Isoastragaloside I inhibits NF-κB activation and inflammatory responses in BV-2 microglial cells stimulated with lipopolysaccharide

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Abstract. The excessive activation of microglia in many neurodegenerative diseases is detrimental to neuronal survival. Isoastragaloside I (ISO I) is a natural saponin molecule found within the roots of *Astragalus membranaceus*, a famous traditional Chinese medicine. In the present study, the anti-inflammatory effects and the mechanisms of action of ISO I on activated BV-2 cells stimulated with lipopolysaccharide (LPS) were investigated. ISO I dose-dependently inhibited the excessive release of nitric oxide (NO) and tumor necrosis factor (TNF)-α in the LPS-stimulated BV-2 cells. Moreover, it decreased the production of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), and mitigated the gene expression of interleukin (IL)-1β, TNF-α and iNOS induced by LPS. Further experiments revealed that ISO I decreased the phosphorylation levels of nuclear factor-κB (NF-κB), and suppressed its nuclear translocation and transactivation activity. In addition, it inhibited the activation of signaling pathway molecules, such as PI3K, Akt and mitogen-activated protein kinases (MAPKs). Taken together, our findings suggest that ISO I prevents LPS-induced microglial activation probably by inhibiting the activation of the NF-κB via PI3K/Akt and MAPK signaling pathways, indicating its therapeutic potential for neurological diseases relevant to neuroinflammation.

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Introduction

As resident innate immune cells in the central nervous system (CNS), similar to peripheral macrophages, microglial cells help to enhance host defence by eliminating pathogen attack (1). However, the sustained and uncontrolled activation of microglia leads to the accumulation of excessive detrimental substances, most notably nitric oxide (NO), free radicals and pro-inflammatory cytokines [e.g., interleukin (IL)-1β and tumor necrosis factor-α (TNF-α)] that may finally lead to neuronal destruction (2). Indeed, microglial activation is one of the main pathological characteristics in the processes of many neurological diseases associated with neuroinflammation, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis. The suppression of the activated microglia has been suggested to benefit the prevention of these neurodegenerative disorders (3,4).

NF-κB, one of the Rel family transcription factors, is a primary regulator of genes of many pro-inflammatory cytokines or mediators, such as TNF-α, IL-1β and inducible NO synthase (iNOS), as well as cyclooxygenase-2 (COX-2) (5,6). Once activated by phosphorylation, NF-κB is translocated to the nucleus, and transactivates downstream gene expression. Inhibitors of NF-κB have been suggested in the treatment of many inflammatory diseases (7). Lipopolysaccharide (LPS), a prototypical endotoxin, is able to stimulate the inflammatory responses in microglia by activating the NF-κB signaling pathway (8,9). The LPS-induced activation of BV-2 microglial cells has been used widely for the studies of anti-microglial activation drugs (10-12).

Astragalosides are the main active saponins of *Astragalus membranaceus* (*A. membranaceus*), a well-known traditional Chinese medicine with antioxidative, immunoregulatory, anti-inflammatory and neuroprotective properties (13,14), that have been demonstrated to benefit both the peripheral nervous system and CNS (15-17). Isoastragaloside I (ISO I) is one of the saponin molecules found within *A. membranaceus* (18), which has been demonstrated to alleviate insulin resistance and glucose intolerance in obese mice (19). However, little is known about whether it can inhibit microglial activation and whether it is beneficial in neurological diseases. In this

study, the inhibitory effects of ISO I on inflammatory mediator production in BV-2 microglial cells stimulated with LPS were explored. Moreover, the possible underlying molecular mechanisms were investigated. Our findings indicated that ISO I may be an effective candidate for use in the treatment of various neurodegenerative disorders characterized with microglial activation.

Materials and methods

Drugs and reagents. ISO I (HPLC purity ≥98%) was purchased from Shanghai Lightgoal Industry Co., Ltd. (Shanghai, China). Antibodies to p-NF-κB (no. 3033), NF-κB (no. 6956), p-IκB (no. 2859S), IκB (no. 4812S), Akt (no. 2920), p-phosphoinositide 3-kinase (PI3K; no. 4228), p-p38 mitogen-activated protein kinase (MAPK; no. 9215), p38 MAPK (no. 9212), p-JNK (no. 9255), JNK (no. 9252), p-extracellular signal-regulated kinase (ERK)1/2 (no. 4377), ERK1/2 (no. 4348) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; no. 5174) were all obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Lamin B1 (no. 6581-1) and PI3K antibodies (no. 3838-1) were both obtained from Abcam (Cambridge, UK). The TNF-α ELISA kit was from eBioscience, Inc. (San Diego, CA, USA).

