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Suppressive effects of flavonoid fisetin on lipopolysaccharide-induced microglial activation and neurotoxicity

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Abstract

Microglia are innate immune cells in the central nervous system. Activation of microglia plays an important role in the processes of several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and HIV dementia. Activated microglia can produce various proinflammatory cytokines and nitric oxide (NO), which may exert neurotoxic effects. Inhibition of microglia activation may alleviate neurodegeneration under these conditions. To search for the novel therapeutic agents against neuroinflammatory diseases, we have screened a series of flavonoid compounds using a cell-based assay. Our studies showed that fisetin markedly suppressed the production of tumor necrosis factor (TNF)-α, NO, and prostaglandin (PG) E_2 in lipopolysaccharide (LPS)-stimulated BV-2 microglia cells or primary microglia cultures. Fisetin also inhibited the gene expression of TNF-α, interleukin (IL)-1β, cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) at both mRNA and protein levels. Fisetin significantly suppressed IκB degradation, nuclear translocation of NF-κB, and phosphorylation of p38 mitogen-activated protein kinase (MAPKs) in the LPS-stimulated BV-2 microglia cells. In addition, fisetin reduced cytotoxicity of LPS-stimulated microglia toward B35 neuroblastoma cells in a co-culture system. These results indicate that fisetin has a strong anti-inflammatory activity in brain microglia, and could be a potential therapeutic agent for the treatment of neuroinflammatory diseases.

1. Introduction

Neuroinflammation is closely related with pathogenesis of several neurodegenerative diseases such as Alzheimer's diseases, Parkinson's diseases, multiple sclerosis, and HIV-associated dementia [1]. Microglia, as the primary immune effector cells in the central nerves system (CNS), are considered to be the

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major cell type responsible for inflammation-mediated neurotoxicity [2]. Like monocytes/macrophages, activated microglia can secrete proinflammatory cytokines and neurotoxic mediators after stimulation with lipopolysaccharide (LPS), interferon (IFN)-γ or β-amyloid *in vitro* [3,4]. The proinflammatory cytokines and neurotoxic mediators, which include tumor necrosis factor (TNF)-α, prostaglandin (PG) E_2, interleukin (IL)-1 and IL-6 and free radicals like nitric oxide (NO) and superoxide anion, are thought to contribute to neuronal injuries and progression of the neurodegenerative diseases [5,6]. Recent studies have indicated that microglia activation can be caused by various signaling molecules produced by damaged neuronal cells [7,8]. These findings suggest that neuroinflammation may be a consequence of neurodegeneration processes. However, over-activation of microglia also promotes ongoing neurodegeneration by releasing various proinflammatory factors [9–11]. Microglia activation is often observed in neuronal injuries and actively involved in the initiation and progression of several neurodegenerative diseases [1,9–11]. Thus, inhibition of microglia activation and neuroinflammation may be an effective therapeutic approach against neurodegenerative diseases.

Flavonoids are a group of small molecular weight polyphenolic compounds in plant kingdom, many of which have been found to exhibit a wide range of biological activities including anti-oxidant, anti-inflammatory and anti-tumor effects [12–14]. Fisetin (3, 3, 4, 7-tetrahydroxy flavone) is a naturally occurring flavonoid commonly found in strawberries and other fruits and vegetables. Studies have demonstrated that fisetin exhibits a wide variety of activities, including anti-cancer [15], anti-angiogenic [16], neuroprotective [17], neurotrophic [18] and anti-oxidant [19] effects. Recent studies have demonstrated that fisetin has anti-inflammatory property in macrophages and mast cells. Fisetin inhibited NO production and TNF-α secretion in RAW 264.7 macrophage and human peripheral blood mononuclear cells [20]. It was also reported that fisetin inhibited TNF-α, IL-1, and IL-4 production in human mast cells stimulated with phorbol-12-myristate 13-acetate (PMA) plus calcium ionophore A23187 [21]. In C6 glia cells, fisetin attenuated NO production induced by LPS/IFN-γ treatment [22].

