Antiinflammatory Effects of Orientin-2′-O-Galactopyranoside on Lipopolysaccharide-Stimulated Microglia

Xiang Zhou,a,b Ping Gan,a,# Lili Hao,b Li Tao,a Jia Jia,a Bo Gao,a Jiang-yun Liu,b Long Tai Zheng,b,* and Xuechu Zhen*b,a

bDepartment of Pharmacology, College of Pharmaceutical Sciences, Soochow University; and aDepartment of Traditional Chinese Medicine, College of Pharmaceutical Sciences, Soochow University; Suzhou 215123, P. R. China.

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Microglia activation-mediated neuroinflammation plays an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, and human immunodeficiency virus (HIV)-associated dementia. Inhibition of microglia activation may alleviate neurodegeneration under neuroinflammatory conditions. In the present study, we compared three flavone C-glycosides extracted from Trollius chinensis Bunge using a cell-based assay to evaluate their antiinflammatory effects on microglial cells. The results showed that orientin-2′-O-galactopyranoside (OGA) significantly inhibited the production of nitric oxide and tumor necrosis factor (TNF-α) in lipopolysaccharide (LPS)-stimulated microglial cells. OGA also markedly inhibited the LPS-induced expression of TNF-α, interleukin-1β, inducible nitric oxide (NO) synthase, and cyclooxygenase-2, which was accompanied by suppression of the activation of nuclear factor (NF)-κB and the extracellular signal-regulated kinase (ERK) signal pathway. In addition, OGA decreased LPS-induced reactive oxygen species generation, which appears to be related to the activation of the NF-E2-related factor2 (NRF2)/heme oxygenase-1 (HO-1) pathway in BV-2 microglial cells. Furthermore, OGA reduced the cytotoxicity of activated microglia toward HT-22 neuroblastoma cells in a co-culture system. Taken together, the present study demonstrated that the induction of HO-1-mediated inhibition of the NF-κB and ERK pathways contributes significantly to the antineuroinflammatory and neuroprotective effects elicited by OGA.

Key words orientin-2′-O-galactopyranoside; microglia; lipopolysaccharide; inflammation; neuroprotection

Neuroinflammation is actively involved in the pathological process of neurodegenerative diseases such as Parkinson’s disease (PD), Alzheimer’s disease (AD) and multiple sclerosis (MS). Microglia, resident macrophages, participate in the innate immunity in the brain, and is also known as the major cell types responsible for inflammation-mediated neurotoxicity. Upon neuronal injury or inflammatory stimuli such as lipopolysaccharide (LPS), interferon (IFN)-γ or β-amyloid, microglia is over-activated and release various pro-inflammatory mediators and cytokines such as nitric oxide (NO), prostaglandin E2 (PGE2), reactive oxygen species (ROS), tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β). These pro-inflammatory mediators and cytokines may regulate neuronal survival and are subsequently involved in the processes of neurodegeneration. Therefore, inhibition of microglia activation may be an effective approach for the treatment of neurodegenerative diseases.

Trollius chinensis Bunge is a perennial herb that is widely distributed in northern China and Mongolia. Its flowers have been used to treat upper respiratory infections, pharyngitis, tonsillitis, and bronchitis as traditional Chinese medicine. These effects were suggested to associate with the flavonoids, a major chemical composition of Trollius chinensis Bunge. Trollius chinensis Bunge has a high content of orientin and vitexin which belong to the flavone C-glycoside class of flavonoids. Orientin and vitexin have a variety of biological activities such as antioxidant, antiviral, antibacterial, anticancer, antithrombus and against myocardial ischemic injuries. In addition, vitexin was also reported to prevent N-methyl-D-aspartate (NMDA) receptor mediated mouse cerebral cortical neurons cell death, indicating a potential role in neuroprotection. However, the anti-inflammatory activity of these flavone C-glycosides in microglia has not been investigated. Therefore, in the present study, we investigated the anti-inflammatory effects of orientin-2′-O-galactopyranoside (OGA) isolated from the fruits of Trollius chinensis Bunge in LPS-stimulated microglia and analyzed molecular mechanisms. Here, we reported that OGA exerts anti-inflammatory and neuroprotective effects, mainly by modulating heme oxygenase-1 (HO-1) expression mediated inhibition of nuclear factor (NF)-κB and extracellular signal-regulated kinase (ERK) signaling pathway.

