nuclear translocation of nuclear factor E2-related factor 2 (Nrf2). In addition, SG inhibited the cytotoxicity of conditioned medium prepared by LPS-activated BV2 microglia to neuronal PC12 cells and improved cell viability.

Conclusion: S. alopecuroides and SG displayed anti-neuroinflammatory activity in LPS-activated BV2 microglia. SG was able to inhibit the neuroinflammation by MAPKs, PI3K/AKT, JAK/STAT and Nrf2/HO-1 signaling pathways and might act as a natural therapeutic agent to be further developed for the treatment of various neuroinflammatory conditions.

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Introduction

Neuroinflammation plays an important role in AD and other neurodegenerative conditions (Woo et al., 2015). In neuroinflammation, cytokines, chemokines, reactive oxygen species and reactive nitrogen species, and PGE2, have pivotal functions in the development and maintenance of inflammatory responses (Ma et al., 2015). Microglia, the resident macrophages of the central nervous system (CNS), act as the prime effector cells in mediating neuroinflammation (Kumar et al., 2014). They can be activated by LPS, an endotoxin released from the outer membranes of gram-negative bacteria, to cause neuroinflammatory effects through accelerating the production of pro-inflammatory mediators such as NO, PGE2,
and pro-inflammatory cytokines TNF-α, IL-6 and IL-1β (Hou et al., 2014; Song et al., 2014). Therefore, modulation of microglial activation and production of pro-inflammatory mediators and cytokines is considered to be a promising strategy in the onset and progression of neurodegenerative diseases.

*S. alopecuroides*, a traditional medicinal herb of Uighur medicine in China, has been widely used for treating fever, inflammation, edema and pain for many years (Gao et al., 2012). SG (Fig. 1B), a major flavonoid found in the *S. alopecuroides* (Wan et al., 2015), has also been reported to exhibit anti-inflammatory activity both in vitro and in vivo (Han et al., 2006; Kim et al., 2002; Rollinger et al., 2004; Tashiro et al., 2002; Wun et al., 2013). However, the effect of *S. alopecuroides* and SG on microglia-mediated neuroinflammation has not been investigated. Thus, this study was designed to estimate the capability of *S. alopecuroides* and SG on LPS-activated BV2 neuroinflammation in vitro and to explore the underlying molecular mechanisms.

**Materials and methods**

**Preparation of *S. alopecuroides* extract and SG**

Roots of *S. alopecuroides* were collected at Alar in Xinjiang, China. The crude extract preparation and SG isolation were as described before (Wan et al., 2015). Briefly, air-dried roots of *S. alopecuroides* were homogenized and successively extracted with 75% ethanol for three times under conditions of reflux. The ethanol extract of *S. alopecuroides* was collected and filtered. The supernatant was condensed in a rotary evaporator system and the combined ethanol extract was dried by lyophilization overnight. The extract of *S. alopecuroides* (abbreviated as SG) was stored at 4°C prior to use. SG was separated by using successive open column chromatography over MCI, silica and Sephadex LH-20.

**HPLC analysis of SAE and SG**

HPLC analysis of the SAE and SG was carried out using an Agilent 1290 HPLC instrument (Agilent, Waldbronn, Germany). Chromatographic separation was performed on an Agilent ZORBAX SB-C18 column (4.6 × 250 mm, 5 μm). The mobile phase consisted of water containing 0.1% formic acid (A) and methanol (B). The solvent gradient adopted was 0–5 min, 10–45% B; 5–10 min, 45–60% B; 10–15 min, 60–80% B; 15–25 min, 80–90% B; 25–30 min, 90% B. The flow rate was 1 ml/min. The DAD wavelength was set at 292 nm. The column temperature was maintained at 30°C and injection volume was 5 μl. The purity of SG was more than 95% (Fig. 1B) and the content of SG in SAE was 9.10 ± 0.18μg/ml (Fig. 1A).

