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Icariin targets Nrf2 signaling to inhibit microglia-mediated neuroinflammation

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ABSTRACT

Microglia-mediated neuroinflammation is an important contributor to the pathogenesis of neurodegenerative diseases. Inhibition of neuroinflammation has been proved to be effective in neurodegenerative diseases treatment. Nuclear factor erythroid 2 related factor 2 (Nrf2) is a key mediator of endogenous inducible defense systems in the body. In response to oxidative stress, Nrf2 translocates to the nucleus and binds to specific DNA sites termed as anti-oxidant response elements to initiate transcription of cytoprotective genes, such as hemeoxygenase-1 (HO-1) and nicotinamide adenine dinucleotide phosphate: quinine oxidoreductase-1 (NQO1). However, insufficient Nrf2 activation has been closely associated with the progress of neurodegenerative diseases. New findings have linked activation of Nrf2 signaling to anti-inflammatory effects. Icariin (ICA), a natural compound derived from Herba Epimedii, possesses amounts of pharmacological activities, such as anti-aging, anti-oxidation and anti-inflammatory effects. Recent studies have confirmed that ICA exerted neuroprotection against neurodegenerative diseases. However, the mechanisms underlying ICA-mediated neuroprotection were not fully understood. In the present study, microglia BV-2 cell lines were performed to investigate the anti-neuroinflammatory effects of ICA and the mechanisms of actions. Results showed that ICA suppressed lipopolysaccharide (LPS)-induced microglial pro-inflammatory factors production. In addition, activation of Nrf2 signaling pathway participated in ICA-mediated anti-neuroinflammation, as evidenced by the following observations. First, Nrf2 siRNA reversed ICA-reduced microglial activation and pro-inflammatory factors release. Second, a selective inhibitor of HO-1 abolished ICA-mediated anti-neuroinflammatory actions. This study will give us an insight into the potential of Nrf2 and neuroinflammation in terms of opening up an alternative therapeutic strategy for neurodegenerative diseases.

1. Introduction

Neuroinflammation is an important and inevitable pathological process implicated in all types of damage and disorders in the brain [1]. The hallmark of neuroinflammation is glial activation, especially microglial activation. As immune-surveillance cells, microglia are well-established major components of neuroinflammatory reactions in the brain [2]. In response to stimuli, such as brain injury, inflammation and pathogens, microglia become activated and excrete a battery of inflammatory cytokines and neurotoxic factors. These factors could result in lesions in neighboring neurons, which elicit more microglia re-activation as feedback [3]. Far from solving the problem, the low-level but continuous activation of the immune system is also involved in brain injury. Recently, a large number of studies emphasized the application of anti-inflammatory drugs to ameliorate neurodegeneration [4,5]. However, the fact is that classical non-steroidal anti-inflammatory drugs, generally designed to halt acute inflammation, yielded mixed or inconclusive outcomes [6]. Thus, a novel approach towards a neuroprotective therapy need consider the restoration of homeostatic functions, such as control of undesirable neuroinflammation.

Nuclear factor erythroid 2 related factor 2 (Nrf2) is a key mediator of endogenous inducible defense systems in the body. Upon physiological and conditions, Nrf2 is anchored by Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and degraded by the ubiquitin proteasome pathway [7]. In response to oxidative stress, Nrf2 is excreted from Keap1 and translocated to the nucleus and binds to specific DNA sites termed as anti-oxidant response elements (ARE) to initiate transcription of cytoprotective genes, such as hemeoxygenase-1 (HO-1) and nicotinamide adenine dinucleotide phosphate: quinine oxidoreductase-1 (NQO1) [8,9]. However, insufficient Nrf2 activation has

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been closely associated with the progress of neurodegenerative diseases. Recently, new findings have linked activation of Nrf2 signaling to anti-inflammatory effects. Activation of Nrf2 has been suggested to attenuate lipopolysaccharide (LPS)-induced inflammatory response via the key innate immunity-regulating adaptor, Myeloid differentiation primary response gene 88 (MyD88) [10]. Furtherly, in Nrf2-deficient mice, the brain neuroinflammation and microglia activation are much more prominent under LPS stimulation [11]. Therefore, a functional Nrf2 signaling is identified as an important regulator of neuroinflammation during oxidative stress in the brain.

Icariin (ICA), a flavonoid derived from *Herba Epimedii*, possesses a large number of pharmacological properties, such as anti-aging, anti-oxidation and anti-inflammatory effects [12]. Pharmacokinetically, ICA could pass through the blood-brain barrier (BBB). Recent studies have confirmed that ICA exerted neuroprotection against neurodegenerative diseases [13]. In PC12 cells, ICA exerted neuroprotection against oxygen-glucose deprivation-induced neurotoxicity through activating the anti-oxidative defense [14]. In addition, ICA ameliorated rat spatial learning and memory abilities in LPS-induced brain dysfunction via the decreased pro-inflammatory factors expressions in hippocampus [15]. However, the mechanisms underlying ICA-mediated neuroprotection were not fully understood.

In the present study, microglia BV2 cell lines were performed to investigate the anti-neuroinflammatory effects of ICA and further elucidate whether ICA-mediated actions were dependent on Nrf2 signaling activation. Particularly, these findings might confer a potential beneficial candidate for neuroinflammation-related diseases treatment.