MATERIALS AND METHODS

Sample Preparation The fruits of Trollius chinensis Bunge were collected from northern China. Three flavone C-glycosides, OGA, orientin and vitexin (Fig. 1) (>95%, usually require 87% or more) were isolated from the fruits of Trollius chinensis Bunge and identified by various spectroscopic analysis (including different one dimensional (1D) and 2D NMR spectroscopes, high-resolution electro spray ionization mass spectrometry) and chemical evidences as described previously. These flavone C-glycosides were dissolved in dimethyl sulfoxide (DMSO) at 100 mM stock solution. All compounds used were completely dissolved in DMSO. The final concentration of DMSO in the culture media was less than 0.5%. Chemical compounds studied in this article: Orientin (PubChem CID: 5280441); Vitexin (PubChem CID: 5281675); Orientin-2′-O-galactopyranoside (CAS: 1377947–82–4P).

Materials and Cell Culture Bacterial LPS (Escherichia coli, serotype 055:B5) were used as ligands in the following experiments. Human primary microglia were isolated from the frontal cortex of the human subject within 6 h after death and maintained in a monolayer culture with the growth medium RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), 200 units/mL penicillin, 200 μg/mL streptomycin, 1 mM L-glutamine and 1% non-essential amino acids (Gibco). All experiments were performed at least in triplicate; statistical analysis was performed by one-way analysis of variance (ANOVA). The authors declare no conflict of interest. *To whom correspondence should be addressed. e-mail: zhengxuechu@suda.edu.cn; zhenglongtai@suda.edu.cn © 2014 The Pharmaceutical Society of Japan
Review Board of Soochow University.

Laboratory Animals. The study was approved by Institutional National Institutes of Health Guide for the Care and Use of and cared in accordance with the guidelines published in the than 95% as determined by CD11b immune staining (data not

triplicate at the density of $2 \times 10^4$ cells/well. After incubation with compounds in presence or absence of LPS at 37°C for 24 h, 50 µL of culture medium were used to test the expression of TNF-α.

Quantitative Real-Time Polymerase Chain Reaction (PCR) and Reverse Transcription (RT)-PCR For mRNA quantification, total RNA was isolated using TRIzol reagent (TaKaRa Biotechnology Co., Ltd., China) and cDNA was synthesized from 1 µg of total RNA using Moloney murine leukemia virus (M-MLV) and oligo (dt) primer to reverse transcription according to the manufacturer’s instruction. cDNA was amplified using specific primers inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-1β or glyceraldehyde-3-phosphate dehydrogenase (GAPDH): iNOS forward, 5'-TAG GCA GAG ATT GGA GGC CTT G-3'; iNOS reverse, 5'-GGG TTG TTG CTG AAG TCG -3'; COX-2 forward, 5'-CAG GCT GAG GGA CAG AAA CTC CA-3'; COX-2 reverse, 5'-GCT CAC GAG GCC ACT GAT ACC TA-3'; TNF-α forward, 5'-CAG GAG GGA GAA CAG AAA CTC CA-3'; TNF-α reverse, 5'-CCT GGT TGG CTT GCT TGT CTT GCT T-3'; IL-1β forward, 5'-TCC AGG ATG AGC AGA TGA GCA C-3'; IL-1β reverse, 5'-GGA GTT CAC ACA CCA GCA GGT TA-3'; GAPDH forward, 5'-TGG TGC CGT CGT GGA TCT GA-3'; GAPDH reverse, 5'-TTG CTG TTG AAG TCG CAG GAG-3'. Quantitative real-time PCR was performed using SYBR Premix II on CFX96 PCR instrument (BIORAD, U.S.A.). The values obtained for target gene expression were normalized to GAPDH and quantified relative to expression in control samples. For RT-PCR, cDNA was amplified using specific primers HO-1 and GAPDH: HO-1 forward, 5'-AGA GTT TCC GCC TCC AAC CA-3'; HO-1 reverse, 5'-CGG GAC TGG TCT AGT TCA GG-3'; GAPDH forward, 5'-ACC ACA GTC CAT GCC ATC AC-3'; GAPDH reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3'. GAPDH was used as an internal control to evaluate relative expression of HO-1.