BV-2 cell culture and treatment. BV-2 microglial cells were supplied by the American Type Culture Collection (ATCC; Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin. The cells were seeded in 6-, 12- or 96-well plates at a density of $2x10^5$ cells/ml according to the requirement of the different experiments.

For the examination of iNOS and COX-2, and mRNA expression pattern assay, the cells were pre-treated with ISO I (100 μM) or dexamethasone (Dex; 10 μM). Two hours later, they were incubated with LPS (200 ng/ml) for 20 h, and were harvested for further western blot analysis and quantitative PCR (qPCR) assay.

For the analysis of activated PI3K and Akt, the cells were pre-treated with ISO I (100 μM) for 2 h followed by LPS (200 ng/ml) stimulation for 5, 15 and 30 min, respectively. The cells were then lysed in CelLytic™ MT mammalian tissue lysis reagent with phosphatase and protease inhibitor cocktail (all from Sigma-Aldrich, St. Louis, MO, USA) on ice for 0.5 h. Following centrifugation at 12,000 rpm for 15 min at 4°C, the supernatant of the lysate was subjected to western blot analysis.

For the nuclear translocation assay of p-NF-κB, nuclear extract of the cells was collected using a respective kit (NE-PER® Nuclear and Cytoplasmic Extraction Reagents; Thermo Fisher Scientific, Inc., Rockford, IL, USA), and further used for western blot analysis.

Cytotoxicity and pro-inflammatory factor assay. To examine the effects of ISO I on cell viability, the BV-2 cells were treated with ISO I (0, 25, 50 and 100 μM) for 24 h, the cell viability of which was assessed using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). For the subsequent experiments, following pre-treatment with ISO I (0, 25, 50 and 100 μM) or Dex (10 μM) for 2 h, BV-2 cells were stimulated with LPS (200 ng/ml) for 20 h, and the culture medium was collected and subjected to further analysis.

The release of NO in the medium was evaluated by Griess assay as previously described (20). In brief, following incubation with nitrate reductase for 1 h at 37°C, the medium was mixed with an equal volume of Griess reagent (Sigma-Aldrich). The optical density of the mixture was measured immediately at 540 nm. The concentration of nitrate in the medium was calculated by a standard curve of sodium nitrite. The concentration of TNF-α was determined using a TNF-α ELISA kit according to the manufacturer's instructions.

Western blot analysis. Proteins were extracted from the cells by sonication in CelLytic™ MT mammalian tissue lysis reagent with protease and phosphatase inhibitor cocktails. Following centrifugation at 12,000 x g for 15 min at 4°C, the supernatant of the lysate was collected. Proteins in the supernatant were separated by 10% SDS-PAGE and transferred onto PVDF membranes. After blocking in 5% skim milk for 0.5 h, the membranes were incubated with respective antibodies against p-NF-κB (1:1,000), NF-κB (1:1,000), iNOS (1:1,000), COX-2 (1:1,000), p-IκB (1:1,000), IκB, Lamin B1 (1:1,000), Akt (1:1,000), p-Akt (1:1,000), p-PI3K (1:1,000), PI3K (1:1,000), p-ERK1/2 (1:1,000), ERK1/2 (1:1,000), p-JNK (1:1,000), JNK (1:1,000), p-p38 MAPK (1:1,000), p38 MAPK (1:1,000) and GAPDH (1:2,000) overnight at 4°C. Thereafter, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (1:5,000). The blots were developed using the ECL prime kit (GE Healthcare, Little Chalfont, UK). Protein bands were quantified using ImageJ 1.46r (National Institutes of Health, Bethesda, MD, USA).

qPCR. Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and were reverse transcribed into cDNA using PrimeScript RT reagent (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using the TaqMan SYBR-Green qPCR kit (Life Technologies). The quantity of target genes was normalized to that of GAPDH in the same sample. The primer sequences were listed as follows: iNOS forward, 5'-AACATCAGGTCGGCCATCAC-3' and reverse, 5'-CCAGAGCCTGAAGTCATGTTTG-3'; TNF-α forward, 5'-AACCTCCTCTCTGCCGTCAAG-3' and reverse, 5'-CCTCCCAGGTATATGGGCTCAT-3'; IL-1β forward, 5'-TGGGCCTCAAAGGAAAGAATC-3' and reverse, 5'-GGT ATTGCTTGGGATCCACACT-3'; GAPDH forward, 5'-ATG TGTCCGTCGTGGATCTGA-3' and reverse, 5'-ATGCCTGC TTCAC CACCTTCT-3'.

