Isaindigotone as an inhibitor of the lipopolysaccharide-induced inflammatory reaction of BV-2 cells and corresponding mechanisms

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Abstract. Isaindigotone possesses extensive pharmacological activities, including anti-inflammatory effects. The present study investigated the role of isaindigotone in the inhibition of neuroinflammation. Mouse BV-2 cells were incubated with lipopolysaccharide (LPS; 1 mg/l) for 24 h in a microglial inflammatory model *in vitro*. The effects of isaindigotone on BV-2 cell proliferation were observed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Following co-incubation, an enzyme-linked immunosorbent assay and western blot analysis were used to analyze cellular levels of cytokines and associated protein expression, including the phosphorylation of nuclear factor (NF)-κB. The effects of isaindigotone concentration on LPS-mediated cell chemotaxis behavior were assessed using a chemotaxis assay. The results indicated that isaindigotone is non-toxic towards BV-2 cells. Compared with the LPS group, isaindigotone significantly reduced the secretion of tumor necrosis factor-α and interleukin-1β in BV-2 cells and reduced the cell chemotaxis caused by LPS; it also reversed morphological changes in the BV-2 cells and inhibited the phosphorylation of NF-κB. The results of the present study suggest that isaindigotone can inhibit inflammatory reactions in LPS-induced BV-2 cells, and provides a theoretical basis and experimental evidence for examining the mechanism underlying the isaindigotone-induced inhibition of neuroinflammation.

Introduction

Numerous studies have reported that several chronic diseases with a high incidence are associated with inflammation. As documented, the progression of certain central neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and multiple sclerosis, involve neuroinflammation (1-3). Inflammation is also important in the development of circulatory diseases, including atherosclerosis, blood viscosity and primary hypertension (4-6). Certain types of malignant tumors are known to be closely associated with the inflammatory mechanism (7-9). Therefore, the development of novel anti-inflammatory drugs is of ongoing interest. Isaindigotone is an alkaloid extracted from *Radix isatidis* (10). The parent compound of isaindigotone is formed by the connection of pyrroquinolone with conjugated benzylidene (Fig. 1) (10). Isaindigotone possesses extensive pharmacological activities, including antibiotic, antiviral, anti-endotoxin, anti-inflammatory and antitumor activities (11-14). It has been previously shown that isaindigotone is able to pass through the blood-brain barrier and be transferred to the central nervous system (15). Therefore, understanding whether isaindigotone can inhibit the inflammatory response of the central nervous system is of significance for preventing and relieving central nervous system diseases.

Microglia (MG) are resident immune cells in the central nervous system. The continuous activation of MG is a key factor in the induction and intensification of central neuroinflammation (16,17). Therefore, in the present study, an *in vitro* inflammation model of lipopolysaccharide (LPS)-induced BV-2 cells (an MG line) was selected to examine the effects of isaindigotone on LPS-induced inflammatory responses. The conclusions provide a theoretical and experimental basis for further investigation and clinical development.

Materials and methods

Materials. Isaindigotone was purchased from J&K Scientific, Ltd. (Shanghai, China) and was dissolved in 100% dimethyl sulfoxide (DMSO). A stock solution of 10 mmol/l isaindigotone was prepared and stored as small aliquots (5 μl) at -20˚C for future use. The BV-2 cell line was obtained from the Animal Experimental Center of Sun Yat-Sen University (Guangzhou, China). MTT and DMSO were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) ELISA kits were purchased from Cell Signaling Technology, Inc. (Danvers,

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MA, USA). All antibodies were purchased from Cell Signaling Technology, Inc.

MTT assay. The BV-2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Husbio, Inc., Shanghai, China) supplemented with 10% fetal calf serum at 37˚C. The cells were adjusted to $1x10^5$ cells/ml and were inoculated onto a 96-well culture plate with 100 μl of cell suspension in each well. After 24 h, isaindigotone (5, 10, 20, 40 and 80 mg/l), LPS (1 mg/l), and LPS (1 mg/l) + isaindigotone (80 mg/l) were added into each well respectively. The MTT (10 μl of 5% MTT) was added into each well at 24, and 48 h during culture, following which the cells were further incubated for 4 h at 37˚C. Subsequently, the culture solution was removed and 100 μl DMSO was added to each well for dissolution. The optical density values were read at 490 nm following vibration mixing.