Although the anti-inflammatory effects of fisetin in peripheral macrophages, mast cells, and astrocytes have been reported, its possible anti-inflammatory effect in brain microglia has not been investigated. Here, we determined the effect of the flavonoid fisetin on the inflammatory activation and neurotoxicity of BV-2 microglia cells. We found that fisetin inhibited the production of inflammatory mediators, and suppressed NF-κB and p38 mitogen-activated protein kinase (MAPK) activation in LPS-stimulated BV-2 microglia cells. In addition, the flavonoid showed a neuroprotective effect by attenuating microglial neurotoxicity in the microglia–neuron co-culture assay.

2. Materials and methods

2.1. Reagents and cell culture

Bacterial lipopolysaccharide (LPS) (*E. coli* serotype 055:B5) and flavonoid compounds of high purity (>98%) [galangin (Catalog number, 282200), 7-hydroxyflavone, flavanone (Catalog number, 102032), morin (Catalog number, M4008), morin hydrate (Catalog number, M87630), fisetin (Catalog number, F4043)] were purchased from Sigma-Aldrich (St. Louis, MO). These flavonoid compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml stock solution. All compounds used were completely dissolved in DMSO. The final concentration of DMSO in the culture media was less than 0.25%. BV-2 murine microglia cell line was grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), gentamicin (50 μg/ml) at 37 °C, 5% CO_2. B35 rat neuroblastoma and HAPI rat microglia cell line were grown and maintained in DMEM supplemented with 10% heat-inactivated FBS, penicillin (10 U/ml) and streptomycin (10 μg/ml) at 37 °C, 5% CO_2. Mouse primary microglial cultures were prepared by mild trypsinization as described previously with minor modifications [23]. Mixed glial cultures were prepared from cerebral cortices of 1-day-old Institute of Cancer Research (ICR) mice. The cortices were chopped and dissociated by mechanical disruption using a nylon mesh. The dissociated cells were seeded in poly-D-lysine-coated flasks at 2.5×10^5 cells/ml and cultured at 37 °C, 5% CO_2. Cultures were replaced every 4–5 days with DMEM with 10% FBS. After culture for 10–14 days, microglial cells were isolated from mixed glial cultures by mild trypsinization. Mixed glial cultures were incubated with a trypsin solution [0.25% trypsin, 1 mM EDTA in Hank's balanced salt solution (HBSS)] diluted 1:4 in phosphate-buffered saline containing 1 mM $CaCl_2$ for 30–60 min. This resulted in the detachment of an upper layer of astrocytes in one piece, whereas microglia remained attached to the bottom of the culture flask. The detachment typically striated in the periphery of the culture flask after approximately 15 min in trypsin solution. The detached layer of astrocytes was aspirated, and the remaining microglia were used for experiments. After isolation by trypsinization, adherent microglia cells were transferred into multi-well plates. The prepared primary microglial cultures were more than 95% pure, as determined by isolectin B4 staining (data not shown). It has been reported that microglia cells obtained by this method are morphologically and functionally equivalent to microglial cells isolated by shaking (in terms of proliferation, phagocytosis, NF-κB translocation, and nitric oxide production), and that trypsinization method provides a greater than fivefold increase in microglial yield when compared with the shaking method [23]. Animals used in the current research were acquired and cared for in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Cell viability test

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The BV-2 microglia cells were seeded in triplicate at the density of 5×10^4 cells/well on 96-well plate. BV-2 microglia cells or primary microglia cultures were treated with flavonoids and LPS for 24 h. MTT was added to each well, and the cells were incubated for 4 h at 37 °C. After culture media were discarded, DMSO was added to dissolve the formazan dye. The optical density was measured at 540 nm.

2.3. Nitrite quantification

NO secreted in microglial culture supernatants was measured by Griess reagent as described [24]. After BV-2 microglia cells or primary microglia cultures were treated with stimulating agents in 96-well plates, NO_2^- concentration in culture supernatants was measured to assess NO production in microglial cells. Fifty microliters of sample aliquots were mixed with 50 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in a 96-well plate and incubated at 25 °C for 10 min. The absorbance at 550 nm was measured on a microplate reader. $NaNO_2$ was used as the standard to calculate NO_2^- concentrations.

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

Total RNA was isolated using TRIzol reagent (Molecular Research Center Inc, Cincinnati, OH) according to the manufacturer's instruction. Reverse transcription was carried out using Moloney murine leukemia virus (M-MLV) and oligo (dT) primer. PCR amplification using primer sets specific for inducible nitric oxide synthase (iNOS), TNF-α, IL-1β,