2. Materials and methods

2.1. Reagents

ICA (purity > 98%) was obtained from Nanjing Ze Lang Medical Technology Co., Ltd. (Nanjing, China). LPS (*Escherichia coli* O111:B4) was purchased from Sigma Aldrich (St. Lewis, CA, USA). Protoporphyrin IX zinc (II) (ZnPP IX) was available from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). MTT assay kit was from Beijing Solarbio science and Technology Co., Ltd. (Beijing, China). SYBR green polymerase chain reaction (PCR) master mix was purchased from Bio-Rad Laboratories (CA, USA). Trizol reagent was obtained from Takara Biotech Co., Ltd. (Dalian, China). The enzyme-linked immunosorbent assay (ELISA) kits were purchased from Elabscience biotechnology Co., Ltd. (Wuhan, China). Griess reagent was obtained from Beyotime Biotechnology (Shanghai, China). Lipofectamine™ 2000 transfection reagent was purchased from Thermo Fisher Scientific Co., Ltd. (Waltham, MA, USA). Anti-HO-1 (Catalog No. Ab13243), NQO1 (Catalog No. Ab34173) and CD16 (Catalog No. Ab25235) antibodies were from Abcam (Cambridge, MA, USA). Anti-Nrf2 (Catalog No. 16396-1-AP), PCNA (Catalog No. 10205-2-AP), β-actin (Catalog No. 20506-1-AP) and rabbit IgG (Catalog No. SA00001-2) antibodies were obtained from Proteintech Group (Chicago, IL, USA).

2.2. BV2 cell culture

Mouse microglial BV2 cell lines were obtained from China Center for Type Culture Collection (Wuhan, China). Cultures were maintained in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in the humidified atmosphere of 5% CO_2 and 95% air.

2.3. Primary microglia cell culture

Primary rat mixed-glia cultures were prepared from the whole brains of 1-day-old rat pups. The meninges and blood vessels were isolated and the brain tissues were homogenized and the cells (1×10^6/well) were planted in poly-D-lysine-coated 75 cm^3 culture flask [16]. After a confluent monolayer of glia cells had been obtained, microglia were shaken off. Cells were seeded at 5×10^5/well in 24-well plates, and used for treatment the next day.

2.4. MTT assay

Cell viability was evaluated by MTT assay. BV2 cells were cultured in 1×10^5/well in 96-well plates and incubated in an environment with 5% CO_2 at 37 °C for 24 h. Afterwards, cells were treated with different concentrations of ICA for 30 min followed by LPS (1 μg/ml) treatment for 24 h and then incubated with MTT solution (5 g/l) for 4 h. Formazan crystals in the cells were solubilized using 200 μl dimethyl sulfoxide (DMSO) and the absorbance was detected by an automated microplate reader within 490 nm wavelength.

2.5. Real time RT-PCR

Total RNA was extracted by Trizol reagent. Single-stranded DNA was synthesized from total RNA using the RT Reagent kit. The SYBR green PCR Master Mix was performed for real-time PCR analysis. The relative mRNA expression was measured via cycle time (Ct) values normalized with β-actin of the same sample. The primer sequences were designed by Sangon Biotech (Shanghai, China). PCR primer sequences were as follows: Nrf2: F: 5′- GTTCTCCGCTGCTCGGACTA -3′, R: 5′-GGTGGCAACTCCAAGTCCAT -3′; HO-1: F: 5′- GGTGGCAACTCC AAGTCCAT -3′; R: 5′- TCTGACGAAGTGACGCCATC -3′; NQO1: F: 5′- AGCCAATCAGCGTTCGGTAT -3′, R: 5′- GCCTCCTTCATGGCGTAGTT −3′;

β-actin: F:5′- GTGCTATGTTGCTCTAGACTTCG -3′, R:5′- ATGCCAC AGGATTCCATACC-3′.

2.6. IL-18, IL-1β and nitrite assay

NO production was determined by measurement of the accumulated levels of nitrite in the culture medium with Griess reagent. The levels of IL-1β and IL-18 released from cells in the culture supernatants were detected by ELISA kits.

2.7. Immunofluorescence staining

Cells were fixed with paraformaldehyde (4%) for 30 min. Later, cells were permeabilized using triton X-100 (0.3%) for 15 min. Then, cells were blocked using goat serum blocking solution for 60 min at 37 °C. Thereafter, cells were incubated with 1:300 dilution of anti-Nrf2 antibody or 1:50 dilution of anti-CD16 antibody overnight at 4 °C. Following overnight incubation, cells were incubated in dark for 30 min with goat anti-rabbit secondary antibody (1:1000) or goat anti-mouse secondary antibody (1:1000). Cells were also counterstained with DAPI for 5 min. After rinsing cells with PBS, representative fluorescence images were obtained using EVOS® Floid® Cell imaging station.

2.8. RNA transfection

BV2 cells were cultured and seeded in a 6-well plate at a density of 1×10^5 cells/ml. The transfection of siRNA was performed complying with the manufacturer's protocol. In one tube, 2 μl of Nrf2 siRNA duplex was diluted into 100 μl of transfection medium. In a second tube, 2 μl of Lipofectamine™ 2000 transfection reagent was diluted into 100 μl of transfection medium. Two tubes were allowed to stand for 5 min. The contents of both tubes were gently mixed and incubated for 15 min at room temperature. Next, Nrf2 siRNA transfection cocktail was added to BV2 cells and further incubated for 6 h at 37 °C. Cells were recovered in fresh media containing 2% FBS for 18 h. Transfection efficiency was determined using western blot assay. Effects of ICA on NO, IL-1β and IL-18 production in LPS-stimulated BV2 cells transfected by Nrf2-siRNA

were determined.

2.9. Western blot analysis

For the cell lysates extraction, cell cultures were washed with cold PBS and lysed with RIPA cell lysis buffer. Cell lysates were incubated on ice for 10 min and then centrifuged at 12,000 rpm for 15 min. The protein levels were quantified by the BCA assay. Protein (10 μg) from each sample was subjected to SDS-PAGE gel under the reduced conditions. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk for 2 h at room temperature and then incubated overnight at 4 °C with the primary antibodies: Nrf2, HO-1, NQO1, Iba-1 and β-actin. After extensive washing, protein was detected by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies at room temperature for 1 h. The blot films were developed with enhanced ECL reagent.