**Chemicals and reagents**

SAE and SG were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mg/ml and 50 mM, respectively, and further diluted in cell culture media so that the final DMSO concentration was less than 0.1% v/v. LPS (Escherichia coli 055: B5) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). The ERK inhibitor U0126 and the AKT inhibitor LY294002 were got from Cell Signaling Technology (Beverly, MA, USA). The JNK inhibitor SP600125, the P38 inhibitor SB203580 and the JAK2 inhibitor AG490 were purchased from Beyotime institute of Biotechnology (Jiangsu, China). The HO-1 inhibitor SnPn and inducer CoPP were bought from Santa Cruz Biotechnology (San Diego, CA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Gibco (Gibco-BRL, Grand Island, NY, USA). HO-1 pAb and Keap1 pAb were purchased from Bioworld Technology (Nanjing, Jiangsu, China) and other antibodies used were bought from Cell Signaling Technology (Beverly, MA, USA).

**Cell culture and treatment**

BV2 microglial cells were obtained from National Platform of Experimental Cell Resources for Sci-Tech, Cell Resource Center (Beijing, China) and PC12 dopaminergic neurons were bought from the Cell Bank of the Shanghai Institute of Cell Biology and Biochemistry, Chinese Academy of Sciences (Shanghai, China). The murine BV2 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and rat pheochromocytoma PC12 cells were cultured in DMEM supplemented with 10% FBS and 5% horse serum. To keep sterile, 100 U/ml penicillin and 100 μg/ml streptomycin were added into the culture mediums. Both of the two cell lines were grown at 37°C in humidified 5% CO2.

**MTT assay for cell viability**

BV2 cells were plated at a density of 5 × 10⁴ cells/ml in 96-well plates and incubated with SAE (10–100 μg/ml) or SG (2.5, 5, 10, 15 and 20 μM) for 1 h, followed in the presence or absence of LPS (100 ng/ml) for 24 h. Cell viability was evaluated by the conventional MTT reduction assay as previously described (Guo et al., 2012).

**NO assay**

BV2 cells were seeded into 96-well plates at a density of 2.5 × 10⁵ cells/ml and cultured overnight. After 18 h of treatment with LPS (100 ng/ml) alone or LPS + SAE or LPS + SG, the culture supernatants were collected and the NO production was determined as previously described (Guo et al., 2012).

**Measurement of PGE2, TNF-α, IL-6 and IL-1β release**

For the measurements of PGE2, TNF-α, IL-6 and IL-1β, BV2 were pretreated with SAE or SG for 1 h and then stimulated with LPS (100 ng/ml) for 18 h. PGE2, TNF-α, IL-6 and IL-1β in the cell supernatants were assayed using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

**RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)**

BV2 cells (5 × 10⁵ cells in a 6-well plate) were treated with SGE for 1 h prior to stimulation with LPS for 6 h and then total RNA was extracted with an EASYspin Plus Cell/Tissue RNA Isolation Kit (Aidlab Biotechnologies Co., Beijing, China). Then 1 μg total RNA...
was reverse-transcribed using a ReverTra Ace qPCR RT Kit (TOYO-OBO Ltd., Osaka, Japan) according to the manufacturer’s instructions. Aliquots of diluted cDNA (1:5) were amplified with SYBR Green Real-Time PCR Master Mix (TOYOBO Ltd., Osaka, Japan) in a final volume of 20 μL. QRT-PCR was carried out using the LightCycler 480 (Roche Molecular Biochemicals, Mannheim, Germany). PCR cycles consisted of initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 15 s. The Δ cycle threshold method was used to calculate relative differences in mRNA abundance with the LightCycler 480. Data were normalized to the expression of GAPDH. The results were expressed as fold-changes. The normalized value of the target mRNA of the LPS control group is arbitrarily presented as 1. Primers used for real-time PCR reactions were listed as follows:

- iNOS 5′–3′(sense) GAATCTGGACGGAGTGTGCA
  5′–3′(antisense) GTGAGCCGTGCTGATGAG
- COX-2 5′–3′(sense) CTTGCCTGCTGATGATGT
  5′–3′(antisense) AGTCTGCTGGTTGGAAATGTTGCT
- TNF-α 5′–3′(sense) CTTGTCCTCCTCCTTGCTTA
  5′–3′(antisense) CTTTATCTCCTAATGACCCTAG
- IL-6 5′–3′(sense) AAGGATGGCTAAGGACCAAGAC
  5′–3′(antisense) AGTGAAGGATGTCACAAACTGATA
- IL-1β 5′–3′(sense) TTCTGCTTCTCTCCTCTCGAT
  5′–3′(antisense) TGCTGCTAATGTTCCCTGCTGAT
- GAPDH 5′–3′(sense) CTTTGCCATGGGAGGGCTC
  5′–3′(antisense) GCAGGGATGATGTTGTCGGGAC

