Research article

Anti-inflammatory effects of novel polygonum multiflorum compound via inhibiting NF-κB/MAPK and upregulating the Nrf2 pathways in LPS-stimulated microglia

Sun Young Park, Mei Ling Jin, Nam Jun Kang, Geuntae Park, Young-Whan Choi

HIGHLIGHTS

- CRPE56IGIH is a natural compound isolated from Polygonum multiflorum.
- CRPE56IGIH inhibits LPS induced neuroinflammatory response in microglia.
- CRPE56IGIH inhibits LPS-induced NF-κB and JAK-STATs activation in microglia.
- Nrf2 mediates the anti-neuroinflammation of CRPE56IGIH in microglia.

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ABSTRACT

The incorporation of Polygonum multiflorum into the diet can result in anti-aging effects owing to its wide range of biological and pharmaceutical properties. We investigated the anti-neuroinflammatory properties of CRPE56IGIH isolated from P. multiflorum by focusing on its role in the induction of phase II antioxidant enzymes and the modulation of upstream signaling pathways. In microglia, CRPE56IGIH significantly inhibited lipopolysaccharide (LPS)-stimulated nitric oxide and prostaglandin E2 production with nonspecific cytotoxicity. CRPE56IGIH also markedly inhibited LPS-inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 protein and mRNA expression in the same manner as it inhibited nitric oxide and prostaglandin E2 production. In the control cells, NF-κB transactivation and nuclear translocation occurred at a baseline level, which was significantly increased in response to LPS. However, pretreatment with CRPE56IGIH concentration-dependently inhibited the LPS-induced NF-κB transactivation and nuclear translocation. The phosphorylation of Janus kinase-signal transducers and activators of transcription and mitogen-activated protein kinases was markedly upregulated by LPS, but considerably and dose-dependently inhibited by pretreatment with CRPE56IGIH. Furthermore, CRPE56IGIH induced...
1. Introduction

The microglia of the brain play a crucial role in providing immunological protection against virulent pathogenic factors in the central nervous system (CNS). Under ordinary conditions, microglia are in an inactive state and exhibit a ramified morphology. In this state, they repeatedly monitor the neighboring environment for any changes in the homeostasis of the CNS that may be harmful to the neurons or induce injury in the CNS [4,5]. However, in the presence of positive stimuli, the microglia are activated and facilitate the appropriate brain development processes or repair of damaged sites via the secretion of various inflammatory mediators and phagocytosis. This protects the neuronal tissue from secondary damage in the healthy CNS [3]. After activation of the microglia, intracellular signaling cascades, including NF-κB, JAK-STATs, and MAPKs, are activated. Abnormal levels of microglial activation induce chronic neuroinflammatory environments through the sustained activation of pro-inflammatory mediators. This ultimately leads to damage, rather than protection, of neighboring neurons and causes CNS injury [10].

Bacterial lipopolysaccharide (LPS) is a major antigenic agent against innate immune cells, such as microglia and macrophages. LPS is also a major component of the outer membranes of gram-negative bacteria. The surfaces of immune cells include Toll-like receptors (TLRs), which are engaged in the recognition of various bacterial products such as LPS, peptidoglycan (PGN), and lipoteichoic acid (LTA). Recognition by TLRs triggers the immune or innate signaling pathways that are involved in the cellular defense against external or internal pathogens [12]. Numerous studies have demonstrated that TLR-4 performs an essential role in LPS-induced microglial activation. The signal transduction cascade pathway via TLR-4 is mediated by diverse adaptor proteins, including MyD88, NF-κB, JAK-STAT, and MAPK signaling pathways, which are triggered by the activation of pro-inflammatory mediators [2]. In addition, TLR-4 has also been reported to be involved in neuroinflammatory-related neurodegenerative diseases and was recently reported to be involved in neuronal injury following the LPS-induced inflammatory response in microglia [9].