2.10. Statistical analysis

Results were indicated as mean ± standard error of the mean (SEM) from three independent experiments performed in triplicate. Statistical significance was analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Upon ANOVA demonstrating the significant differences, pairwise comparison between means was evaluated by Bonferroni's post hoc *test* with correction. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. ICA attenuated LPS-induced microglia activation and pro-inflammatory factors release in BV2 cells

In our previous study, we had shown that ICA (0.1 μM) reduced dopaminergic neuronal loss in vivo and in vitro [17]. Therefore, BV2 cells were treated with ICA (0.01 and 0.1 μM) for 30 min and then incubated with LPS (1 μg/ml) for 24 h. Cell viability was evaluated by MTT assay. As shown in Fig. 1A, no significant difference among the control, ICA (0.1 μM) alone, LPS (1 μg/ml), and LPS + ICA (0.01 and 0.1 μM) treatment was indicated. The total cell protein was collected and the protein expression of Iba-1, the β-integrin marker of microglia, was detected by western blot assay. As shown in Fig. 1B and C, ICA suppressed LPS-induced microglial activation. Later, the production of NO, IL-1β, and IL-18 in primary microglia and BV2 culture medium was detected. As shown in Fig. 1D and E, ICA decreased LPS-induced production of NO, IL-1β and IL-18 in primary microglia and BV2 culture medium, respectively.

3.2. ICA activated Nrf2 signaling pathway in LPS-treated BV2 cells

BV2 cells were pre-incubated with ICA (0.1 μM) for 30 min followed by the stimulation with LPS (1 μg/ml) for 2 h, 6 h and 24 h, respectively. The mRNA expressions of Nrf2, HO-1 and NQO1 were determined by real-time RT-PCR. As shown in Fig. 2A, LPS led to an apparent increase in mRNA expressions of Nrf2 at 2 h, HO-1 and NQO1 at 6 h in BV2 cells. ICA increased the LPS-induced elevated transcripts of Nrf2 at 2 h, HO-1 and NQO1 at 6 h. Moreover, immunofluorescence staining assay indicated that ICA elicited Nrf2 activation in BV2 cells (Fig. 2B). In addition, after LPS treatment for 24 h, the protein expressions of Nrf2, HO-1 and NQO1 was detected by western blot assay. As shown in Fig. 2C, ICA induced an increase in the translocation of Nrf2 from cytoplasm to nucleus. Also, ICA enhanced the protein expressions of HO-1 and NQO1 (Fig. 2D).

3.3. Silence of Nrf2 abolished the inhibitory effects of ICA on microglia-mediated neuroinflammation

To investigate the role of Nrf2 on ICA-inhibited microglia-mediated neuroinflammation, Nrf2-siRNA was applied to determine whether the anti-inflammatory effects of ICA were dependent on Nrf2 activation. First, the RNA silence efficiency was assessed. As shown in Fig. 3A, a marked deletion of Nrf2 protein in Nrf2 siRNA-transfected BV2 cells was demonstrated. Also, MTT assay revealed that Nrf2-siRNA (50 nM) had no cytotoxicity on BV2 cells. Second, BV2 cells were treated with ICA in the presence of Nrf2-siRNA and then exposed to LPS for 2 h, 6 h and 24 h, respectively. As shown in Fig. 3B, LPS led to an apparent increase in mRNA expressions of HO-1 and NQO1 at 6 h in BV2 cells. ICA further increased LPS-elevated transcripts of HO-1 and NQO1 at 6 h. However, the mRNA expressions of HO-1 and NQO1 were decreased after Nrf2-siRNA application. Consistent with mRNA detection, ICA enhanced the protein expressions of HO-1 and NQO1, which could be reduced by Nrf2-siRNA treatment (Fig. 3C).

Next, the effects of Nrf2-siRNA on ICA-suppressed microglia-mediated neuroinflammation were explored. As shown in Fig. 4A and B, ICA inhibited LPS-induced microglial activation and these inhibitory effects were abolished by Nrf2-siRNA. In addition, Nrf2-siRNA eliminated ICA-decreased production of NO, IL-1β and IL-18 in the culture medium (Fig. 4C).

3.4. HO-1 inhibitor neutralized ICA-attenuated microglia-mediated neuroinflammation

To further confirm Nrf2 signaling pathway participated in ICA-reduced microglia neuroinflammation, the selective Nrf2 pathway downstream HO-1 inhibitor (ZnPP) was employed to investigate the role of Nrf2 pathway on ICA-mediated anti-neuroinflammatory effects. As shown in Fig. 5A, ZnPP decreased HO-1 protein expression, while ZnPP didn't have neurotoxicity on BV2 cells. In addition, ZnPP had no significant effects on ICA-induced Nrf2 activation (Fig. 5B and C).

Furthermore, the effects of ZnPP on ICA-conferred anti-neuroinflammatory actions were analyzed. As shown in Fig. 6A and B, ICA suppressed LPS-induced microglial activation, which could be neutralized by ZnPP treatment. Also, ZnPP weakened ICA-reduced pro-inflammatory factors release (Fig. 6C).

4. Discussion

The present study demonstrated that ICA suppressed microglia-mediated neuroinflammation induced by LPS. In addition, activation of Nrf2 signaling pathway participated in ICA-conferred anti-neuroinflammation, as evidenced by the following observations. First, Nrf2 siRNA reversed ICA-reduced microglial activation and pro-inflammatory factors release. Second, a selective inhibitor of HO-1 abolished ICA-mediated anti-neuroinflammatory actions. Together, these results suggested ICA-exerted anti-neuroinflammation was dependent on Nrf2 signaling activation.