Chemotaxis assay. BV-2 cells in the logarithmic phase were selected and pre-incubated in a 35-mm culture dish for 24 h. The BV-2 cells were then dislodged, centrifuged (300 x g for 5 min at room temperature) and resuspended in fresh complete culture solution. A blank group and LPS group were set; the LPS group was further divided into five subgroups according to isaindigotone concentrations (0, 10, 20, 40 and 80 mg/l). The cells were adjusted to $1x10^5$ cells/ml, and 200 μl of cells were collected and placed in the upper chambers of a Transwell assay plate, and 20 nmol/l of amide compound MMK-1 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was placed in each of the lower chambers as a chemoattractant. The BV-2 cells were cultured in a CO_2 incubator for 24 h. The culture solution was then removed and BV-2 cells were rinsed three times with PBS, followed by 10 min of fixation in methyl alcohol at room temperature. The BV-2 cells were then incubated with 5 mg/l of 4,6-diamino-2-phenylindole for 20 min at room temperature and rinsed three times with PBS. The cells were observed and counted under a light microscope. The experimental results are expressed as the chemotaxis index, which is the ratio of cell numbers between the treatment group and the blank group.

Evaluation of IL-1β and TNF-α levels by ELISA. The BV-2 cells were cultured in high-glucose DMEM (10% fetal calf serum) and were adjusted to $1x10^5$ cells/ml. The BV-2 cells were inoculated onto a cell culture plate with 96 wells. The samples were divided into a blank group, isaindigotone group (80 mg/l), blank + LPS group (1 mg/l), and LPS (1 mg/l) + isaindigotone groups (10, 20, 40 and 80 mg/l). Each group comprised six complex wells, with 100 μl of sample per well. In the LPS group, the BV-2 cells were incubated with 1 mg/l LPS for 24 h. In the isaindigotone group, the BV-2 cells were incubated with isaindigotone (80 mg/l) for 24 h. In the LPS + isaindigotone group, the BV-2 cells were incubated with different concentrations of isaindigotone (10, 20, 40 and 80 mg/l) and 1 mg/l LPS for 24 h. The secretory levels of IL-1β and TNF-α in the supernatant were assessed by ELISA.

Observation of cell morphology. BV-2 cells in the logarithmic phase were selected and incubated in a 6-well culture plate. The BV-2 cells were divided into a blank group, isaindigotone group (40 mg/l), LPS group, and LPS + isaindigotone group (40 mg/l) after 24 h. In the LPS group, the BV-2 cells were co-incubated with 1 mg/l of LPS for 24 h. Subsequent morphological changes in the BV-2 cells were observed using an inverted phase-contrast microscope.

Western blotting. BV-2 cells in the logarithmic phase were selected and divided into blank, LPS, and LPS + isaindigotone groups. In the LPS group, the BV-2 cells were incubated with 1 $\mu g. L^{-1}$ LPS for 20 min. In the LPS + isaindigotone group, the BV-2 cells were co-incubated with 20 mg/l isaindigotone and 1 mg/l LPS for 20 min. The BV-2 cells from all groups were collected, and total proteins were extracted using RIPA lysis buffer. The protein concentrations were determined using the bicinchoninic acid method. Subsequently, the proteins were prepared with 5X loading buffer; the loading quantity was 20-40 μg. A 10% SDS polyacrylamide gel electrophoresis was performed, following which the proteins were transferred onto a PVDF membrane through the semi-dry method. The PVDF was blocked using 5% powdered skim-milk for 2 h, and then incubated with the primary antibodies against NF-κB (cat. no. 8242; 1:1,000; Cell Signaling Technology, Inc.), phospho-NF-κB (cat. no. 3033; 1:1,000; Cell Signaling Technology, Inc.), β-actin (cat. no. 3700; 1:1,000; Cell Signaling Technology, Inc.) overnight at 4˚C, followed by incubation with HRP-linked anti-mouse (cat. no. 7076; 1:1,000; Cell Signaling Technology, Inc.) or HRP-linked anti-rabbit (cat. no. 7074; 1:1,000; Cell Signaling Technology, Inc.) at room temperature for 30 min. The signals were detected using an enhanced chemiluminescence substrate (Bioss Biotechnology, Beijing, China), and the optical density of the bands was measured by a BandScan imaging analysis system.