HO-1 and NQO-1 have been extensively studied in the CNS for their neuroprotective and anti-neuroinflammatory effects; these studies have alluded to their potential as therapeutic targets for many neuroinflammatory-related diseases [11]. The induction of HO-1 is a rate-limiting step in the catabolic activity during heme degradation. In this reaction, the oxidation of free heme produces ferrous iron and biliverdin, which are then converted into ferritin, carbon monoxide, and bilirubin. HO-1 and its by-products show anti-oxidant and anti-inflammatory properties [13]. The induction of phase II antioxidant enzymes, such as HO-1 and NQO-1, is regulated through the activation of transcription factors, including Nrf2. The promoter regions of the HO-1 and NQO-1 genes contain antioxidant responsive elements (AREs), which directly bind to Nrf2 and regulate the expression of many genes involved in the antioxidant and anti-inflammatory responses [7]. Several studies have demonstrated the therapeutic potential of targeting the Nrf2/ARE pathway in neuronal injury after abnormal neuroinflammation. Previous studies have demonstrated that the gene silencing of Nrf2 exaggerated the inflammatory response and impaired HO-1 induction, which aggravated the neighboring neuronal damage. As Nrf2/ARE plays a regulatory role in the neuroinflammatory response, it is considered a promising therapeutic target for the treatment of neurodegenerative diseases [7,11].

In several Asian countries, Polygonum multiflorum has been used in traditional medicine for the treatment of age-related cognitive dysfunction and for its ability to aid learning and memory. Researchers have also discovered that P. multiflorum was also useful for the treatment of neurodegenerative disorders, such as Alzheimer’s disease (AD) and Parkinson’s disease (PD). Moreover, extracts of P. multiflorum have been shown to exert beneficial effects on hippocampal neurons and in mouse focal cerebral ischemia [1,14]. Although many compounds have been isolated from P. multiflorum, anthraquinone and stilbenes are considered to be the active compounds. In addition, extracts of P. multiflorum were shown to inhibit LPS-induced inducible nitric oxide synthase (iNOS) expression via MAPKs, and the results of various studies have demonstrated that CRPE56IGIH exerted its anti-neuroinflammatory responses.

2. Materials and methods

2.1. Materials

The cell culture medium, Dulbecco’s Modified Eagle’s Medium/Ham’s Nutrient Mixture F-12 (DMEM/F12), Dulbecco’s modified Eagle’s medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco BRL (now Invitrogen Corporation, Carlsbad, CA, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA). siRNAs against Nrf2, HO-1, and NQO1, and the antibodies against iNOS, COX-2, HO-1, Nrf2, HO-1, NQO-1, c-Jun, c-Fos, NF-κB, IkBα, TATA-binding protein (TBP), ERK, JNK, p38, and α-tubulin were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against phosphorylated (p)-p38, p-JNK, p-ERK, p-IκBα, p-NF-κB (p65), p-STAT-1, STAT-1, p-STAT-3, and STAT-3 were supplied by Cell Signaling Technology (Beverly, MA). The FuGENE 6 transfection reagent and the X-treme GENE siRNA Transfection Reagent were obtained from Roche (Indianapolis, IN).