Luciferase activity assay. The BV-2 cells were seeded in a 24-well plate at a density of $2x10^5$ cells/well and cultured overnight. The cells were then transiently transfected with NF-κB reporter vector pGL6 (luc2P/NF-κB-RE/Hygro) (Beyotime Institute of Biotechnology, Shanghai, China) and *Renilla* luciferase plasmid with Lipofectamine™ LTX and Plus reagent (Life Technologies). Twelve hours later, the cells were treated with ISO I (100 μM) for 2 h followed by stimulation with LPS (200 ng/ml) for 20 h. Afterwards, the cells were harvested and subjected to luciferase activity assay according to the manufacturer's instructions (Promega, Madison, WI, USA). The final NF-κB activity was presented
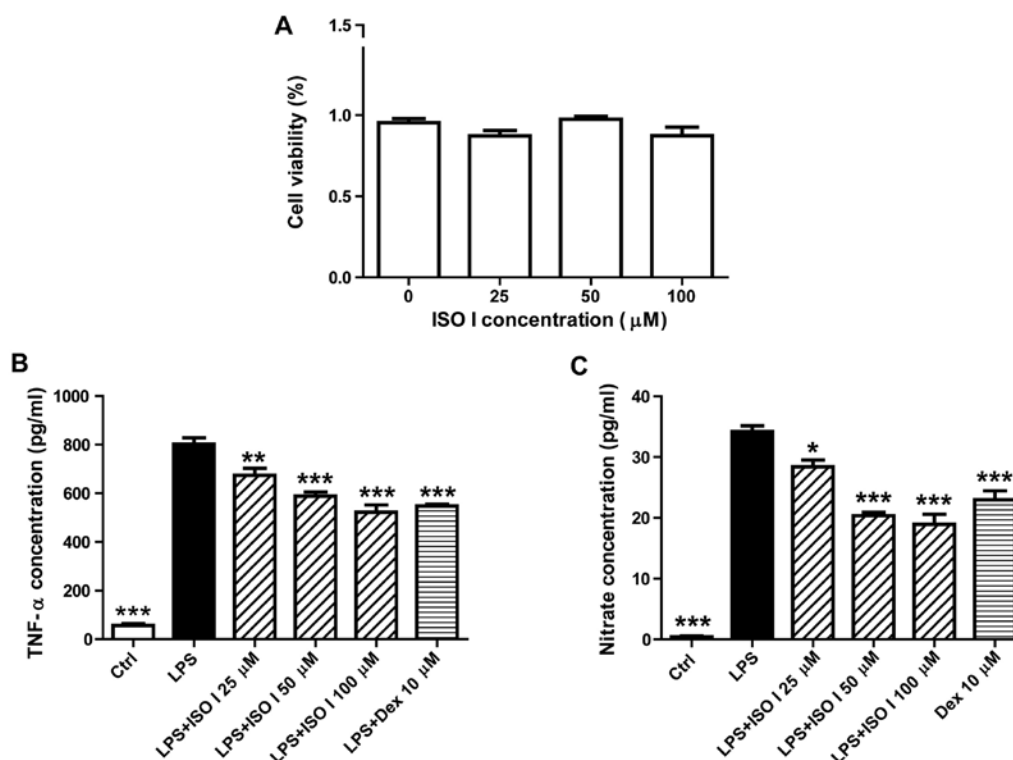
Figure 1. ISO I inhibits the release of inflammatory mediators from BV-2 cells upon lipopolysaccharide (LPS) stimulation. (A) Effect of ISO I on cell viability. (B) Effect of ISO I on tumor necrosis factor (TNF)-α secretion. (C) Effect of ISO I on nitric oxide (NO) release. *p<0.05; **p<0.01; ***p<0.001 vs. LPS group, n=4/group, mean ± SEM. The data shown are one of the representative results from at least 3 independent experiments.

as the ratio of the activity of firefly luciferase to that of *Renilla* luciferase.