**Protein extraction and Western blot analysis**

The BV2 cells were harvested and lysed, and the protein concentrations were determined using the Enhanced BCA Protein Assay Kit (Beyotime institute of Biotechnology, Jiangsu, China). In a parallel experiment, nuclear and cytosolic proteins were prepared using Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) according to the manufacturer’s protocol. Western blot analysis was carried out as previously described (Geng et al., 2014).

**Microglia-conditioned medium preparation, treatments and detection**

BV2 microglial cells were pretreated with different concentrations of S. alopecuroides (5–20 μM) for 1 h, then stimulated with LPS (100 ng/ml) for 18 h. The culture media were collected as conditioned media (CM) and clarified by centrifugation at 12,000 g for 5 min to remove cellular debris. PC12 cells were plated in 96-well plates at a density of 1 × 10^4 cells/well and allowed to settle for 24 h at 37 °C. The conditioned media were then transferred to PC12 neuronal cells which were further incubated at 37 °C for 24 h. PC12 cells viability was then measured by MTT assay.

**Statistical analysis**

Results are expressed as mean ± SEM. Statistical analysis of experimental data was performed using one-way analysis of variance (one-way ANOVA) on the results of at least three independent biological replicates. All calculations were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). p < 0.05 was considered statistically significant.

**Results**

**SAE inhibited LPS-induced neuroinflammation in BV2 cells**

Before evaluating the anti-neuroinflammatory activity of S. alopecuroides, we first performed an MTT assay to determine the cytotoxicity of SAE. The result showed that SAE didn’t impact the cell viability within 80 μg/ml (Fig. 2A). Subsequently, three no-cytotoxic dosages of SAE were chosen to test the anti-neuroinflammatory activity and results showed that SAE dose-dependently inhibited LPS-induced overproduction of pro-inflammatory mediators NO and PGE2, and cytokines TNF-α, IL-6 and IL-1β (Fig. 2B-F).
The major flavonoid of S. alopecuroides, SG, was further examined for its anti-neuroinflammatory activity on LPS-induced NO and PGE₂ production in BV2 microglial cells. As expected LPS stimulation led to a dramatic increase in NO and PGE₂ production. Pretreatment with SG significantly inhibited the LPS-induced NO and PGE₂ production in a dose-dependent manner. While SG treatment alone did not apparently alter the basal NO and PGE₂ production (Fig. 3A and B), MTT assay showed that SG didn’t evidently affect the viability of BV2 cells up to 20 μM in the presence or absence of LPS (Fig. 3C). Western blot analysis indicated that LPS treatment prominently up-regulated iNOS and COX-2 expressions while pretreatment with SG led to a concentration-dependent suppression (Fig. 3D–F). Further qRT-PCR experiment demonstrated that SG attenuated LPS-induced iNOS and COX-2 mRNA levels in a concentration-dependent manner, but SG alone didn’t change the basal expressions (Fig. 3G and H).

**SG inhibited LPS-induced NO and PGE₂ production in BV2 cells through suppression of iNOS and COX-2**

SG inhibited the production of TNF-α, IL-6 and IL-1β and down-regulated the expression of cytokine mRNA in LPS-stimulated BV2 microglia

As shown in Fig. 4A–C, the productions of TNF-α, IL-6 and IL-1β were all dramatically increased in response to LPS stimuli. However, the production was significantly inhibited by SG. Furthermore, SG down-regulated LPS-induced pro-inflammatory cytokine mRNA expression in a concentration-dependent manner in BV2 cells (Fig. 4D–F).

SG inhibited MAPKs inflammatory signaling pathways in LPS-stimulated BV2 microglia

Based on the reported roles of the MAPKs in neuroinflammation, we sought to determine possible effects of SG on the activities of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPKs in LPS-activated BV2 microglia. Stimulation with LPS resulted in marked phosphorylation of ERK, JNK and p38. SG (5–20 μM) produced a concentration-dependent and prominent inhibition of all the MAPK phosphorylation (Fig. 5A–D), especially for the phosphorylation of p38. While total ERK, JNK and
p38 MAPKs expressions did not have any significant change. The result showed that SG produced robust effects on the MAPKs.