Statistical analysis. The results are expressed as the mean ± standard deviation. Statistical comparisons were performed using one-way analysis of variance followed by the least significant difference test the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of isaindigotone on BV-2 cell survival. Different concentrations of isaindigotone with or without LPS (1 mg/l) were applied to BV-2 cells for 24 and 48 h. Isaindigotone (<80 mg/l) was observed to have little toxic effect on BV-2 cells *in vitro* in the presence or absence of LPS (Fig. 2A and B). This provided a reference for determining drug concentration in the follow-up experiments.

Effects of isaindigotone on the chemotaxis of BV-2 cells. Following the abnormal activation of MG, the density of surface receptors increased markedly after 24 h, mediating the secretion of inflammatory factors and cell chemotaxis. The agonist of formylpeptide receptor-2 (FPR2), MMK-1, induced chemotaxis of the LPS-stimulated BV-2 cells (Fig. 3). Following co-incubation of the BV-2 cells with different concentrations (40 and 80 mg/l) of isaindigotone, the BV-2 cell chemotaxis induced by MMK-1 was reduced significantly compared with that in the LPS group.

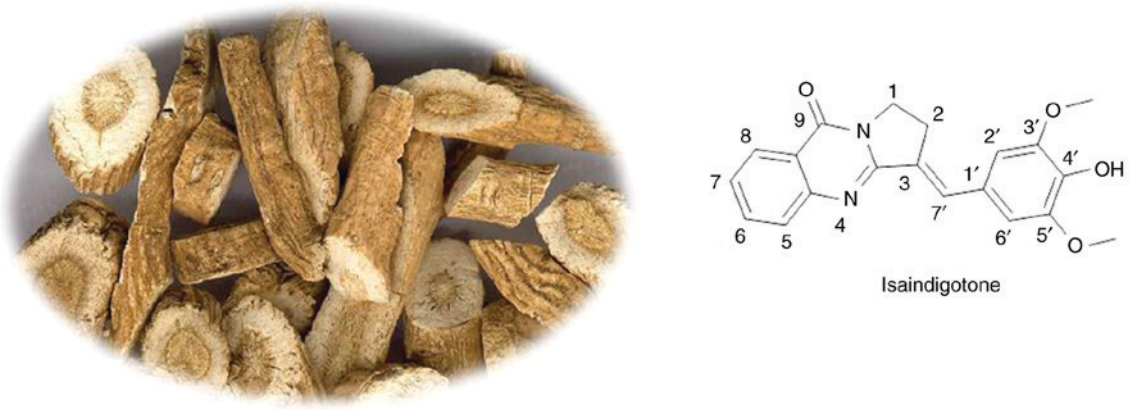

Figure 1. Structures of *Radix isatidis* and isaindigotone.