Immunocytochemistry (ICC). The BV-2 cells were seeded in 8 chamber slides (BD Biosciences, San Diego, CA, USA) at a density of $2x10^5$ cells/ml. Tweety-four hours later, the cells were treated with or without ISO I (100 μM) for 2 h followed by stimulation with LPS (200 ng/ml) for 20 h. Thereafter, they were washed with phosphate-buffered saline (PBS), fixed with 4% PFA, permeabilized and blocked in PBS with 5% normal donkey serum and 0.1% Triton X-100. Half an hour later, cells were incubated with p-NF-κB p65 antibody overnight at 4˚C. After washing in PBS, they were further incubated with Alexa-594 conjugated secondary antibody at room temperature for 1 h. At last, cells were sealed in medium with DAPI. Fluorescence images of the cells were acquired using an Olympus DX81 fluorescent microscope (Olympus, Tokyo, Japan).

Statistical analysis. All data are presented as the means ± SEM. Differences among 3 or more groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Moreover, an unpaired Student's t-test was used to compare the differences between 2 groups. Differences were considered statistically significant at a value of P<0.05.

Results

ISO I suppresses the production of pro-inflammatory mediators induced by LPS. Prior to the assessment of the anti-inflammatory

effects of ISO I, the cytotoxic effects of ISO I on BV-2 cells were first examined by CCK-8 assay. As shown in Fig. 1A, treatment with ISO I at 25, 50 and 100 μM did not affect the viability of the BV-2 cells. We then examined the effects of ISO I on LPS-stimulated BV-2 cells. As shown in Fig. 1B and C, LPS significantly induced the release of pro-inflammatory factors, such as TNF-α and NO (P<0.001). Moreover, it also increased the protein expression of iNOS and COX-2 (Fig. 2; P<0.001). Following treatment with ISO I (25, 50 or 100 μM), the production of all the pro-inflammatory factors was decreased (P<0.05, P<0.01 or P<0.001). Similarly, Dex, the positive control drug, also suppressed the production of all of the pro-inflammatory factors in the BV-2 cells when used at 10 μM (P<0.01 or P<0.001). These results indicated that ISO I attenuated the inflammatory response in microglia upon LPS stimulation. Since ISO I at 100 μM exhibited the optimal inhibitory effect, this concentration was selected for use in the subsequent experiments.

ISO I downregulates the mRNA expression of pro-inflammatory mediators induced by LPS. To determine the effects of ISO I on the mRNA expression of pro-inflammatory mediators in BV-2 cells upon LPS stimulation, qPCR analysis was performed. As shown in Fig. 3, the mRNA levels of TNF-α, IL-1β and iNOS were markedly upregulated by LPS (P<0.001). However, treatment with ISO I (100 μM) significantly inhibited the overproduction of TNF-α, IL-1β and iNOS mRNA (P<0.01 or P<0.001), which was consistent with its effects on the protein levels of these pro-inflammatory mediators.