**SG reduced LPS-induced PI3K/AKT signaling activation**

To confirm whether SG alters LPS-stimulated activation of PI3K/AKT signaling, we examined AKT, the key protein in the PI3K/AKT signaling pathway. As shown in Fig. 5E and F, LPS notably enhanced phosphorylated AKT protein expression in BV2 cells. Whereas, AKT phosphorylation was reduced by Sophoraflavanone G at 20μM. This result implied that Sophoraflavanone G also had a modestly repressive effect on the LPS-stimulated PI3K/AKT pathway signaling.
SG inhibited JAK/STAT signaling pathway in LPS-induced BV2 cells

To expand our understanding of the anti-neuroinflammatory mechanism of SG, we further explored the regulatory effects of SG on JAK/STAT inflammatory signaling pathway in BV2 cells. Here, we observed that LPS remarkably up-regulated phosphorylation of JAK and STAT in BV2 cells. However, SG markedly reversed this increase (Fig. 6), suggesting that JAK/STAT signaling may be, at least in part, one of potential anti-inflammatory mechanisms of SG in BV2 cells.

Effects of SG on HO-1 expression via nuclear translocation of Nrf2 in BV2 microglia

We examined the effects of SG on HO-1 expression in BV2 microglia. SG (5–20 μM) up-regulated HO-1 protein expression in the presence or absence of LPS (Fig. 7A–D). Activated Nrf2 is released by Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and translocated to nuclear fraction, which is an important event in upstream of HO-1 expression. Treatment of BV2 microglia with SG significantly reduced the Keap1 expression at 20 μM and while increased nuclear translocation of Nrf2 (Fig. 7C,E and F).

Effect of inhibitors and inducers of relevant signaling pathways on NO production and mRNA levels of pro-inflammatory genes

To confirm the effects of several signaling pathways on SG against LPS-induced neuroinflammation, inhibitors and inducers of relevant signaling pathways were applied to evaluate the effects on LPS-induced NO production and mRNA levels of pro-inflammatory genes (Fig. 8). The results showed that MAPK inhibitors (U0126, SP600125 and SB203580), AKT inhibitor (LY294002) and JAK2 inhibitor (AG490) reduced not only the NO production (Fig. 8A) but also the mRNA levels of iNOS, COX-2, TNF-α, IL-6 and IL-1β (Fig. 8B–F). Similarly, CoiPP, the inducer of HO-1, exerted the anti-inflammatory effect on LPS-stimulated BV2 cells. Nonetheless, the HO-1 inhibitor SnPP efficiently reversed the effect of SG when pretreatment with SnPP before adding SG. All these results, directly or indirectly, validated the importance of MAPKs, PI3K/AKT, JAK/STAT and HO-1/Nrf2 signaling pathways in SG against LPS-induced BV2 neuroinflammation.

Effects of SG on microglia-mediated neurotoxicity

As shown in Fig. 9, when conditioned media from LPS-stimulated microglia (L-CM) was added to cultured PC12 cells, the viability of PC12 cells was significantly decreased by about 30% compared with control conditioned media (C-CM) group, suggesting the neurotoxicity of microglia mediated inflammation. However, the conditioned media from BV2 cells with SG pretreatment prior to LPS stimulation, reduced PC12 neuronal cell death in a concentration-dependent manner, which provided evidence that SG exerted a neuroprotective effect at least partly by suppressing microglial activation and subsequent neurotoxicity.

Discussion

In the current study, we demonstrated that S. alopecuroides and its major flavonoid SG could inhibit LPS-activated microglia neuroinflammation and further elucidated the underlying molecular mechanisms. Furthermore, we found that SG could protect PC12 neuronal cells from the cytotoxicity of conditioned medium from LPS-stimulated BV2 microglia.

