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Icariin attenuates lipopolysaccharide-induced microglial activation and resultant death of neurons by inhibiting TAK1/IKK/NF-κB and JNK/p38 MAPK pathways

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ABSTRACT

Microglia in the central nervous system (CNS) play an important role in the initiation of neuroinflammatory response. Icariin, a compound from *Epimedium brevicornum* Maxim, has been reported to have anti-inflammatory effect on the macrophage cell line RAW264.7. However, it is currently unknown what anti-inflammatory role icariin may play in the CNS. Here, we reported the discovery that icariin significantly inhibited the release of nitric oxide (NO), prostaglandin E (PGE)-2, reactive oxygen species (ROS) and mRNA expression of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in lipopolysaccharide (LPS)-activated microglia. Icariin also inhibited the protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in a dose-dependent manner. Further mechanism studies revealed that icariin blocked TAK1/IKK/NF-κB and JNK/p38 MAPK pathways. It was also found that icariin reduced the degeneration of cortical neurons induced by LPS-activated microglia in neuron–microglia co-culture system. Taken together these findings provide mechanistic insights into the suppressive effect of icariin on LPS-induced neuroinflammatory response in microglia, and emphasize the neuroprotective effect and therapeutic potential of icariin in neuroinflammatory diseases.

1. Introduction

Inflammation usually occurs in the CNS in response to various cerebral bacterial infections [1,2]. One of the most important characteristics of the neuroinflammatory response is the activation of resting resident immune cells in the brain, particularly microglia [3]. In bacterial infection conditions, the uncontrolled activation of microglia can cause neuronal injury via the overproduction of neurotoxic proinflammatory mediators, such as nitric oxide (NO), prostaglandin E-2 (PGE-2), reactive oxygen species (ROS), tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β [4–8]. These proinflammatory mediators are usually induced from activated microglia, by treatment with bacterial endotoxin lipopolysaccharide (LPS) in experimental conditions, with further resulting detrimental effect on neurons [9].

The NF-κB is an important signaling pathway involved in inflammatory response. The activation of NF-κB requires phosphorylation of upstream IκB kinase (IKK), which contains two catalytic subunits, IKKα and IKKβ [10]. Upon stimuli with LPS and proinflammatory cytokines, IKK is phosphorlated and activated via upstream of TGF-β activated kinase 1 (TAK1), resulting in further phosphorylation and degradation of IκB in the ubiquitination pathway [11–14]. Then, NF-κB releases from the IκB/NF-κB dimer and translocates from cytoplasm into the nucleus, inducing further proinflammatory gene expression and inflammatory response [6–8,15]. Therefore, the inhibition of the NF-κB pathway may have a potential therapeutic effect in neurodegenerative diseases that are accompanied by microglial activation.

In addition, mitogen-activated protein kinases (MAPKs) also contribute to activated microglia-related neuroinflammation. Extracellular signal-regulated protein kinase (ERK) and p38 MAPKs regulate, at least in part, NO production, iNOS expression, and TNF-α secretion in activated microglia [16]. Moreover, a rapid and lasting activation of c-Jun NH(2)-terminal kinase (JNK) MAPK in rat primary microglia can be induced by LPS, and the inhibition of JNK reduces the LPS-induced activity of COX-2, TNF-α, IL-6 and monocyte chemoattractant protein-1 [17,18].

Epimedium brevicornum Maxim (Berberidaceae) is an important traditional Chinese herbal medicine originally used as a tonic effect in ancient China. Icariin (ICA, $C_{33}H_{40}O_{15}$, MW: 676.65), with the chemical name (2-(4′-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone), is a major component isolated from *E. brevicornum*. As a highly interesting natural flavonoid compound for drug development, icariin has a broad spectrum of established pharmacological functions, including antioxidant effect [19], immunoregulatory effect [20], antidepressant-like effect [21], stimulation of angiogenesis [22] and induction of cardiomyocyte differentiation [23]. Recent studies have reported that icariin inhibits inflammatory response by decreasing the production of TNF-α, IL-6, NO and adhesion molecules (CD11b) both in macrophage cell line RAW264.7 and in mouse serum [24]. In addition, icariin also shows an anti-inflammatory effect on LPS-treated murine chondrocytes through

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inhibition of NO and MMP synthesis [25]. However, the effect of icariin on neuroinflammation in the CNS remains to be elucidated. As icariin is not only a small molecule, but also may pass through blood–brain barrier and improve the learning and memory of rats [26,27], the present study was thus undertaken to investigate the anti-neuroinflammatory activity of icariin on inflammagen LPS-activated microglia for exploring the potential therapeutic effect on bacterial infection-related neuroinflammatory diseases.

2. Materials and methods

2.1. Materials

Icariin (standard compound) was from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, China) (Fig. 1). 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharide (LPS from *Escherichia coli*, serotype 055:B5), 2′,7′-dichlorofluorescin diacetate (DCF-DA), ammonium pyrrolidine-dithiocarbamate (PDTC), SP600125, SB202190 and N-Acetyl-L-cysteine (NAC) were purchased from Sigma Chemical Co. (USA). Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), Neurobasal Medium and B-27 Supplements were obtained from Gibco (USA). Cocktail protease inhibitor was purchased from Roche (Germany). RIPA buffer, iNOS, COX-2, p-TAK1, TAK1, p-IκB, IκB, p-IKKα/β, IKKα/β, p-NF-κB (Ser276 and Ser468), NF-κB, p-ERK, ERK, p-JNK, JNK, p-p38, p38, β-actin, α-tubulin, HDAC1 polyclonal antibodies and HRP-conjugated anti-rabbit IgG were purchased from Cell Signaling Technology (CST, USA). Alexa Fluor 488-labeled goat anti-rabbit IgG antibody, Superscript II reverse transcriptase and Trizol were obtained from Invitrogen (USA). The ELISA kit for PGE-2 assay was obtained from R&D Systems (USA). ECL Advance Western Blotting Detection Reagents was obtained from GE Healthcare (UK).