As the first line of the CNS defense, neuroinflammation is considered to be a complex biochemical process in which brain and spinal cord react to diverse pathogenic and harmful stimuli, such as host-derived danger signal of cellular damage [18]. However, uncontrolled neuroinflammation may result in tissue injury and neuronal loss and has been recognized as a causative factor of multiple neurological disorders [19]. Furtherly, microglial activation- mediated neuroinflammatory reactions are well-known to play a pivotal role in neuroinflammation and subsequent neuronal damage. Activated microglia could excrete excess production of pro-inflammatory and neurotoxic factors. The accumulation of these factors contributed to the surrounding neuronal injuries [20]. However, the continuing dying/dead neurons, in turn led to the secondary activation of microglia and the activated microglia further released pro-inflammatory factors and thus

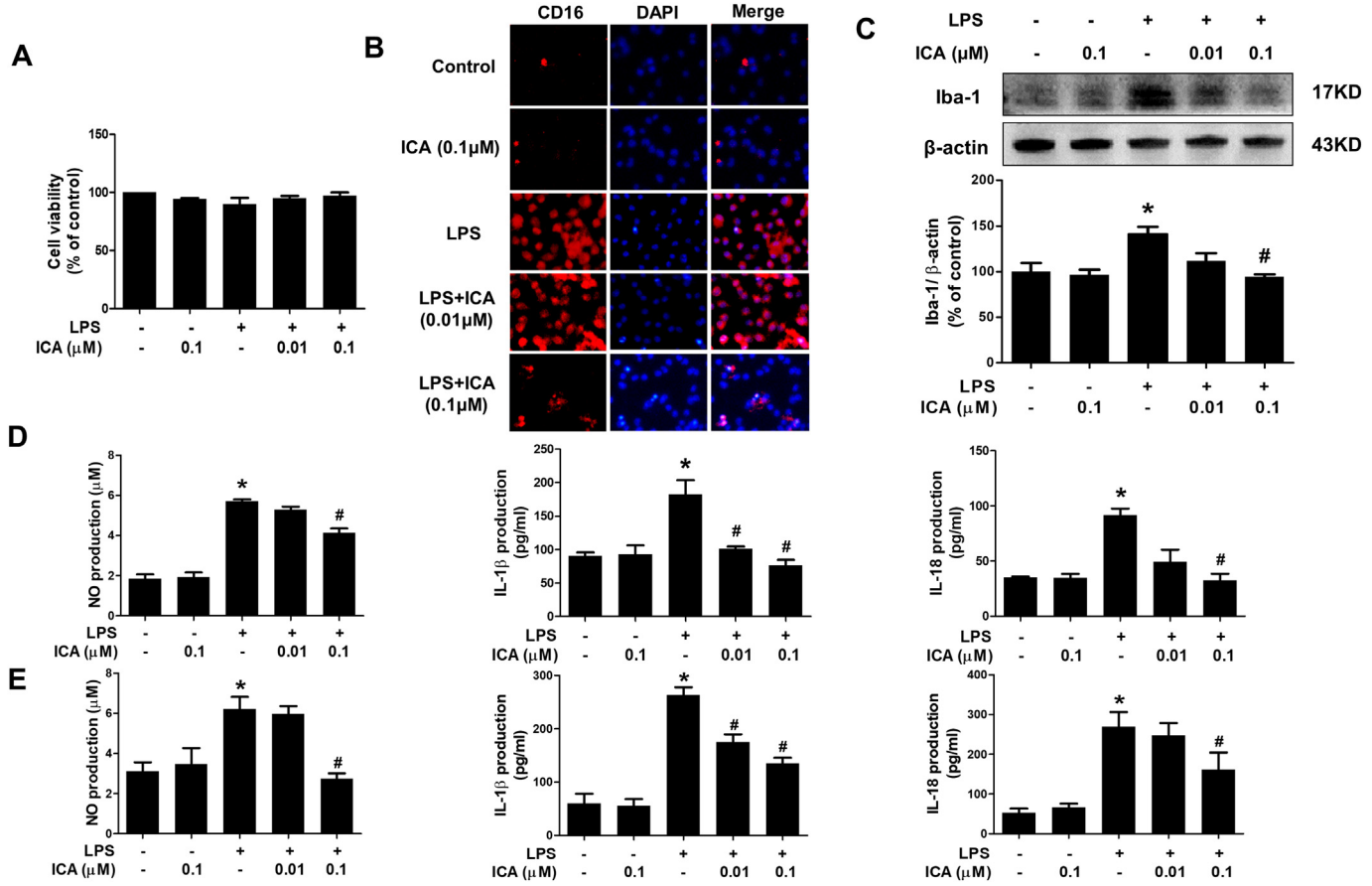

Fig. 1. ICA attenuated LPS-induced microglia activation and pro-inflammatory factors release in BV2 cells. BV2 cells were treated with ICA (0.01 and 0.1 μM) for 30 min and then incubated with LPS (1 μg/ml) for 24 h. Cell viability was determined by MTT assay (A). Microglial activation was evaluated by immunostaining (B) with an anti-CD16 antibody and quantitated by western blot analysis with an anti-Iba-1 antibody (C). Scale bar = 100 μm. The ratio of densitometry values of Iba-1 with β-actin was analyzed and normalized to each respective control cultures. The levels of NO, IL-1β and IL-18 in primary microglia and BV2 culture medium were detected by the Griess reagent and ELISA (D and E), respectively. Results were the mean ± SEM from three independent experiments performed in triplicate. $^{*}P < 0.05$ compared with the control cultures; $^{\#}P < 0.05$ compared with LPS-treated cultures.

caused neuronal damage [21]. Collectively, a vicious cycle to result in the prolonged neuroinflammation and the progressive neuronal loss was created [22]. Therefore, attenuating activation of microglia-elicited neuroinflammation might be a promising therapeutic approach for neuroinflammation-related disorders. This study indicated that ICA reduced LPS-induced microglial TNFα, IL-1β and NO production. These results were consistent with the previous studies that ICA inhibited microglia–producing TNFα and IL-1β via the depression of nuclear factor kappa B (NF-κB) signaling pathway activation in the hippocampus [12].