2.2. Isolation and structure elucidation of active CRPE56IGIH

CRPE56IGIH was isolated from Polygonum multiflorum Thunb. Briefly, the dried roots of P. multiflorum Thunb. were purchased from Dongnam Co. (Busan, Korea) and were authenticated by Professor Y.W. Choi and the Department of Horticultural Bioscience, Pusan National University. A voucher specimen was deposited at the Plant Drug Research Laboratory of Pusan National University (Mirang, Korea). The dried roots (1 kg) were ground to a fine powder, extracted with 70% EtOH at room temperature, and evaporated in vacuo. The 70% EtOH extract was successively
fractionated with n-hexane, ethyl acetate (EtOAc), and butanol (BuOH) to obtain fraction weights of 3.33 g, 32.80 g, and 30.74 g, respectively. The EtOAc fraction (CRPE, 32.80 g) was evaporated in vacuo and separated on a silica gel column (80 cm × 5.0 cm) using a stepwise gradient of 5% and 25% acetone in CH2Cl2 and 5%, 25%, and 50% MeOH in CHCl3; this resulted in 95 fractions (CRPE1–CRPE95). The subfraction CRPE56 (4594.5 mg) was separated on a Sephadex gel column (90 cm × 3.0 cm) using MeOH to obtain CRPEIGIH (464.3 mg) and separated again, using the same conditions, to obtain pure CRPEIGIH (62.4 mg). The active molecule isolated from the roots of P. multiflorum Thunb. was identified by the 1H, 13C, DEPT, HSQC, and HMBC NMR spectra in pyridine-d5. From the calculated molecular ion signal of 432 for CRPEIGIH, an elemental composition of C21H20O10 was ascertained. This finding was confirmed by the 1H, 13C, and DEPT NMR techniques, which showed one primary (δC 22.87), one secondary (δC 63.61), nine tertiary (δC 125.92, 121.17, 111.39, 110.93, 104.39, 80.50, 79.40, 76.13, 72.26), and ten quaternary carbons (δC 188.89, 184.27, 167.68, 164.28, 163.70, 148.24, 138.95, 134.37, 116.78, 115.51). From these data and the elemental composition, the presence of emodin and one glucopyranoside was determined. The 1H NMR spectrum of CRPEIGIH showed one methyl singlet at δH 2.22 (C-3-CH3), two methine doublets at δH 7.83 (H-5, d, J=1.8) and 7.67 (H-4, d, J=3.6), and two methine singlets at δH 7.14 (H-2, s) and 7.59 (H-7, s). The absorptions of four olefinic proton were detected at δH 7.83 (H-5), 7.67 (H-4), 7.59 (H-7), and 7.14 (H-2). Six protons of the sugar moiety showed resonances at δH 5.71 (H-1′), 4.54 (H-2′), 4.40 (H-5′), 4.37 (H-4′ and H-6′) and 4.08 (H-3′). The connectivity of the olefinic protons was established through two-dimensional NMR experiments. The downfield signals at δC 188.89 (C-9) and 184.27 (C-10) were assigned to the two carbonyl carbons. This finding was confirmed by the HMBC spectrum, in which protons H-4 (δH 7.67) and H-5 (δH 7.83) were simultaneously coupled to δC 184.27 (C-10). Similarly, proton δH 7.67 (H-4) was also coupled to methyl carbon (C-3), C-2 (δC 125.92), and C-11 (δC 116.78) and H-5 were coupled to C-7 (δC 110.93) and C-13 (δC 115.51). As H-1′ was also coupled to the quaternary carbon C-1 (δC 164.26), this suggested the connection of the D-glucopyranoside group was at the C-1 position of the phenyl ring. Therefore, CRPEIGIH was deduced as emodin-1-B-β-glucopyranosyl (Fig. 1A and B).

2.3. Isolation of mouse primary microglia and cell culture

ICR mice were individually housed in polypropylene cages in a temperature-controlled environment (22 °C), with a reversed-phase 12-h light:dark cycle, fed a commercial diet, and given ad libitum access to tap water. All studies were approved by the Pusan National University Animal Care and Use Committee. To obtain primary microglia, we used an isolation method from a previously published protocol [6]. To monitor the purity, the cells
were immunostained with a CD11b antibody, which resulted in the positive staining of more than 90% of the cells. The immortalized mouse BV-2 cell line has been used as a model to investigate the neuroinflammatory response. The cells were maintained in DMEM supplemented with 5% heat-inactivated FBS and 0.1% penicillin-streptomycin in a 37°C incubator with a humidified atmosphere of 5% CO2 and 95% air.

2.4. MTT cell viability assay

The cytotoxicity of CRPE56IGH was assessed using the MTT assay. The cells were treated with various concentrations of CRPE56IGH for 24 h and then MTT solution was added to each well. After incubation for 8 h at 37°C in an atmosphere of 5% CO2, the supernatant was removed and the formazan crystals formed in the viable cells were solubilized in dimethylsulfoxide (DMSO). The absorbance of each well was measured at 570 nm by using a microplate reader (Wallace 1420, PerkinElmer, Waltham, MA, USA).