Immunofluorescence Assay The intercellular location of p65 subunit of NF-κB was measured as described. 20) BV-2 cells were seeded on sterile cover glass in 24-well plates at the density of $2 \times 10^4$ cells per well, and then treated with compound and LPS. After treatment with LPS for 1 h, the medium was removed and cells were fixed using −20°C methanol for 20 min and washed three times with PBS for 5 min. The fixed cells were treated with 0.5% Triton X-100/1.5% albumin from

mouse TNF-α antibody as capture antibody and goat biotinylated polyclonal anti-mouse TNF-α antibody as detection antibody (ELISA development reagents; R&D Systems, Minneapolis, MN, U.S.A.). The biotinylated anti-TNF-α antibody was detected by sequential incubation with streptavidin-horseradish peroxidase conjugate and chromogenic substrates. BV-2 cells were seeded in 96-well plates at the density of $2 \times 10^4$ cells/well. After incubation with compounds in presence or absence of LPS at 37°C for 24 h, 50 µL of culture medium were used to test the expression of TNF-α.

Nitrite Quantification NO secreted in microglia culture supernatants was measured by Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine hydrochloride/2% phosphoric acid). 18) Fifty microliter of culture medium was mixed with 50 µL of Griess reagent in a new 96-well plate and absorbance was measured at 540 nm using a microplate absorbance reader (Multiskan MK3, Thermo Scientific, U.S.A.). The values obtained for target gene expression were normalized to GAPDH and quantified relative to expression in control samples. For RT-PCR, cDNA was amplified using specific primers HO-1 and GAPDH: HO-1 forward, 5'-AGA GTT TCC GCC TCC AAC CA-3'; HO-1 reverse, 5'-CGG GAC TGG TCT AGT TCA GG-3'; GAPDH forward, 5'-ACC ACA GTC CAT GCC ATC AC-3'; GAPDH reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3'. GAPDH was used as an internal control to evaluate relative expression of HO-1.
bovine serum for 1 h at room temperature. Cells were treated with a 1:100 dilution of mouse monoclonal anti-human NF-κB p65 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) over night at 4°C. Followed the washes with 0.05% Tween-20/1.5% bovine serum albumin in PBS for 5 min, cells were treated with Alexa Fluor 488-labeled goat anti-mouse immunoglobulin G (IgG) antibody (Invitrogen) for 1 h at room temperature, and washed three times with 0.05% Tween-20 in PBS for 5 min. After washes, cells were stained with 0.5 µg/mL of Hoechst staining solution for 30 min at 37°C and then washed. Finally, the cover glass with cells were dried in room temperature for 30 min and mounted in a 1:1 mixture of xylene and malinol. The number of cells with p65 nuclear translocation was determined under a fluorescence microscope.