Encouraged by the activation of Nrf2 signaling by ICA in LPS-stimulated BV2 cells, we further investigated the role of Nrf2 pathway on ICA-inhibited neuroinflammation. Recent studies indicated that Nrf2 activation contributed to the suppression of microglial activation and further protection from microglia-elicited neuronal loss [23]. Moreover, it was found in the nucleus of the Nrf2 activation, represented by HO-1 and NQO1 expressions, was up-regulated, implying an attempt to enhance neuroprotection via this pathway [24]. On the other hand, accumulating evidence demonstrates that Nrf2 activation not only exerts anti-oxidative properties, but also regulates redox homoeostasis and alleviates neuroinflammatory responses in activated microglia [25–27]. Furthermore, activated microglia are composed of two cell populations which have distinct and even opposing functions. The two microglial polarization extremes are termed as the classically activated M1 (pro-inflammatory) and alternatively activated M2 (anti-inflammatory) phenotypes [2]. More prominently, activation of Nrf2 signaling has also been revealed to promote microglial polarization towards the M2 phenotype, therefore suppressing neuroinflammation [26,28]. It is noteworthy that several confirmed compounds/agents, such as curcumin, ellagic acid, sulforaphane and dimethyl fumarate, exhibit promising anti-inflammatory activities through the activation of Nrf2 signaling in microglia [29]. Here, this study indicated that the Nrf2 siRNA and selective HO-1 inhibitor neutralized ICA-produced inhibitory properties on neuroinflammatory reactions in LPS-activated BV2 microglia, suggesting that the activation of Nrf2 signaling was involved in the anti-neuroinflammatory responses of ICA. Therefore, activating Nrf2 signaling pathway by ICA might be a considerable avenue for ameliorating neuroinflammation.

At present, an increasing clinical interest of using Nrf2 activators for therapeutic purposes is emerged. One approach to activate the Nrf2 signaling is to use drugs that are already used clinically. Melatonin could activate Nrf2 and is applied to treat sleep disorders in children and adolescents [30]. In addition, a number of Nrf2 inducers have been tested clinically. Sulforaphane-rich broccoli sprout extract is undergoing clinical assessment in the treatment of autism disorders and schizophrenia [31]. Experimental studies show that the fumaric acid ester Dimethyl fumarate (DMF) produced neuroprotection against oxidative stress in a Nrf2-dependent manner [32]. Besides, DMF was approved for psoriasis treatment and was discerned to be beneficial for relapsing-remitting multiple sclerosis in recent placebo-controlled phase II and III studies [33]. Triterpenoids are potent inducers of Nrf2-regulated genes [34], and new synthetic triterpenoids are displayed to

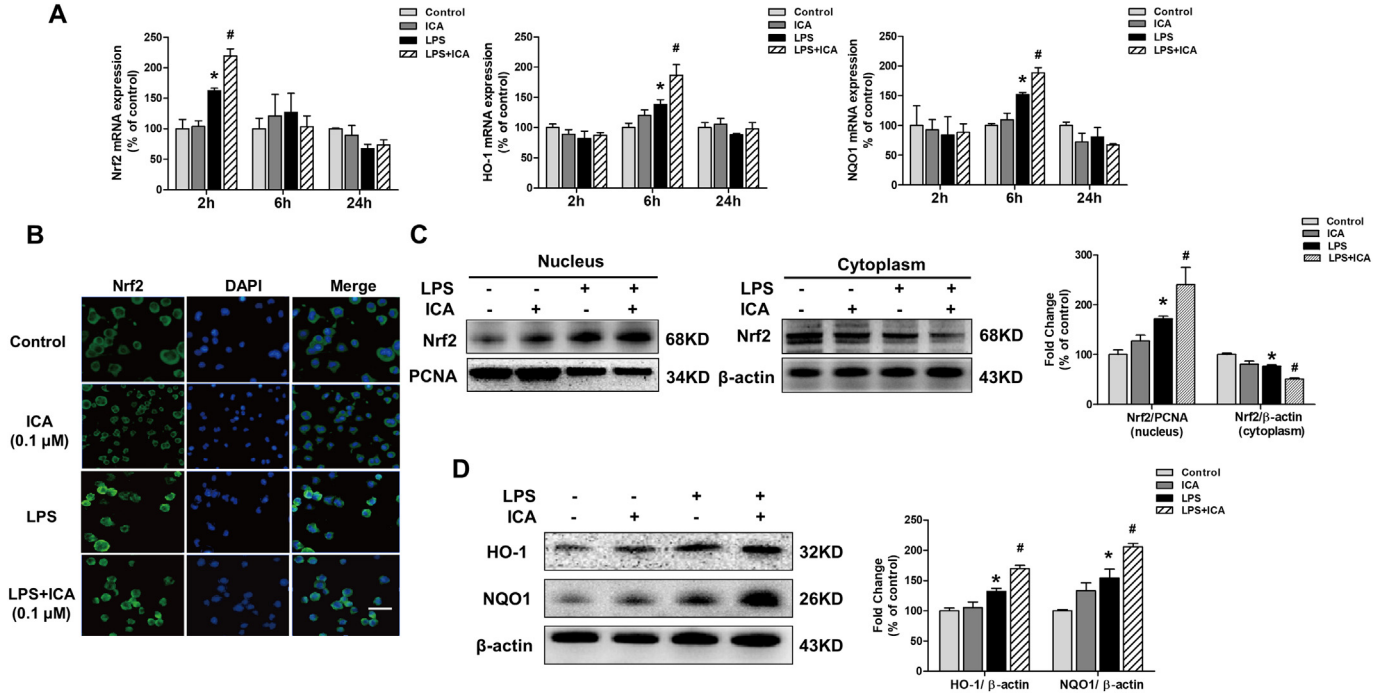

Fig. 2. ICA activated Nrf2 signaling pathway in LPS-treated BV2 cells. BV2 cells were incubated with ICA (0.1 μM) for 30 min followed by the stimulation with LPS (1 μg/ml) for 2, 6 and 24 h, respectively. The mRNA expressions of Nrf2, HO-1 and NQO1 were determined by real-time RT-PCR (A). In addition, after LPS treatment for 24 h, immunofluorescence staining was performed to detect Nrf2 protein localization (B). Cells were counterstained with DAPI. Scale bar = 100 μm. Cytoplasm and nucleus lysates were collected and analyzed for Nrf2 protein expressions via western blot assay (C). The protein levels of HO-1 and NQO1 in whole cells were also measured by western blot assay (D). Results were the mean ± SEM from three independent experiments performed in triplicate. $^{*}P < 0.05$ compared with the control cultures; $^{\#}P < 0.05$ compared with LPS-treated cultures.