ISO I prevents the activation of NF-κB induced by LPS. To explore the role of ISO I on NF-κB activation, a luciferase
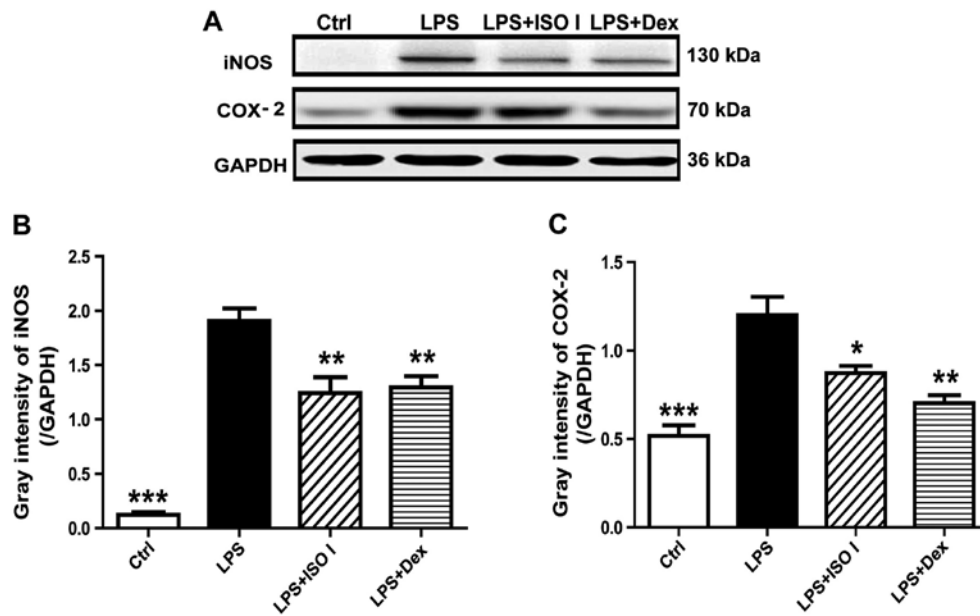
Figure 2. ISO I prevents the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-stimulated BV-2 cells. BV-2 cells were pre-treated with ISO I 2 h prior to incubation with LPS (0.2 μg/ml) for 20 h. (A) Western blot analysis. (B) Gray intensity analysis of iNOS. (C) Gray intensity analysis of COX-2. *p<0.05; **p<0.01; ***p<0.001 vs. LPS group, n=3/group, mean ± SEM. The data shown are one of the representative results from at least 3 independent experiments.
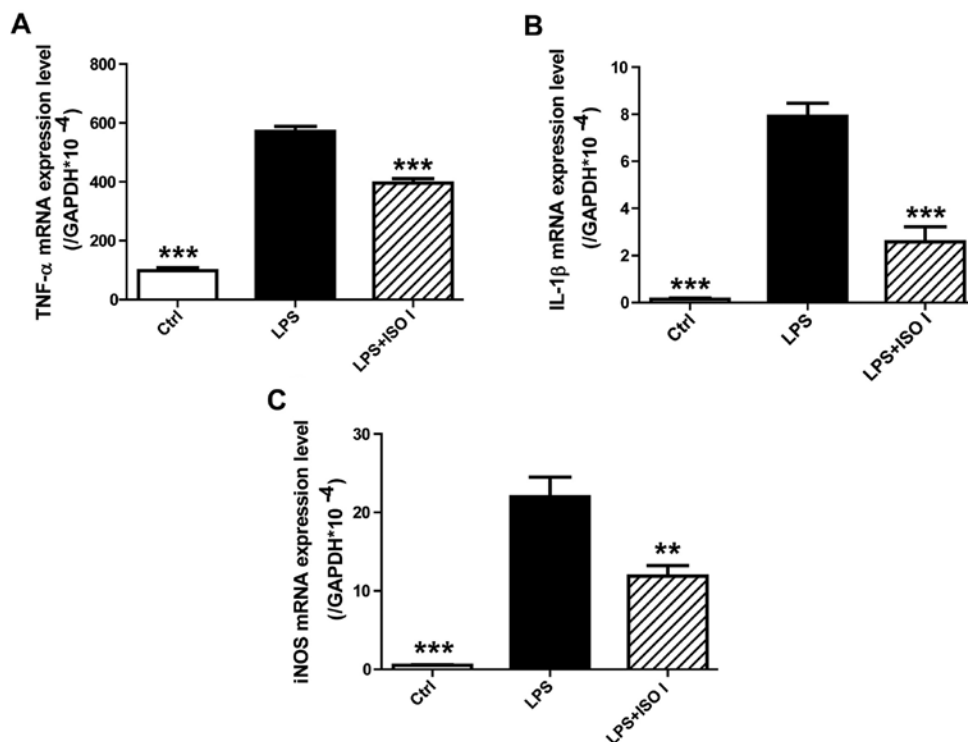

Figure 3. ISO I suppresses the mRNA expression of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-induced BV-2 cells. Cells were pre-treated with ISO I for 2 h prior to LPS stimulation (0.2 μg/ml) and total RNA was isolated 20 h after LPS stimulation. (A) Effect of ISO I on mRNA expression of TNF-α. (B) Effect of ISO I on mRNA expression of IL-1β. (C) Effect of ISO I on mRNA expression of iNOS. **p<0.01; ***p<0.001 vs. LPS group, n=4/group, mean±SEM. The data shown are one of the representative results from at least 3 independent experiments.