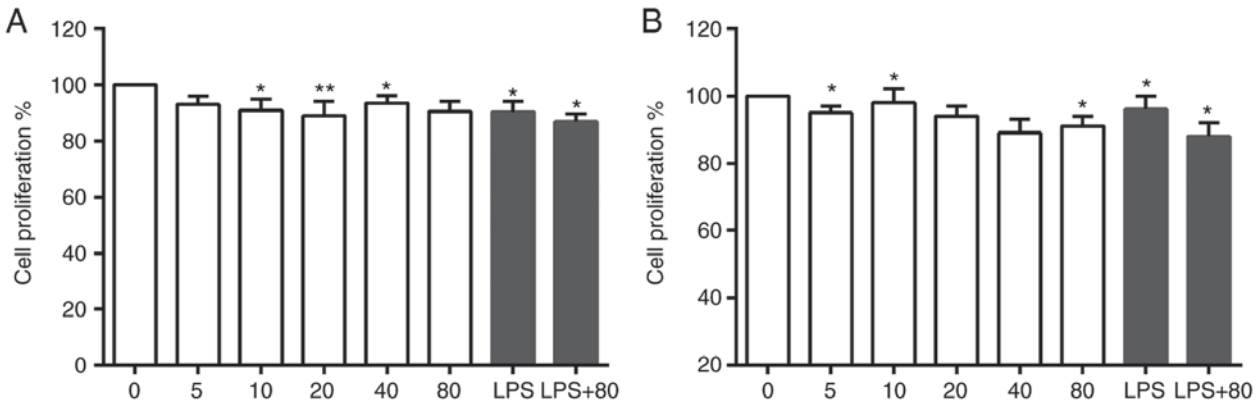

Figure 2. Isaindigotone exhibits little toxic effect towards BV-2 cells. Data are expressed as the mean ± standard deviation. Three samples in each group were incubated for (A) 24 h and (B) 48 h. *P<0.05 vs. 0 group; **P<0.01 vs. 0 group. LPS, lipopolysaccharide.

Effects of isaindigotone on the secretion of inflammatory factors from BV-2 cells. Compared with the blank group, incubation with LPS significantly upregulated the secretion of IL-1β and TNF-α in the cell supernatants. Following incubation of the BV-2 cells with different concentrations of isaindigotone (40 and 80 mg/l), the levels of IL-1β and TNF-α secretion were significantly inhibited compared with those in the LPS group (Fig. 4).

Effects of isaindigotone on the morphology of BV-2 cells. BV-2 cell morphology altered significantly following treatment with 1 mg/l of LPS. Prior to treatment, the MG were in a relative static state, accompanied by small thin and long soma. Following treatment, morphological changes of the BV-2 cells included protuberance, shrinkage and coarsening into an amoebic appearance (Fig. 5A-D). The BV-2 cells were treated simultaneously with 40 mg/l of isaindigotone and LPS after 24 h, which revealed that isaindigotone reversed the LPS-induced morphological changes in BV-2 cells (Fig. 5E).

Effects of isaindigotone on the phosphorylation of NF-κB in BV-2 cells. The phosphorylation of NF-κB in BV-2 cells intensified following stimulation with 1 mg/l of LPS, but was significantly downregulated following treatment with 20 and 40 mg/l isaindigotone (Fig. 6A and B).

Discussion

Isaindigotone, found widely in *R. isatidis*, is a natural antioxidant (14,15). This compound has multiple functions, including cancer preventative, anticancer and neuroprotective activities, and is suitable for the treatment of cardiovascular diseases. Methods for the extraction and the assessment of content and purity of isaindigotone have been well developed and quality-controlled (11-14). The *in vivo* reaction mode, mechanism of action and metabolism of isaindigotone have been investigated extensively. The long-term and in-depth chemical and clinical data also reflect the lack of toxic side effects of isaindigotone and its preparation, its high level of safety, low accumulation potential and minimal residues (15). These findings improve current understanding of isaindigotone, facilitating the development and utilization of this compound. In the present study, the effects of isaindigotone on the LPS-induced activation of BV-2 cells were preliminarily examined from the perspective of inflammation. The results provide a basis for further *in vivo* experiments and provide a foundation for the development of isaindigotone-based drugs, indicated for the prevention and treatment of central nervous system degradation diseases caused by neuroinflammation.

MG are regarded as 'inspectors' of the central nervous system (16). They are rapidly activated by foreign matter or detrimental stimuli. As a result, MG release abundant