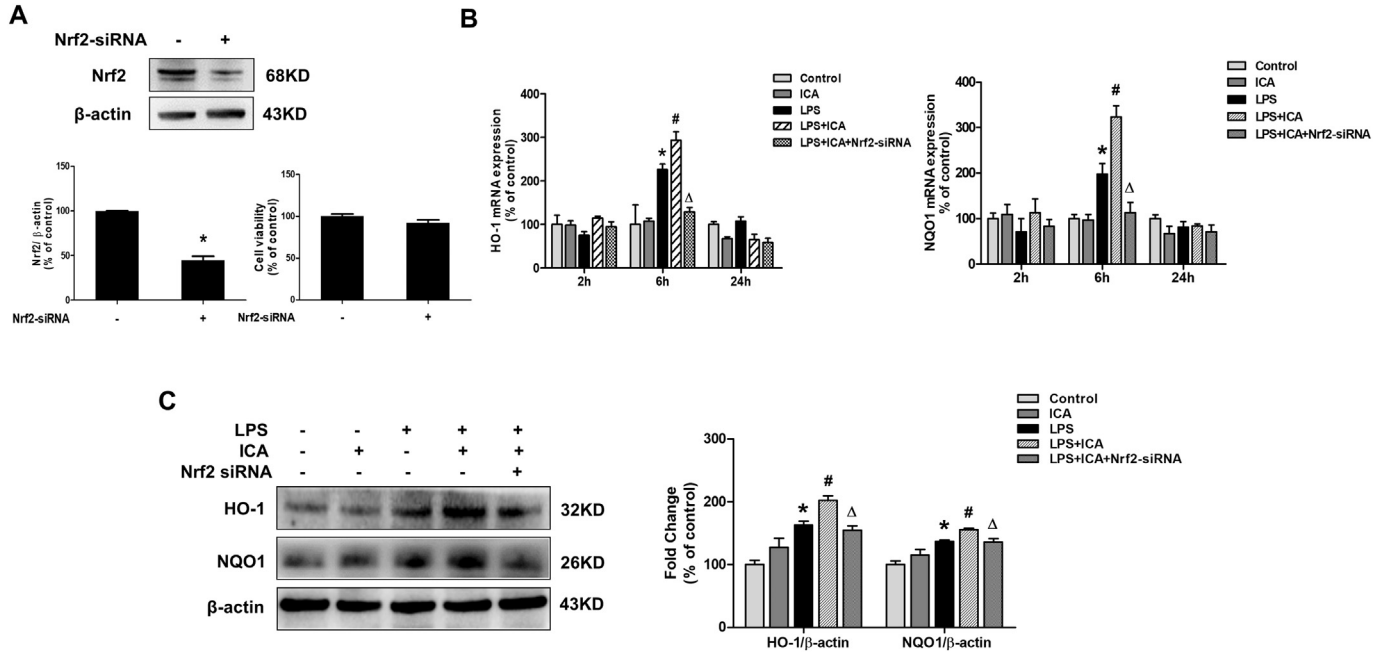

Fig. 3. Silence of Nrf2 abolished the effects of ICA on Nrf2 signaling pathway activation. BV2 cells were treated with Nrf2-siRNA (50 nM) for 24 h. The silence efficiency was assessed via Nrf2 protein expression detection and the effects of Nrf2-siRNA on cell viability was also determined by MTT assay (A). Moreover, BV2 cells were treated with ICA (0.1 μM) in the presence of Nrf2-siRNA and then exposed to LPS for 2, 6 and 24 h. The HO-1 and NQO1 mRNA expressions were determined by real-time RT-PCR (B). In addition, upon cells transfected with Nrf2-siRNA followed by LPS administration for 24 h, the protein levels of HO-1 and NQO1 were detected by western blotting (C). Results were the mean ± SEM from three independent experiments performed in triplicate. $^{*}P < 0.05$ compared with the control cultures. $^{\#}P < 0.05$ compared with LPS-treated cultures; $^{\Delta}P < 0.05$ compared with LPS + ICA-treated cultures.

enter the CNS through BBB to alleviate microglial activation and pro-inflammatory factors production in PD animal model [35]. Thus, the clinical use of Nrf2 activators is still an open innovation field of discussion and introduction of novel Nrf2-based therapies would be perhaps considered to be a more potential viable strategy in future.