2.5. Determination of NO production

The presence of NO in the cell culture media was measured by the Griess method. The cells were seeded in 24-well plates and stimulated for 16 h. The culture medium was removed and mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H3PO4). The NO concentration was determined by measurement of the absorbance at 540 nm with a microplate reader (Wallace 1420, PerkinElmer, Waltham, MA, USA) and comparison with a standard curve of sodium nitrate absorbance.

2.6. Measurement of prostaglandin E2 (PGE2)

The cells were first incubated with various concentrations of CRPE56IGH for 1 h and then incubated with LPS for 24 h. After the incubation, the PGE2 level was quantified in the culture media by using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. The PGE2 concentration was determined by the measurement of the absorbance at 490 nm by using a microplate reader (Wallace 1420, PerkinElmer, Waltham, MA, USA).

2.7. Reverse transcription (RT)-real-time polymerase chain reaction (PCR)

Total cellular RNA was isolated by using a RNeasy mini kit (QIAGEN, Valencia, CA, USA) in accordance with the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using Maxime RT PreMix (Bioneer, Daejeon, Korea) and anchored with oligo-dT15-primers. Real-time PCR was performed using a Chromo4™ instrument (Bio-Rad) with the SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The following primers were used: iNOS-sense, 5′-agatccgaaaagcctcact-3′; iNOS-antisense, 5′-cttctgagcctgttag-3′; COX-2-sense, 5′-tacctacagggccctc-3′; COX-2-antisense, 5′-catattgagctggggtc-3′; GAPDH-sense, 5′-agtggtcctctcgtact-3′; and GAPDH-antisense, 5′-tacgaggaataggctgac-3′.

2.8. Transient transfection with siRNA

The cells were transfected with siRNA specifically targeting mouse HO-1 (sc-35555), mouse Nr2f (sc-37049) (Santa Cruz, Heidelberg, Germany), or negative control siRNA using the X-tremeGENE siRNA transfection reagent in accordance with the manufacturer’s instructions. Briefly, the X-tremeGENE siRNA transfection reagent (10 μL) was added to 100 μL of serum-free medium containing 2 μg of each siRNA and incubated for 20 min at room temperature. The impact of gene silencing was evaluated by western blotting analysis of the relevant protein expression at 48 h after transfection.

2.9. Transient transfection and dual luciferase assay

The cells were seeded in 24-well plates, grown to 70% confluence, and each well was co-transfected with the iNOS-luc, COX-2-luc, and NF-κB-luc reporter plasmid, the firefly luciferase gene, and either an ARE-reporter plasmid, or an HO-1 promoter reporter plasmid (Stratagene, Grand Island, NY, USA) by using the FuGENE-HD reagent (Roche Applied Science), in accordance with the manufacturer’s protocol. The Renilla luciferase control plasmid, pRL-CMV (Promega, Madison, WI, USA), was co-transfected as an internal control to ensure transfection efficiency. The luciferase activity was assayed using a dual-luciferase assay kit (Promega) in accordance with the manufacturer’s protocol. The luminescence was measured by using a microplate luminometer (Wallace 1420, PerkinElmer, Waltham, MA, USA).

2.10. Western blot analysis

For western blotting, the cells were washed with cold PBS and cell lysates were prepared by using lysis buffer. The protein content of the cell lysates was determined by using the Bradford reagent (Bio-Rad, Hercules, CA, USA). The proteins in each sample (20–50 μg) were resolved by electrophoresis on a 7.5–12% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and incubated with the appropriate antibodies. The horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies were visualized by using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA) and images were acquired by using an ImageQuant 350 analyzer (Amersham Biosciences).

2.11. Statistical analysis

Data were expressed as the mean ± standard error of multiple independent experiments. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software (version 18.0, SPSS Inc., Chicago, IL, USA) to identify significant differences based on one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. The level of statistical significance was set at P < 0.05.