reporter assay was carried out. As shown in Fig. 4A, LPS stimulation led to a marked increase in NF-κB-luciferase activity (P<0.001) in the BV-2 cells, which was reversed by ISO I (P<0.01). Moreover, as revealed by western blot

analysis (Fig. 4B and C), ISO I treatment suppressed the increased phosphorylation of NF-κB induced by LPS (P<0.05). Accordingly, the elevation of phosphorylated IκB was lessened (P<0.05).
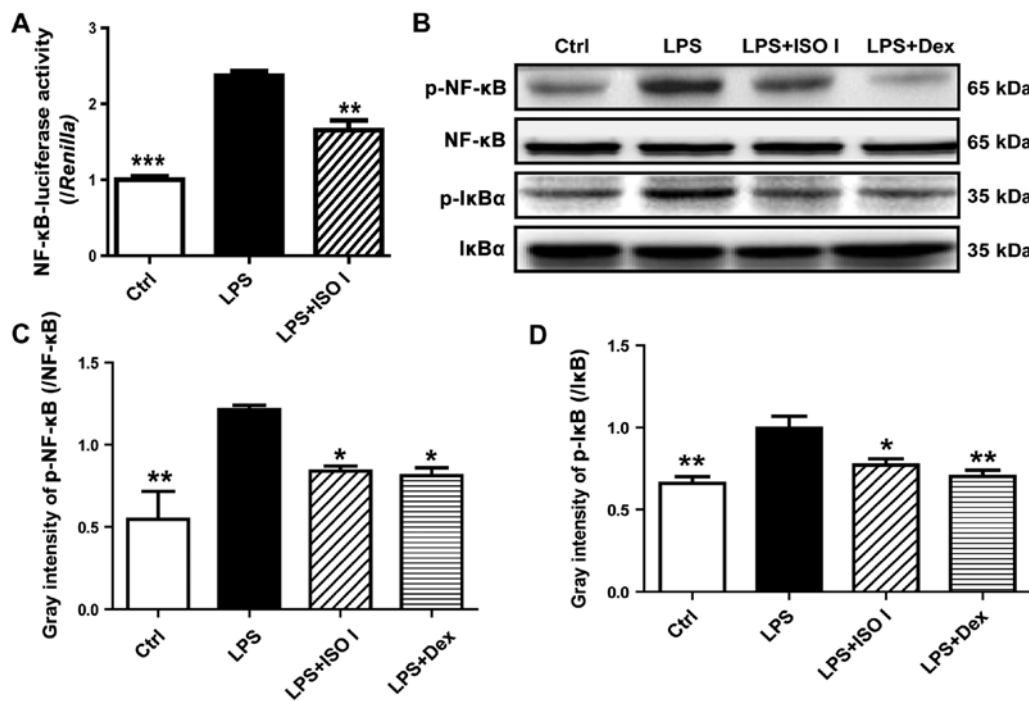
Figure 4. ISO I prevents nuclear factor-κB (NF-κB) activation in lipopolysaccharide (LPS)-stimulated BV-2 cells. (A) ISO I inhibited NF-κB-luciferase activity after LPS stimulation. $^{**}p<0.01$; $^{***}p<0.001$ vs. LPS group, n=4/group, mean±SEM. (B) Western blot analysis. (C) Gray intensity of phosphorylated NF-κB. (D) Gray intensity of phosphorylated IκB. $^{*}p<0.05$; $^{**}p<0.01$ vs. LPS group, n=3/group, mean ± SEM. The data shown are one of the representative results from at least 3 independent experiments.

To further confirm the inhibitory effects of ISO I on the activation of NF-κB, the nuclear translocation of phosphorylated NF-κB was analyzed by an immunocytochemistry assay in the BV-2 cells. As shown in Fig. 5, in the non-activated cells, the majority of phosphorylated NF-κB was localized in the cytoplasm. Upon LPS stimulation, the fluorescence intensity of phosphorylated NF-κB was enhanced and NF-κB was mostly translocated to the nucleus. Conversely, ISO I treatment prevented the nuclear translocation of phosphorylated NF-κB. All these results suggested that ISO I blocked the activation of NF-κB induced by LPS.

ISO I reduces the enhanced phosphorylation of PI3K/Akt and MAPKs induced by LPS. The PI3K/Akt signaling pathway has been shown to control the transactivation of NF-κB (21). Thus, to examine whether ISO I affects the PI3K/Akt pathway, the phosphorylation of PI3K and Akt in the BV-2 cells was detected. As shown in Fig. 6, LPS stimulation time-dependently increased the phosphorylation of both PI3K and Akt, which was significantly attenuated by ISO I treatment.