NO and PGE2, key inflammatory mediators, play a pivotal role in the pathogenesis of many inflammatory pathophysiological conditions (Dilshara et al., 2014). We found that S. alopecuroides and SG
prevented the LPS-induced increase of NO and PGE$_2$ production in BV2 cells, which preliminarily verified the anti-neuroinflammatory potency of $S$. alopecuroides and SG. Excessive NO is mainly generated from l-arginine by iNOS and PGE$_2$ is derived from arachidonic acid by COX-2. Inhibition of iNOS and COX-2 can afford significant neuroprotective effect (Dilshara et al., 2014; Park et al., 2011). Furthermore, experiment showed that SG suppressed the LPS-induced up-regulation of iNOS and COX-2 both in the protein and mRNA levels, suggesting that inhibition of NO and PGE$_2$ production by SG was caused by inhibition of iNOS and COX-2 expression. Moreover, it supported the neuroprotective effect of SG on PC12 neuronal cells.

Fig. 7. (A and B) SG upregulated HO-1 expression in the presence of LPS. (C-F) SG upregulated HO-1 expression via inhibiting Keap1 expression and increasing the nuclear translocation of Nrf2. Cells were treated with SG in the presence or absence of LPS (100 ng/ml) for 18 h and then determined by Western blot with specific antibodies. All values are expressed as mean ± SEM for three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 in comparison with control.

Fig. 8. Effect of inhibitors and inducers of relevant signaling pathways on NO production (A) and mRNA expressions (B–D) of pro-inflammatory genes. Cells were pretreatment with SG and inhibitors and inducers of relevant signaling pathways and then stimulated with LPS (100 ng/ml) for 18 h (A) and 6 h (B–F). NO production was measured by Griess reagents and mRNA expressions were determined by qRT-PCR. All values are expressed as mean ± SEM for three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 in comparison with LPS control. #p < 0.05, ##p < 0.01, ###p < 0.001 in comparison with LPS + SG 20 μM control.
TNF-α, IL-6 and IL-1β are three main pro-inflammatory cytokines produced by activated microglia during the neuroinflammation (Du et al., 2014; Ramsey and Tansey, 2014). They can initiate and regulate the cytokine cascade during an inflammatory response, promote the cascade of glial cell reactions and contribute directly to ischemic, traumatic brain injury and neurodegenerative disease (Wang et al., 2014). In our study we found that S. alopecuroides and SG inhibited the production of TNF-α, IL-6 and IL-1β in LPS-activated BV2 microglia. A previous study reported that SG could decreases the production of these cytokines in LPS-stimulated RAW264.7 cells (Wun et al., 2013). It confirmed the inhibitory activity of SG on LPS-induced production of pro-inflammatory cytokines. Further experiment showed SG may act through down-regulating the expression of cytokines mRNA. These results indicated that S. alopecuroides and SG could exert anti-neuroinflammatory effect, and SG may be a promising candidate to inhibit the neuroinflammation. Therefore, we carried out the study of molecular mechanisms on the following experiments.

MAPKs, a family of serine/threonine protein kinases including ERK, JNK, and p38, play a crucial role in controlling signaling events that contribute to the production of neuroinflammatory mediators (Velagapudi et al., 2014; Zhao et al., 2014). In our experiment, pretreatment with SG decreased the phosphorylated MAPKs especially the phosphorylated p38. While it didn’t change the total MAPKs levels. Further, the MAPKs inhibitors, U0126, SP600125 and SB203580, suppressed the production of NO and the mRNA expression of the key pro-inflammatory genes. These findings elucidated the fundamental role of MAPKs in the anti-neuroinflammation effect of SG.

PI3K/AKT-dependent signaling pathway promotes inflammatory properties in microglia and is the predominant signaling pathway responsible for the synthesis and production of pro-inflammatory mediators (Dong et al., 2014). PI3K/AKT signaling has also been shown to participate in the regulation of gene expression of iNOS and COX-2 in microglia activated by different stimuli including LPS (Wang et al., 2014). SG significantly suppressed the phosphorylated AKT, a central protein in PI3K/AKT signaling. On the other hand, LY294002 inhibited the production of NO and the mRNA expression of the key pro-inflammatory genes including iNOS and COX-2. It was consistent with the previous report about the importance of PI3K/AKT signaling in neuroinflammation (Wang et al., 2014). Taken together, it suggests that for SG the PI3K/AKT signaling, at least to some extent, plays its role in attenuating the neuroinflammation.