3. Results

3.1. CRPE56IGH suppresses iNOS and COX-2 activation in LPS-stimulated microglia

To investigate the effects of CRPE56IGH on cell viability, the microglia were treated with CRPE56IGH and the viability of the cells was assessed with an MTT-based assay. CRPE56IGH did not exhibit cytotoxicity at 10–80 μg/mL (Fig. 1C and D). To investigate the anti-neuroinflammatory effects of CRPE56IGH, the microglia were stimulated with LPS in the presence or absence of CRPE56IGH. As shown in Fig. 2A, CRPE56IGH significantly inhibited NO and PGE2 production in LPS-stimulated microglia, but did not significantly affect basal NO and PGE2 production. Consistent with previous studies, CRPE56IGH suppressed LPS-induced iNOS and COX-2 protein expression (Fig. 2B). Subsequently, LPS treatment increased the iNOS and COX-2 mRNA levels and promoter activities, whereas pretreatment with CRPE56IGH inhibited the iNOS
Fig. 2. The effect of CRPE56IGIH on iNOS and COX-2 activation in LPS-stimulated microglia. (A) Primary microglia and BV-2 cells were treated with increasing doses of CRPE56IGIH for 1 h and subsequently treated with LPS for 24 h. The amounts of NO and PGE2 released from into the culture media were determined by using Griess reagent and ELISA assay, respectively. (B) The protein expressions of iNOS, COX-2, and α-tubulin were detected via western blot analysis using specific antibodies. (C) The relative iNOS and COX-2 mRNA expressions were determined by using a real-time polymerase chain reaction and calculated by the subtraction of the cycle threshold (Ct) value for GAPDH from the Ct value for iNOS and COX-2. (D) The cells were transfected with iNOS-luc or COX-2-luc reporter plasmids. At 24 h after transfection, the cells were treated with increasing doses of CRPE56IGIH for 1 h, stimulated with LPS for 24 h, and the iNOS and COX-2 promoter activities were then determined. Each bar represents the mean ± standard error of three independent experiments per group. *P<0.05 and **P<0.01 relative to the LPS-treated group.

and COX-2 mRNA levels and promoter activity in comparison with the LPS-treated control cells (Fig. 2C and D). Collectively, these results suggested that CRPE56IGIH suppressed the activation of iNOS and COX-2 in LPS-stimulated microglia without exertion of cellular damage.

3.2. CRPE56IGIH inhibits LPS-induced NF-κB activation in the microglia

To elucidate whether CRPE56IGIH affected the nuclear translocation of NF-κB (p65), the microglia were treated with various concentrations of CRPE56IGIH in the presence of LPS for 1 h and the nuclear extracts were prepared and examined via western blot
analysis. LPS induced the nuclear translocation of NF-κB (p65), which was inhibited by CRPE56IGIH. Simultaneously, CRPE56IGIH suppressed the phosphorylation of NF-κB (p65). We also found that the LPS markedly induced the phosphorylation of IκBα, but that the CRPE56IGIH pretreatment significantly suppressed this phosphorylation (Fig. 3A). Furthermore, the LPS-induced increases in the NF-κB reporter activities were suppressed by CRPE56IGIH in a dose-dependent manner (Fig. 3B). These results indicated that CRPE56IGIH inhibited the LPS-stimulated expression of neuroinflammatory molecules by blocking the activation of NF-κB.

### 3.3. The inhibitory effect of CRPE56IGIH on the LPS-mediated phosphorylation of STATs and MAPKs

The western blot analysis revealed that CRPE56IGIH markedly inhibited the LPS-induced phosphorylation of JAK-1 and STAT-1, but increased the phosphorylation of STAT-3 (Fig. 4A). However, treatment with CRPE56IGIH or LPS did not affect total JAK-1, STAT-1, and STAT-3. To investigate the regulation of MAPKs by CRPE56IGIH in the LPS-stimulated microglia, MAPK phosphorylation was analyzed by western blotting. As shown in Fig. 4B, CRPE56IGIH inhibited the LPS-induced phosphorylation of ERK, JNK, and p38. CRPE56IGIH alone had no effect on JAT-STAT and MAPK phosphorylation in the microglia, which suggested that the JAK-STAT and MAPK pathways were involved in the anti-neuroinflammatory effects of CRPE56IGIH in LPS-stimulated microglia.