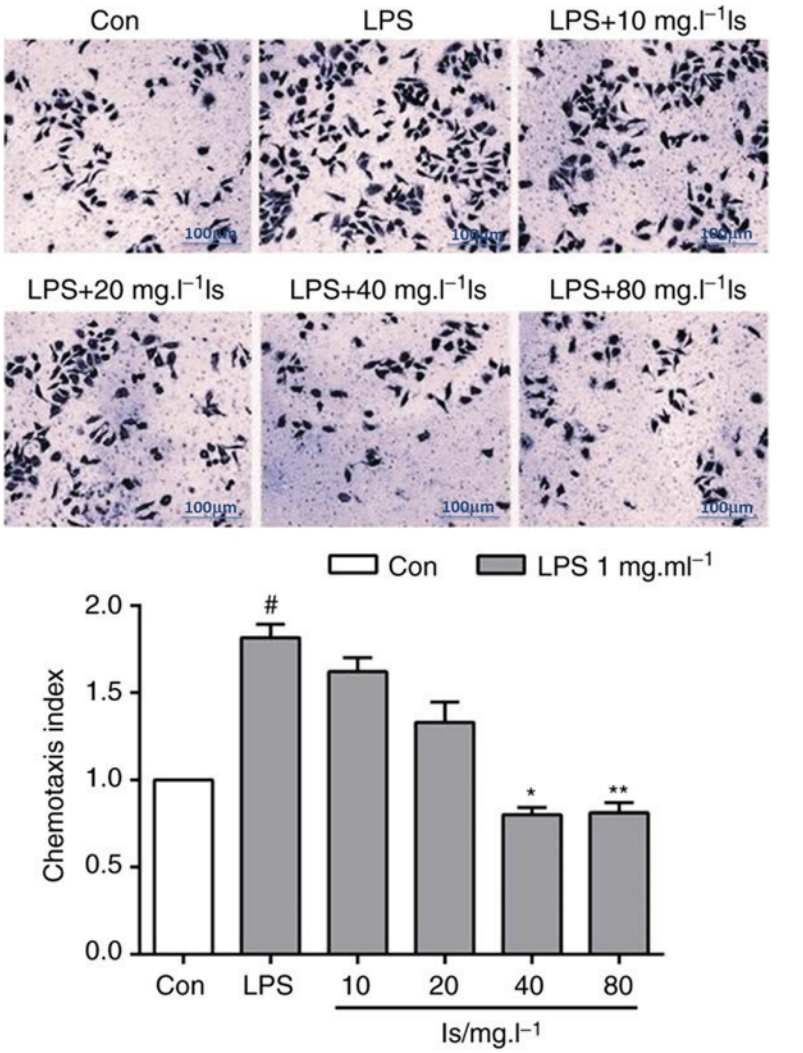

Figure 3. Isaindigotone inhibits MMK-1-induced BV-2 cell chemotaxis. The upper chambers were filled with BV-2 cells in suspension, which had been incubated in culture medium with LPS or LPS + isaindigotone (10, 20, 40 and 80 mg/l). The lower chambers contained 20 nmol/l MMK-1. Data are expressed as the mean ± standard deviation; each group included three samples. Images show cell chemotaxis at concentrations of 10, 20, 40 and 80 mg/l. The graph shows quantitative analysis of cell chemotaxis. $^{\#}$P<0.01 vs. Con; *P<0.05 and **P<0.01 vs. LPS. LPS, lipopolysaccharide; Is, isaindigotone; Con, control.