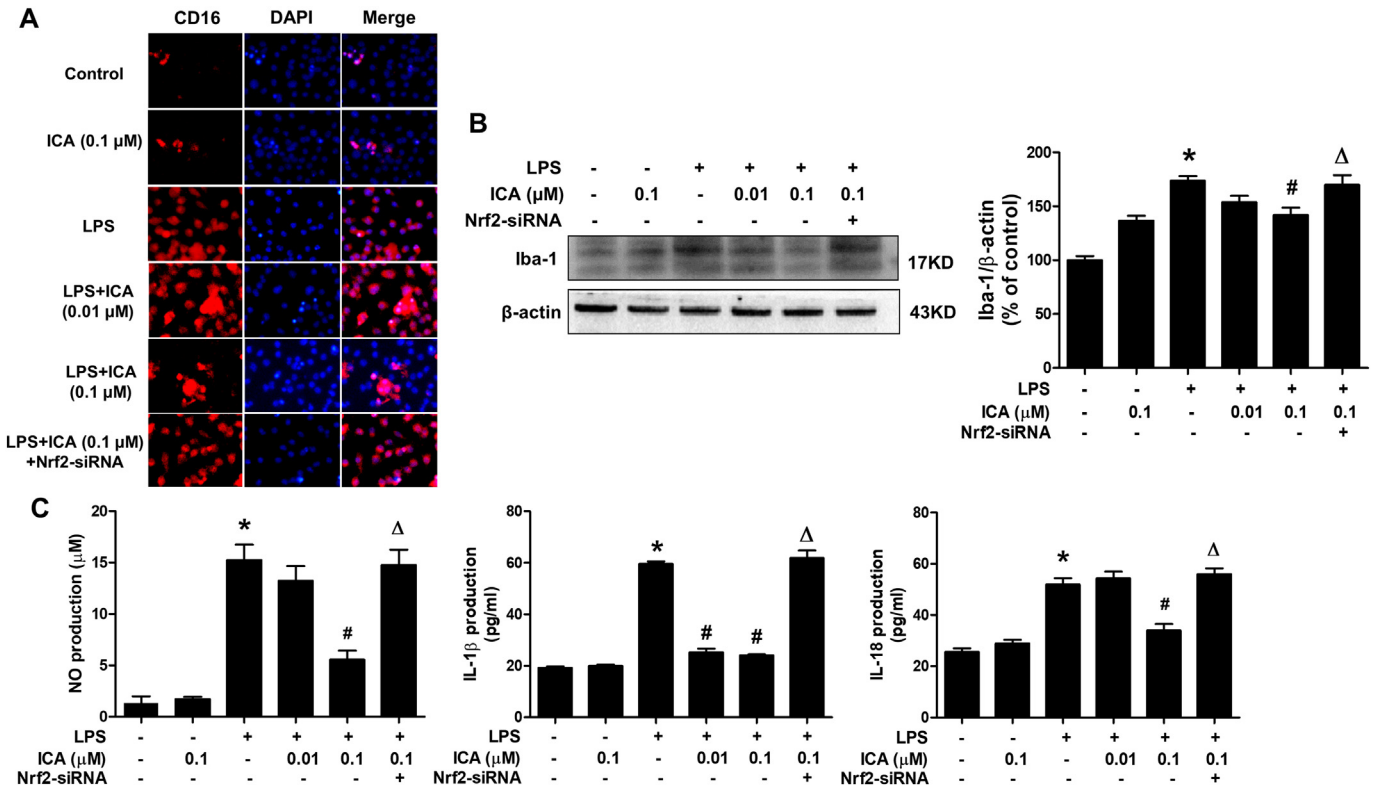

Fig. 4. Nrf2-siRNA reversed the suppressive effects of ICA on LPS-induced microglia activation and pro-inflammatory factors release. BV2 cells were transfected with Nrf2-siRNA. Then, cells were treated with ICA (0.01 and 0.1 μM) and then stimulated by LPS for 24 h. Microglia activation was analyzed by immunostaining (A) with an anti-CD16 antibody and western blotting antibody with an anti-Iba-1 antibody (B). Scale bar = 100 μm. The ratio of densitometry values of Iba-1 with β-actin was assessed and normalized to each respective control cultures. The levels of NO, IL-1β and IL-18 in culture medium were measured by the Griess reagent and ELISA (C). Results were the mean ± SEM from three independent experiments performed in triplicate. $^{*}P < 0.05$ compared with the control cultures. $^{\#}P < 0.05$ compared with LPS-treated cultures; $^{\Delta}P < 0.05$ compared with LPS + ICA-treated cultures.