**Western Blot Analysis**  For the detection of the protein expression, cells were lysed in triple-detergent lysis buffer [50 mM Tris–HCl, PH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany)]. Protein concentration was determined using BCA protein assay kit (TianGen, Beijing, China) according to the manufacturer’s instructions with bovine serum albumin as standard. Protein samples (40 µg) were loaded and electrophoresed on sodium dodecyl sulfate-polyacrylamide (10% gel) and transferred to immobile polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.5% Tween-20 (TBST) for 1 h at room temperature. Then the membranes were incubated separately with primary antibodies for iNOS (1:1000), COX-2 (1:1000),...
IxE-α (1:500), p-IxE-α (1:200) phosphor-ERK (1:1000), phosphor-JNK (1:1000), p-P38 (1:1000), ERK (1:1000), JNK (1:1000), P38 (1:1000), HO-1 (1:500), NF-E2-related factor2 (NRF2) (1:1000), Lamin B (1:1000) and GAPDH (1:10000) were used as loading controls. All band intensities were quantified by the densitometer in the LAS-4000 Image Analyzer (NEB, USA) and analyzed by one-way ANOVA followed by Turkey’s post hoc test using SPSS program (version 16.0).

RESULTS

OGA Inhibits LPS-Induced NO and TNF-α Production in Microglia Cells

We first measured whether these flavone C-glycosides compounds inhibited the production of NO and TNF-α in LPS-stimulated microglia cells. BV-2 cells were treated with flavone C-glycosides (10–40 µM) for 30 min prior to LPS treatment. After stimulation of LPS for 24h, NO and TNF-α production were determined in culture medium. The results showed that OGA, orientin and vitexin significantly decreased the LPS-induced NO and TNF-α production in a dose-dependent manner (Figs. 2A, C). In order to exclude possibility that the decrease in NO or TNF-α production was due to the cytotoxicity of flavone C-glycosides compounds, cell viability was determined by MTT assay. The result showed that the three compounds at the indicated concentrations (10–40 µM) did not alter the cell viability (Fig. 2B). The order of potency on suppression of NO production was arranged as follows based on the IC50: OGA (15.03±1.54 µM)<orientin (16.01±1.67 µM)<vitexin (17.73±2.81 µM), similar for TNF-α production: OGA (27.13±3.41 µM)<orientin (34.50±8.15 µM)<vitexin (41.31±3.63 µM). Further studies were focused on OGA because its anti-inflammatory effects was the strongest and has not been investigated. We also found that OGA dose-dependently inhibited the NO production in LPS-stimulated primary microglia cultures or HAPI rat microglia cells (Fig. 3).

OGA Inhibits the Expression of iNOS, TNF-α, COX-2 and IL-1β

The effects of OGA on the gene expression of pro-inflammatory cytokines such as iNOS, COX-2, TNF-α and IL-1β in activated BV-2 cells were determined by quantitative real time PCR. As shown in Fig. 4, OGA significantly inhibited the LPS-induced gene expression of iNOS, COX-2, TNF-α and IL-1β in a dose-dependent manner. We further confirmed that the inhibitory effects of OGA on iNOS and COX-2 expression at protein levels by Western blot analysis.
OGA Attenuated LPS-Induced NF-κB and ERK Activation

NF-κB play important role in the regulation of various proinflammatory gene expressions such as iNOS, COX-2, IL-1β and TNF-α in activated microglia cells. Thus, we determined whether OGA affected NF-κB and mitogen-activated protein kinases (MAPKs) activation in LPS-stimulated BV-2 microglia cells by immunofluorescence and Western blot. LPS induced the translocation of p65 subunit of NF-κB from cytoplasm into nucleus was detected at 60 min after stimulation. OGA inhibited LPS-induced nuclear translocation of p65 into nucleus in BV-2 microglia cells (Figs. 5A, B). The compounds alone did not affect the nuclear translocation of p65 (data not shown). This is further confirmed by the result of Western blot analysis which indicated that OGA inhibited LPS-induced nuclear accumulation of p65 in BV-2 microglia cells (Fig. 5C). OGA also attenuated LPS-induced IκB-α degradation and increase of IκB-α phosphorylation in a dose-dependent manner (Fig. 5E). To confirm that OGA suppressed LPS-induced gene expression via NF-κB pathway, we next examined effect of OGA on LPS-induced NF-κB luciferase activity. As shown in Fig. 5D, OGA significantly inhibited LPS induced NF-kB luciferase activity in BV-2 microglia cells. We next investigated the effect of OGA on LPS-induced activation of MAPKs (ERK, JNK, p38 MAPK). Activation of MAPKs (ERK, JNK, p38 MAPK) was detected after 20 min stimulation with LPS (200 ng/mL) in BV-2 microglia cells. OGA significantly inhibited LPS-induced phosphorylation of ERK but not the phosphorylation of JNK and p38 MAPK (Fig. 6). Taken together, these results indicated that inhibitory effects of OGA on production of pro-inflammatory mediators and cytokines appeared to be mediated by inhibiting NF-κB and ERK signaling pathway in LPS-stimulated BV-2 microglia cells.