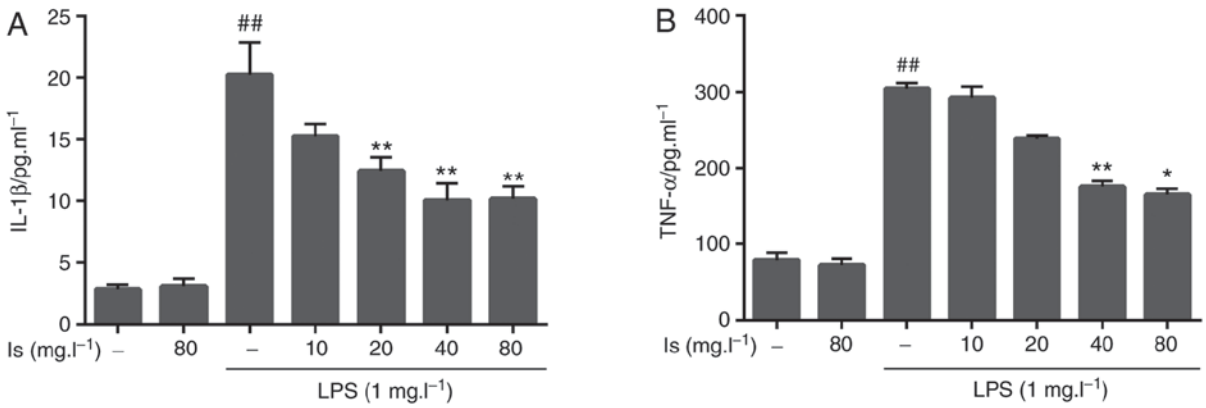

Figure 4. Isaindigotone inhibits the LPS-induced secretion of IL-1β and TNF-α from BV-2 cells. Data are expressed as the mean ± standard deviation; each group included three samples. (A) IL-1β release; (B) TNF-α release. $^{\#\#}$P<0.01 vs. blank group (without LPS); *P<0.05 and **P<0.01 vs. blank group (with LPS). IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; Is, isaindigotone.

cytokines for immunity and inflammation regulation, thereby enabling the elimination of inflammatory agents in nervous tissue or cells with metabolic disorders (17). However, continuously activated MG release excessive inflammatory factors that aggravate neuroinflammation and damage normal nervous tissue (18). LPS can induce MG to generate a series