JAK/STAT signaling plays an essential role in promoting and modulating immune and inflammatory processes (O’Shea and Plenge, 2012). Different expression of the JAK/STAT pathway have been associated with pathological CNS conditions such as cerebral ischemia, traumatic brain injury and brain inflammation (Yang et al., 2010). In our study, we first found that SG could inhibit the protein expressions of phosphorylated STATs including STAT1, STAT3 and STAT5. Phosphorylated JAKs lead to the activation of the full STAT activities (O’Shea and Plenge, 2012). The result showed that SG repressed both of the phosphorylated JAK1 and JAK2 at different levels, though the effect on JAK2 seemed more obvious. AG490, a specific and potent inhibitor of the JAK2, inhibited the NO production and the mRNA expressions of pro-inflammatory genes, validating indirectly the important role of JAK/STAT signaling in SG against neuroinflammation.

Many researchers have reported that induction of HO-1 expression and related signal pathways exerts anti-neuroinflammatory effect, and protects neurons against neurotoxin-induced cell death and protects neurons against neurotoxin-induced cell death (Chen et al., 2012; Lin et al., 2014; Lu et al., 2010). In our study, we found that SG induced HO-1 expression in the presence of LPS or not. Pretreatment with HO-1 inducer CoP markedly reduced LPS-induced pro-inflammatory mediator NO and mRNA expressions of pro-inflammatory genes. While pretreatment with SnPP before adding SG reversed the above observation. HO-1 expression is closely mediated by Nrf2. In the resting state Nrf2 resides in the cytoplasm with an Nrf2-inhibitory protein Keap1. Following oxidative stress, Keap1 releases Nrf2 and sequentially induces its nuclear translocation. Consistent with these findings, SG down-regulated the expression of Keap1 and induced the nuclear translocation of Nrf2. These findings demonstrated the effect of SG on HO-1 expression via nuclear translocation of Nrf2.

NF-κB is another one of the most important transcription factors accounting for mediating the development and maintenance of inflammation. Previous studies have shown the functional cross-talk between NF-κB and Nrf2, which has positive and negative effects on each other (Wardyn et al., 2015). Wun et al. (2013) reported SG inhibited NF-κB subunit p65 proteins to translocate into the nucleus in LPS-stimulated RAW264.7 cells (Wun et al., 2013). In this study, SG-activated Nrf2-HO-1 signaling countered the inflammatory response in LPS-stimulated BV2 microglia. Since the interlaced relationship between two signaling, the question of what signaling pathway plays the predominant role or takes the earlier effect needs to be further investigated.

Neurotoxic microglial-neuronal interactions implicate in the pathogenesis of various neurodegenerative diseases and microglial activation plays a key part in neuronal death by releasing inflammatory mediators (Hou et al., 2014; Song et al., 2014; Wang et al., 2014). In our study, the conditioned medium from LPS-stimulated microglia was potently toxic to PC12 cells. SG pretreatment could increase cell viability in PC12 Cells and exerted neuroprotective effects on neuronal cells exposed to conditioned medium of LPS-stimulated microglia. The result demonstrated that SG may have a neuroprotective function and could probably be further developed for the treatment of various neuroinflammatory conditions.

In summary, our study identified that S. alopecuroides and SG could exhibit anti-neuroinflammatory activity in vitro by suppressing the pro-inflammatory mediators from LPS-induced BV2 microglial cells. Additionally, SG could protect the PC12 against the cytotoxicity of activated BV2 cells. These beneficial effects could be linked to the ability of inhibiting the MAPKs, PI3K/AKT and JAK/STAT inflammatory signaling pathways, and up-regulating HO-1 expression via nuclear translocation of Nrf2 (Fig. 10). To our knowledge, this is the first comprehensive study regarding the in vitro anti-neuroinflammatory effect of S. alopecuroides and SG. Moreover, we explored the mechanisms of SG-mediated attenuation of LPS-induced neuroinflammation. However, the anti-neuroinflammatory effects and mechanisms of S. alopecuroides and
SG in vivo are definitely awaiting investigation and verification in the future research.

Conflict of interest

The authors declare that they have no conflicts of interest.

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