### 3.4. CRPE56IGIH induces the expression of HO-1 and NQO-1 via Nrf2 activation

To assess the effects of CRPE56IGIH on the protein expression of HO-1 and NQO-1, we measured HO-1 and NQO-1 protein expression in primary microglia treated with CRPE56IGIH (5, 10, 20, and 40 μg/mL) or 20 μM of cobalt protoporphyrin (CoPP), an HO-1 activator, for 8 h. A concentration-dependent increase in the expression levels of both proteins was observed; the greatest increase was observed in response to 40 μg/mL CRPE56IGIH (Fig. 5A). Nrf2 is a critical component in the upregulation of phase II antioxidant enzymes, such as HO-1 and NQO-1. We confirmed the effects of CRPE56IGIH on the nuclear accumulation of Nrf2 in the microglia following treatment with CRPE56IGIH. When the cells were treated with varying concentrations of CRPE56IGIH for 2 h, Nrf2 nuclear accumulation was induced in a concentration-dependent manner (Fig. 5B). To clarify the effects of CRPE56IGIH on Nrf2 transactivation, the microglia were transiently transfected with luciferase.
The association of the abnormal activation of microglia with various neurodegenerative disorders has been widely proposed. Microglia are activated by external or internal pathogens, including pathogen invasion, protein aggregation, and signals from damaged neighboring neuronal cells. The activation of the microglia promotes the activation of pro-neuroinflammatory mediators to eliminate abnormal factors and repair the damaged site. Conversely, the abnormal activation of the microglia in the CNS results in abnormal neuroinflammatory responses, which lead to further neurodegenerative disorders [4,5]. We demonstrated the effect of CRPE56IGIH on the microglia using the gram-negative pathogen, LPS, which is known to act on TLR-4. We confirmed the anti-neuroinflammatory effect of CRPE56IGIH, which was shown to inhibit NO and PGE2 via the downregulation of iNOS and COX-2 mRNA and protein expression in LPS-stimulated microglia. CRPE56IGIH also suppressed the upstream signaling pathways involved in the activation of the TLR-4-triggered neuroinflammatory responses in the microglia. We found that CRPE56IGIH reduced the phosphorylation of NF-κB and IKKβ in response to LPS. In addition, CRPE56IGIH decreased the nuclear translocation and transactivation of NF-κB caused by LPS treatment.

JAK-STATs have been shown to play vital roles in neuroinflammatory-related gene expression; they regulate the expression of pro- and anti-neuroinflammatory genes by binding to the promoters of related genes [17]. In the inflammatory response, STAT-1 and STAT-3 act in opposition; STAT-1 is responsible for activation, while STAT-3 is responsible for inactivation. STAT-1 induces the upregulation of pro-inflammatory genes, including IL-6, whereas STAT-3 induces the upregulation of anti-inflammatory genes, such as IL-10 [10]. The present study showed that the LPS-induced phosphorylation of STAT-1 and STAT-3 increased significantly after 2 h, which occurred after the appearance of MAPK phosphorylation. However, the phosphorylation levels of JAK-1 and STAT-1 were dose-dependently decreased by CRPE56IGIH treatment, whereas the phosphorylation levels of STAT-3 were dose-dependently increased by CRPE56IGIH treatment. The CRPE56IGIH-mediated inactivation of STAT-1 or activation of STAT-3 might be related to the pro-inflammatory action of STAT-1 and the anti-inflammatory action of STAT-3.