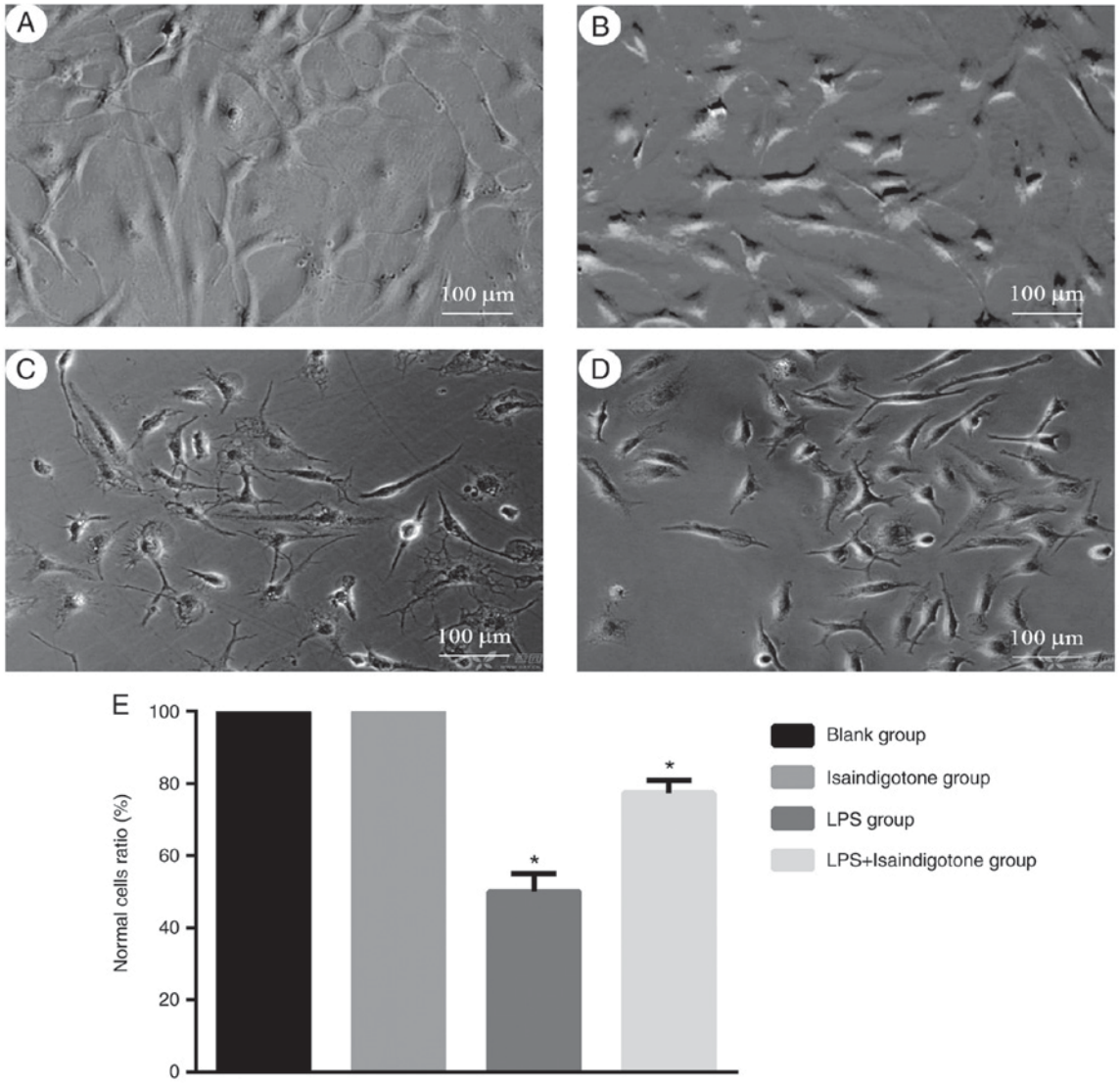

Figure 5. Isaindigotone (40 mg/l) reverses LPS-induced changes in BV-2 cell morphology. (A) Blank group; (B) isaindigotone (40 mg/l) group; (C) LPS group (1 mg/l); (D) LPS (1 mg/l) + isaindigotone (40 mg/l) group. (E) Quantitative analysis of normal BV-2 cells (normal BV-2 cells/all BV-2 cells in each microscopic field). *P<0.05 vs. blank group. LPS, lipopolysaccharide.

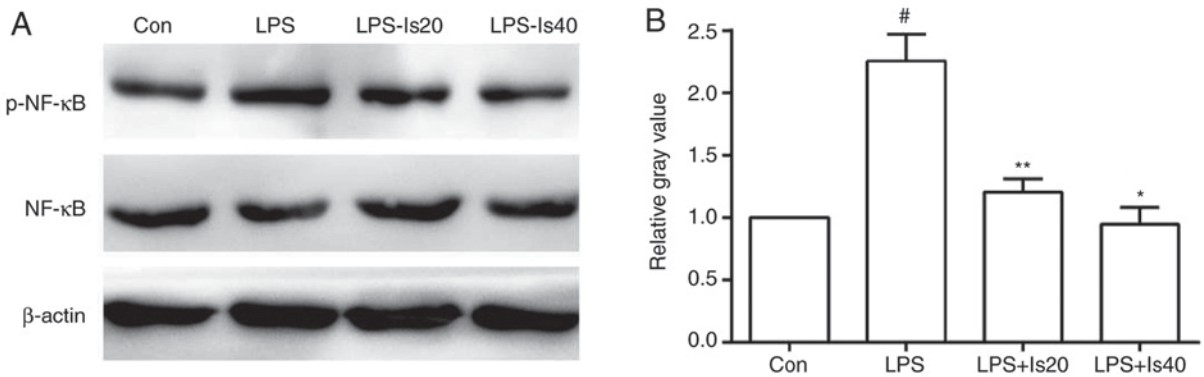

Figure 6. Isaindigotone inhibits LPS-induced phosphorylation of NF-κB in BV-2 cells. Data are expressed as the mean ± standard deviation, and each group included three samples. (A) Representative bands of p-NF-κB. (B) Semi-quantitative analysis of proteins. $^{\#}$P<0.05 vs. Con; *P<0.05 and **P<0.01 vs. LPS. NF-κB, nuclear factor-κB; p-, phosphorylated; LPS, lipopolysaccharide; Is20, 20 mg/l isaindigotone; Is40, 40 mg/l isaindigotone; Con, control.

of inflammatory responses, which significantly increase the expression of inflammation-associated receptors located on MG surface membranes. The secretion of inflammatory factors from MG is also activated to facilitate neuroinflammation following stimulation by LPS (19,20). Therefore, inhibiting the abnormal activation and inflammatory responses of MG bears

clinical significance in terms of improving inflammation in the microenvironment and protecting neurons from damage. The present study showed that isaindigotone significantly inhibited BV-2 MG cells from releasing inflammatory factors, and influenced the functions of inflammatory receptors and phosphorylation of NF-κB. This indicates that isaindigotone can inhibit the abnormal activation of MG, preventing and relieving inflammatory damage to the central nervous system.