MAPKs, including p38, ERK and JNK, are involved in NF-κB activation and the synthesis of pro-inflammatory mediators in activated microglia (22). In our study, we also detected the expression of MAPKs in LPS-stimulated BV-2 cells. As shown in Fig. 7, LPS enhanced the phosphorylation of p38, JNK and ERK1/2. Both ISO I and Dex significantly alleviated the activation of MAPKs.

Discussion

Neuroinflammation with excessively activated microglia has been demonstrated to aggravate the pathophysiology of many neurodegenerative diseases, indicating the beneficial efficacy of microglia regulatory agents in the therapy of these disorders (23,24). As disclosed in our previous study, total astragalosides alleviated neuroinflammation via the inhibition of iNOS and other inflammatory cytokines in mice with experimental autoimmune encephalomyelitis (25). Moreover, astragaloside IV, one of the saponin molecules within total astragalosides, has been shown to inhibit microglial activation via the glucocorticoid receptor-mediated signaling pathway (20). In this study, we demonstrated that another saponin molecule, ISO I, within total astragalosides, also inhibited the activation of microglia. In the BV-2 cells, it mitigated the LPS-induced production of pro-inflammatory mediators, such as NO, TNF-α, iNOS and COX-2. Further experiments revealed that ISO I attenuated NF-κB activation possibly by regulating the PI3K/Akt and MAPK signaling pathways.

The persistent and uncontrolled activation of microglia is one of the characteristics of neurodegenerative disorders (26,27). Pro-inflammatory cytokines, such as NO, IL-1β and TNF-α released from overactivated microglia may lead to progressive neuronal damage and further activate the inflammatory cascade (28). In this study, ISO I treatment significantly reduced the release of NO and TNF-α, decreased the protein levels of COX-2 and iNOS, and downregulated the mRNA expression levels of iNOS, TNF-α and IL-1β, indicating that ISO I suppressed the inflammatory responses in microglia and may thus be beneficial in the treatment of neurodegenerative diseases.

In the inactive state, the inhibitory protein, IκB, binds to NF-κB dimmers that blocks the nuclear translocation of the latter. Upon inflammatory stimulation, IκB is phosphorylated,
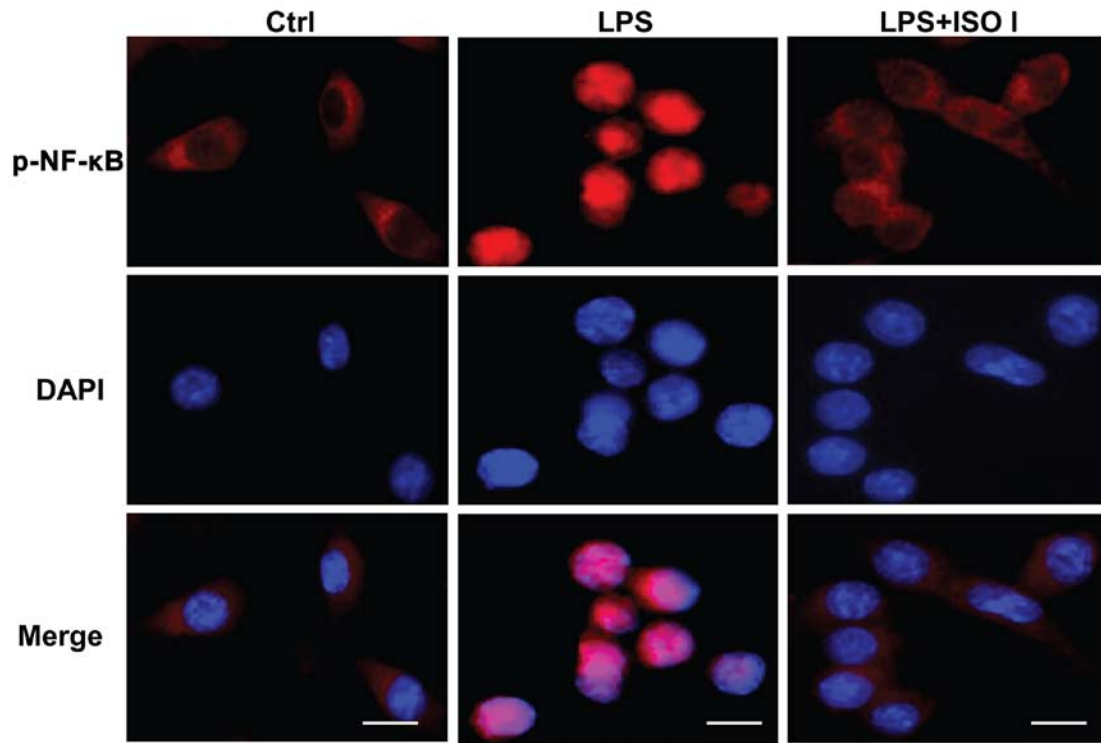
Figure 5. ISO I inhibits the lipopolysaccharide (LPS)-induced nuclear translocation of nuclear factor-κB (NF-κB) in BV-2 cells. Cells were treated with ISO I for 2 h prior to stimulation with LPS (0.2 μg/ml) for 20 h. Scale bar, 20 μm. The data shown are one of the representative results from at least 3 independent experiments.
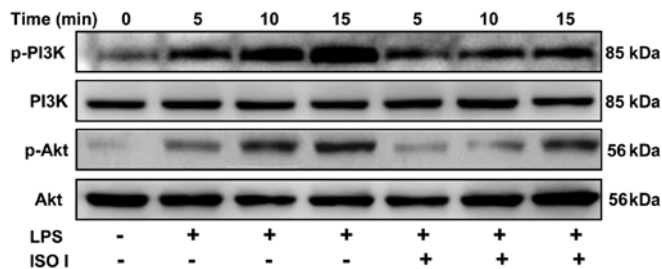