The Relationship between the NF-κB Activation and ERK Pathway in LPS-Stimulated BV-2 Microglia Cells

To determine the relationship between the NF-κB activation and ERK pathway, the BV-2 microglia cells were treated with OGA (10–40 µM) for 30 min, and then treated with LPS (200 ng/mL). After 6 h of LPS treatment, the iNOS (A), COX-2 (B), TNF-α (C) and IL-1β (D) mRNA levels were determined by SYBR green quantitative-RT-PCR. (E) After 16 h of LPS stimulation, the cell lysates (40 µg) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-iNOS or COX-2 antibody (E, left). The α-tubulin was used as an internal control. Quantification of iNOS and COX-2 protein expression was performed by densitometric analysis (E, right). The values were expressed as a percentage of maximal band intensity in the culture treated with LPS alone, which was set to 100% (lane 3). The data were expressed as the mean ± S.D. (n=3), and are representative of three independent experiments. *p<0.05; **p<0.01 as compared with LPS alone treatment.
Fig. 5. Blockade of NF-κB Activation by OGA in BV-2 Microglia Cells

BV-2 microglia cells were pretreated with OGA for 30 min, and then stimulated with LPS (200 ng/mL). After 1 h of LPS treatment, subcellular location of NF-κB p65 subunit was determined by immunofluorescence assay. (A) The NF-κB p65 was detected using anti-NF-κB p65 antibody conjugated with fluorescein isothiocyanate (FITC), and nuclei were visualized by Hoechst staining. Representative images of cells are shown (higher magnification in inset), Scale bar=40 µm. (B) The number of cells with p65 nuclear translocation was determined and the percentage of cells with p65 translocation was calculated. (C) BV-2 microglia cells were pretreated with 40 µM OGA for 30 min, and then stimulated with LPS (200 ng/mL). Nuclear and cytoplasmic protein obtained from 1 h after LPS stimulation were subjected to Western blot to assess the levels of NF-κB p65 in nuclear or cytoplasm (left). Lamin B (nuclear) and α-tubulin (cytoplasm) were used as an internal control. Quantification of p65 protein expression was performed by densitometric analysis (right). (D) BV-2 cells stably expressing an NF-κB reporter were pretreatment with OGA (40 µM) for 30 min, followed by LPS treatment (0.2 µg/mL) for 16 h. Luciferase activity was measured by luminometry. The NF-κB activity is expressed as relative values and the values of control are set to a relative value of 1. (E) BV-2 microglia cells were pretreated with 40 µM OGA for 30 min, and then stimulated with LPS (200 ng/mL) for indicated time, the total cell lysates were subject to Western blot to assess the level of IκB-α (20 min) and phospho-IκB-α (10 min) (left). α-Tubulin was used as an internal control. Quantification of IκB-α and phospho-IκB-α expression was performed by densitometric analysis (right). The data were expressed as the mean±S.D. (n=3), and are representative of three independent experiments. *p<0.05; **p<0.01 as compared with LPS alone treatment.
Fig. 6. Effects of OGA on LPS-Induced MAPKs Activity in BV-2 Microglia Cells

BV-2 microglia cells were seeded at the density of $2.0 \times 10^5$ cells/well in 6-well plates and pretreated with OGA for 30 min, and then stimulated with LPS (200 ng/mL) for 20 min. The total cell lysates were subject to Western blot to assess the levels of phospho-MAPKs (left). Quantification of expression was performed by densitometric analysis (right). Total MAPKs was used as an internal control. The data were expressed as the mean±S.D. ($n=3$), and are representative of three independent experiments. *$p<0.05$; **$p<0.01$ as compared with LPS alone treatment.