Although numerous studies have investigated the neuroprotective effects of phase II antioxidant enzymes against oxidative stress, these enzymes have recently received attention owing to their anti-neuroinflammatory properties [8,11]. In the progression of AD or PD, Nrf2-knockout mice have shown abnormal activation of microglia compared with wild-type cells, which is indicative of the significance of the Nrf2 signaling pathway in the regulation of microglial function [15]. In accordance with the results of these previous studies, we demonstrated that CRPE56IGIH exhibits anti-neuroinflammatory effects in the microglia via the upregulation of Nrf2-mediated HO-1 and NQO-1 expression. The inhibition of these inflammatory molecules by CRPE56IGIH was reversed in cells treated with Nrf2, HO-1, and NQO-1 siRNAs.

In conclusion, CRPE56IGIH isolated from P. multiflorum mediated the suppression of the LPS-induced neuroinflammatory responses through the inactivation of the NF-κB, JAK-1/STAT-1, and MAPK signaling pathways. CRPE56IGIH had anti-neuroinflammatory effects, which were accompanied by the induction of HO-1 and NQO-1 via the Nrf2/ARE pathway. Therefore, CRPE56IGIH isolated from P. multiflorum was shown to be a novel inducer of the Nrf2/ARE pathway and represents a possible pharmaceutical agent for the prevention and treatment of neurodegenerative diseases.

4. Discussion

Table A lists the effects of CRPE56IGIH on the LPS-induced activation of JAK-STATs and MAPK. (A) BV-2 cells were treated with increasing doses of CRPE56IGIH for 1 h, followed by LPS for 2 h. The phosphorylation of JAK-1, STAT-1, and STAT-3 was confirmed via western blot analysis. (B) The cells were treated with increasing doses of CRPE56IGIH for 1 h, followed by LPS for 0.5 h. An equal amount of the cellular protein was analyzed via western blot with anti-p-ERK1/2, anti-jNK, or anti-p-p38 antibodies.

![Diagram](image1)

**Fig. 4.** The effect of CRPE56IGIH on the LPS-induced activation of JAK-STATs and MAPK. (A) BV-2 cells were treated with increasing doses of CRPE56IGIH for 1 h, followed by LPS for 2 h. The phosphorylation of JAK-1, STAT-1, and STAT-3 was confirmed via western blot analysis. (B) The cells were treated with increasing doses of CRPE56IGIH for 1 h, followed by LPS for 0.5 h. An equal amount of the cellular protein was analyzed via western blot with anti-p-ERK1/2, anti-jNK, or anti-p-p38 antibodies.

**reporter genes driven by the ARE, which binds Nrf2. CRPE56IGIH increased the ARE promoter activity in a dose-dependent manner (Fig. 5C) and the HO-1 promoter activity (Fig. 5D). Experiments were conducted to investigate the role of Nrf2, HO-1, and NQO-1 in the observed anti-neuroinflammatory effect of CRPE56IGIH. The transfection with Nrf2, HO-1, and NQO-1 siRNA significantly inhibited the CRPE56IGIH-mediated inhibition of the iNOS and COX-2 promoter activity when compared with control siRNA (Fig. 5E and F), which suggested that Nrf2, HO-1, and NQO-1 were responsible for the anti-neuroinflammatory properties of CRPE56IGIH in microglia.
Fig. 5. The effect of CRPE56IGIH on the activation of Nrf2-mediated HO-1 and NQO-1. (A) Microglia were cultured with increasing doses of CRPE56IGIH for 8 h. HO-1 and NQO-1 expression was determined via western blot analysis. (B) Microglia were incubated with increasing doses of CRPE56IGIH for 2 h. The nuclear localization and whole cell extract of Nrf2 was determined via a western blot analysis. Cells were transfected with the ARE-luciferase (C) or the HO-1 promoter (D)-luciferase constructs and were then treated with increasing doses of CRPE56IGIH. The cells were transfected with the siRNA control and si-RNA to Nrf2, HO-1, or NQO-1. At 24 h after transfection, the cells were treated with CRPE56IGIH (40 μM) for 1 h and then stimulated with LPS for 24 h. iNOS promoter (E) and COX-2 promoter (F) activities were subsequently determined. Each bar represents the mean ± standard error from three independent experiments per group. *P < 0.05 and **P < 0.01 relative to the lipoteichoic acid (LTA)-treated group.

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