Figure 6. ISO I suppresses the phosphorylation of PI3K and Akt in lipopoly-saccharide (LPS)-stimulated BV-2 cells. Cells were incubated with ISO I for 2 h prior to stimulation with LPS (0.2 μg/ml) for 5, 10 and 15 min. ISO I time-dependently prevented the phosphorylation of PI3K and Akt. The data shown are one of the representative results from at least 3 independent experiments.

Figure 7. ISO I prevents the phosphoryaltion of mitogen-activated protein kinases (MAPKs) in lipopolysaccharide (LPS)-stimulated BV-2 cells. BV-2 cells were treated with ISO I for 2 h prior to LPS (0.2 μg/ml) incubation for 20 h. Phosphorylation of p38, JNK and ERK was examined by western blot analysis.

ubiquitinated and degraded sequentially. As a result, NF-κB dimers will be translocated to the nucleus and regulate the transcription of downstream genes (29). In our study, ISO I significantly suppressed NF-κB luciferase activity, reduced the phosphorylation of NF-κB and IκB, and prevented the nuclear translocation of NF-κB in LPS-stimulated BV-2 cells, accordingly, decreasing the downstream gene expression of inflammatory mediators. Therefore, our findings suggested that ISO I inhibited the inflammatory responses in microglia upon LPS stimulation via the NF-κB pathway.

Signaling pathways, such as PI3K/Akt and MAPKs are well known to interact with the NF-κB pathway, and there-fore, are actively involved in the inflammatory cascade in microglia (30). In agreement with the reports (31,32), in our study, LPS induced the prominent activation of PI3K/Akt and MAPKs. The effect of LPS was abrogated by ISO I, suggesting
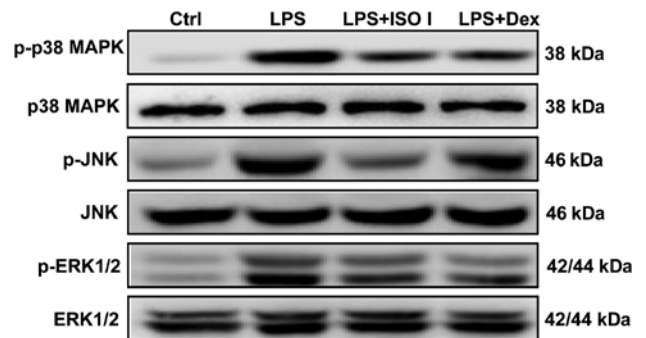
that the anti-inflammatory effects of ISO I may be possibly mediated through these signaling pathways.

In conclusion, the present study demonstrated that ISO I attenuated LPS-induced inflammatory responses in BV-2 microglia, and these effects were probably mediated via the inhibition of NF-κB activation through the PI3K/Akt and MAPK signaling pathways.

Acknowledgements

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