Following abnormal activation, MG secrete inflammatory cytokines which include TNF-α and IL-1β (21). These cytokines are key factors that promote neuroinflammation. TNF-α not only induces the secretion of multiple proinflammatory factors, but also induces cell apoptosis and inflammatory reaction cascades (22). The upregulated expression of TNF-α in the brain can directly reflect the severity of neuroinflammation. IL-1β is one of the important proinflammatory factors in the IL-1 family (23). As a regulatory protein, IL-1β can stimulate the secretion of TNF-α, IL-6, interferon and chemotactic factors through the phosphorylation of NF-κB in MG, thus inducing continuous deterioration through neuroinflammation. In the present study, isaindigotone was shown to inhibit the LPS-induced secretion of TNF-α and IL-1β by BV-2 cells, thus relieving the vascular deterioration caused by persistent neuroinflammation.

FPR2, which is a member of the chemokine receptor family, is a G protein-coupled receptor with seven transmembrane helices. FPR2 possesses a comprehensive set of functions and can mediate inflammatory and immune responses by combining with specific ligands, all of which feature different sources and high structural diversity (24). MMK-1, an agonist with specificity for FPR2, can promote the secretion of IL-1β, IL-6 and TNF-α, and cell chemotaxis, and can activate neutrophil granulocytes, monocytes and T-cells, thus exerting proinflammatory effects (25). In the present study, isaindigotone showed a capacity for inhibiting MMK-1-induced BV-2 cell chemotaxis, indicating that this compound can inhibit the functions of FPR2 and influence inflammation.

Over recent decades, toll-like receptor 4 (TLR4), which is involved in inflammation, immunity adjustment, cell adhesion and chemotaxis, has increased in interest worldwide. The signal transduction pathway of TLR4 can be activated by oxidative stress, LPS and cytokines (particularly IL-1β) to regulate the secretion and activation of cell proinflammatory factors, adhesion molecules and other transcription factors, thus mediating cell behavior. The activation of TLR4 initiates a pro-inflammatory response, which depends on the activation of mitogen-activated protein kinases and NF-κB (26). Subsequently, NF-κB is phosphorylated and the activated NF-κB translocates from cytoplasm to nucleus to promote the transcription of various inflammatory marker genes, including those of interleukins, cytokines, chemokines, inducible nitric oxide synthase and cyclooxygenase-2 (27). As a result, inhibiting TLR4-NF-κB signaling pathway activation can prevent the cell inflammatory responses mediated by NF-κB and reduce inflammation-induced damages in affected nervous cells and tissues (28). Experiments investigating the effect of isaindigotone on the activation of NF-κB signaling, which is the downstream signaling of the TLR4 pathway, showed that isaindigotone pretreatment significantly inhibited the LPS-induced activation of NF-κB in BV-2 cells, indicating that this natural compound can affect cell behavior. An increasing body of data suggests that inflammation, and in particular neuroinflammation, is involved in the pathophysiology of certain types of epilepsy and convulsive disorders. In an epileptic animal model, the TLR4-NF-κB signaling pathway was shown to be activated in separated microglial cells (29). However, the in-depth mechanism and anti-inflammatory effect of isaindigotone *in vivo* require further investigation.

In conclusion, isaindigotone was observed to inhibit the LPS-induced inflammatory reactions of BV-2 cells. The anti-inflammatory action of this molecule was associated with the inhibited release of immune cell inflammatory factors, attenuation of inflammatory cell chemotaxis and decreased phosphorylation of NF-κB. These data provide a theoretical and experimental basis for examining the mechanism of isaindigotone with respect to inhibiting neuroinflammation.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JX conceived the study and the experiments, interpreted the results and prepared the manuscript. HX performed the experiments and helped prepare the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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