2.2. Primary microglia-enriched culture

Primary microglial cultures were prepared from the cerebral cortices of 1 day-old Sprague–Dawley rat pups as described previously with minor modifications [28]. In brief, the brain was removed and the cortices were dissected out. After removing the blood vessels and meninges in cold D-Hank's buffer, the tissues were cut into 1 mm^3 fragments, and incubated in 0.2% trypsin for 20 min in 37 °C. DMEM containing 10% FBS was then added for trypsin inactivation, and the tissues were dissociated by mild mechanical trituration. The cell suspension was seeded into 100 mm culture dishes at a density of 5×10^6 cells per dish. Mixed glial cells were grown in a humidified incubator with 5% CO_2 at 37 °C. The medium was changed every third day. After *in vitro* culture for 10–12 days, culture dishes were shaken at 200 rpm for 1 h at 37 °C in an orbital shaker. The floating cells were then collected and replanted into a new culture dish. After 1 h, when most microglia were adhering to the substrates, the culture dish was gently shaken to remove loosely adhering oligodendrocytes. The prepared primary microglia were more than 98% pure, as determined with microglia-specific marker OX-42.

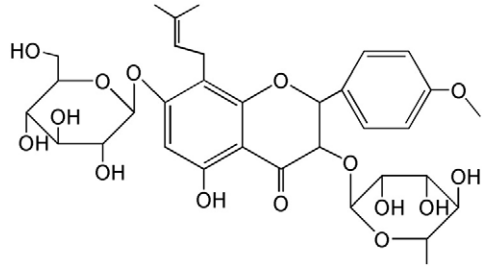

Fig. 1. The chemical structure of icariin (ICA).

2.3. Cell culture treatment

LPS was dissolved in PBS as a stock solution (1 mg/mL) and stored at −20 °C in the dark place. Before use, the stock solution was diluted to 1 μg/mL with culture medium. Primary microglial cultures were pretreated with different concentrations of icariin for 30 min at 37 °C, and then co-treated with LPS (1 μg/mL) at indicated time points.

2.4. MTT assay

Microglia were seeded into 96-well plates at a density of 1.5×10^4 cells per well. After incubation with different drugs for the indicated time points, the medium was removed and MTT (0.5 mg/mL in culture medium) was added and incubated for 4 h at 37 °C. The culture medium was then carefully removed, and the cells were dissolved in dimethyl sulfoxide for 5 min. Absorbance was measured at 550 nm using a microplate reader. Each experiment was performed in triplicate.

2.5. ROS detection

For intracellular ROS detection, cells were pretreated with icariin for 30 min, and subsequently co-treated with LPS (1 μg/mL) for 24 h. Then, cells were washed with PBS and incubated with 50 μM DCF-DA in the dark place for 30 min at 37 °C. After sufficient washing, intracellular levels of ROS were measured by mean fluorescence intensity with a flow cytometer (BD FACSCalibur, USA). Excitation was detected at 480 nm and emission was detected at 520 nm. A minimum of 20,000 events were counted per sample.

2.6. NO assay

The production of NO was determined by measuring the released nitrite (a stable oxidative end-product of NO) in the culture medium based on the Griess reaction [29]. Briefly, 100 μL of culture supernatants (phenol red-free) was then mixed with 100 μL of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in a 96-well plate. Absorbance was measured at 540 nm using a microplate reader. Sodium nitrite was used as a standard and each experiment was performed in triplicate.

2.7. PGE-2 assay

The production of prostaglandin E-2 (PGE-2) in supernatant was measured by a commercial PGE-2 assay kit according to the manufacturer's instructions.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

To investigate the mRNA expression of TNF-α, IL-1β and IL-6 by using RT-PCR, cells were pretreated with icariin for 30 min, and subsequently co-treated with LPS (1 μg/mL) for 6 h. Then total RNA was extracted from cultured microglia with Trizol and converted to cDNA by using a Superscript II reverse transcriptase, according to the manufacturer's instruction. The intended DNA bands were amplified with 0.5 μM of primer pairs and 1 μg of cDNA. The sequences of PCR primers used in the present study were as follows:

TNF-α,	5′-CCC-TCA-CAC-TCA-GAT-CAT-CTT-CTC-AA-3′ (sense), 5′-TCT-AAG-TAC-TTG-GGC-AGG-TTG-ACC-TC-3′ (antisense);
IL-1β,	5′-ATG-GCA-ACT-GTC-CCT-GAA-CTC-3′ (sense), 5′-GTC-GTT-GCT-TGT-CTC-TCC-TTG-3′ (antisense);
IL-6,	5′-GGA-GTT-CCG-TTT-CTA-CCT-GG-3′ (sense), 5′-GCC-GAG-TAG-ACC-TCA-TAG-TG-3′ (antisense);
GAPDH	5′-CCA-TCA-CCA-TCT-TCC-AGG-AG-3′ (sense), 5′-CCT-GCT-TCA-CCA-CCT-TCT-TG-3′ (antisense)