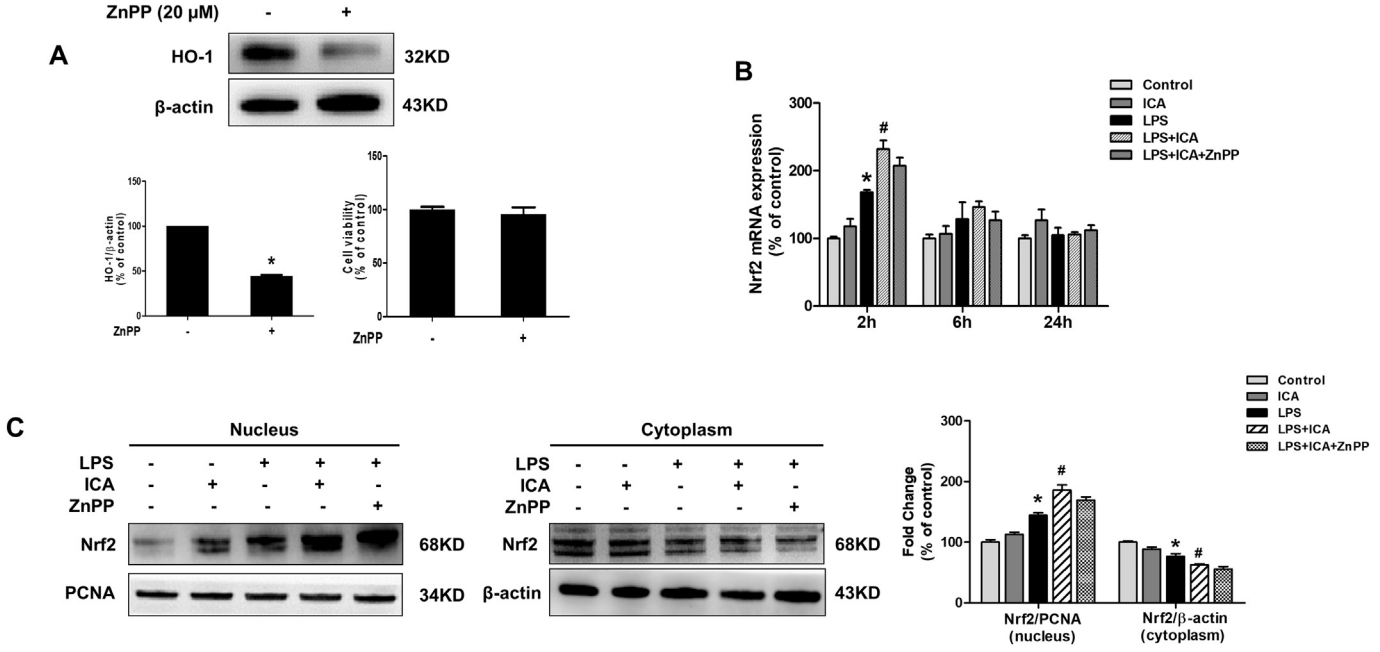

Fig. 5. The effects of HO-1 inhibitor on ICA-induced Nrf2 activation. BV2 cells were treated with ZnPP (20 μM) for 24 h. Lysates were collected and assessed for HO-1 protein expression and cell viability was determined by MTT assay (A). Furtherly, BV2 cells were treated with ICA (0.1 μM) in the presence of ZnPP and then stimulated by LPS for 2, 6 and 24 h. Nrf2 mRNA expressions were measured by real-time RT-PCR (B). In addition, with LPS treatment for 24 h, the protein expressions of Nrf2 in cytoplasm and nucleus lysates were detected by western blot assay (C). Results were the mean ± SEM from three independent experiments performed in triplicate. $^{*}P < 0.05$ compared with the control cultures; $^{\#}P < 0.05$ compared with LPS-treated cultures.

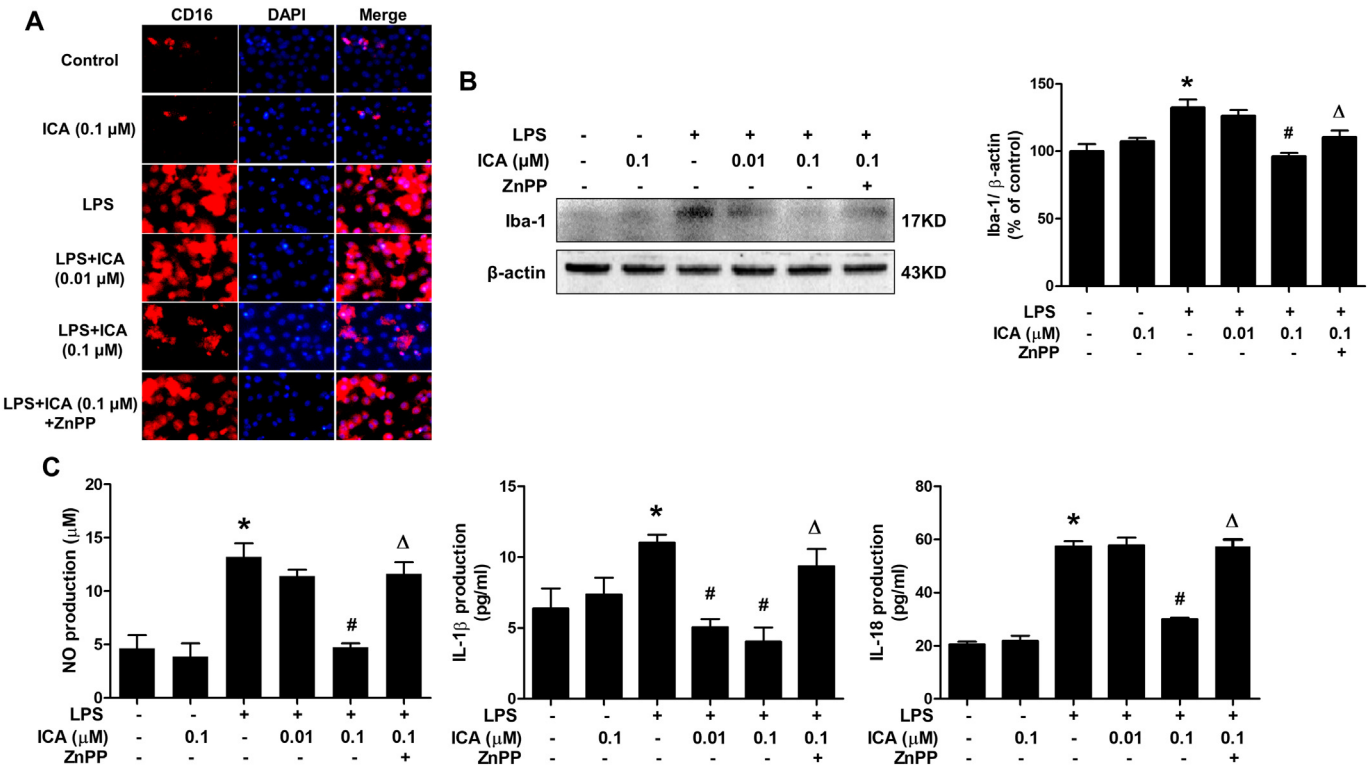

Fig. 6. HO-1 inhibitor neutralized the effects of ICA on LPS-induced microglial activation and pro-inflammatory factors production. BV2 cells were pretreated with ZnPP and then treated with ICA followed by LPS stimulation for 24 h. Microglia activation was evaluated by immunostaining (A) and western blotting (B). Scale bar = 100 μm. The levels of NO, IL-1β and IL-18 in culture medium were detected by the Griess reagent and ELISA (C). Results were the mean ± SEM from three independent experiments performed in triplicate. $^{*}P < 0.05$ compared with the control cultures; $^{\#}P < 0.05$ compared with LPS-treated cultures; $^{\Delta}P < 0.05$ compared with LPS + ICA-treated cultures.

5. Conclusions

This study indicated that ICA inhibited microglia-mediated neuroinflammation via the activation of Nrf2 signaling. This study will give us an insight into the potential of Nrf2 and neuroinflammation in terms of opening up an alternative therapeutic strategy for neurodegenerative diseases.

Conflict of interests

The authors declared no any conflict of interests.

Authors' contributions

FZ conceived and designed the experiments. All the authors participated in the experiments performance and data analysis. FZ and YXZ wrote, revised and checked the article. All authors read and revised and approved the final manuscript.

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