Fig. 7. Regulation of NF-κB and ERK in LPS-Stimulated BV-2 Microglia Cells

(A) BV-2 microglia cells were pretreated with U0126 (10 μM) for 30 min, and then stimulated with LPS (200 ng/mL). Nuclear and cytoplasmic protein obtained from 1 h after LPS stimulation were subjected to Western blot to assess the levels of NF-κB p65 in nuclear or cytoplasm (left). Lamin B (nuclear) and α-tubulin (cytoplasm) were used as an internal control. Quantification of p65 protein expression was performed by densitometric analysis (right). (B) BV-2 microglia cells were pretreated with PDTC (20 μM) for 30 min, and then stimulated with LPS (200 ng/mL). Western blot to assess the levels of phospho-ERK. Quantification of expression was performed by densitometry analysis (right). Total ERK was used as an internal control. The data were expressed as the mean±S.D. ($n=3$), and are representative of results obtained from three independent experiments. **$p<0.01$ as compared with LPS alone treatment.
with either U0126 (ERK-specific inhibitor) or PDTC (NF-κB inhibitor) before LPS stimulation. Then the NF-κB or ERK activation were examined by Western blot. As shown in Fig. 7, U0126 had no significant effect on LPS-induced NF-κB activation. However, PDTC inhibited the LPS-induced phosphorylation of ERK. These results suggest that ERK is not upstream molecule of NF-κB activation in LPS-stimulated BV-2 cells.

OGA Inhibits LPS-Induced Intracellular ROS Generation

There is growing evidence that intracellular ROS is produced by LPS stimulated microglia cells and is known to play important role in inflammatory signaling pathway. Thus, the effect of OGA on LPS induced intracellular ROS generation was determined using ROS sensitive indicator H2DCF-DA. As shown in Fig. 8A, OGA markedly decreased the generation of ROS in LPS-stimulated BV-2 microglia cells. Since ROS production was mediated by NADPH oxidase in LPS stimulated microglia cells, the effect of OGA on expression of NADPH oxidase were examined using RT-PCR. We found that OGA did not affect expression gp91phox, p22phox and p47phox of NADPH in LPS-stimulated BV-2 cells (data not shown). It is well known that intracellular ROS production was also regulated by HO-1 which is an inducible rate-limiting enzyme and exert certain anti-inflammatory and anti-oxidant properties under inflammatory conditions. Therefore, we next examined the effect of OGA on expression of HO-1. The result showed that OGA significantly induced HO-1 expression in a dose-dependent manner at both mRNA and protein levels (Fig. 8B). Since the expression of HO-1 is regulated by (NRF2) in microglia cells, we next examined the effect of OGA on NRF2 activation in microglia cells. The result revealed that OGA induced translocation of NRF2 from cytoplasm into nucleus (Fig. 8C). To test whether inhibition of NF-κB or ERK are involved in OGA induced HO-1 expression, the specific inhibitors are used for determining the HO-1 expression. As shown in Fig. 8D, neither U0126 nor PDTC had any significant effect on the HO-1 expression in BV2 cells.

HO-1 Mediates the Suppressive Effect of OGA on NO Production in LPS-Stimulated BV-2 Microglia Cells

Fig. 8. Effects of OGA on LPS-Induced Intracellular ROS Generation and Expression of HO-1 in BV-2 Cells

BV-2 cells were pretreatment with OGA for 30min, then stimulated with LPS (200ng/mL). (A) After 6h of LPS stimulation, cells were incubated with 30nm DCF-DA for 30min. Fluorescence was measured by flow cytometry. The results were expressed as the mean fluorescence intensity. (B) The total RNA was isolated at 6h after OGA treatment. The mRNA expression of HO-1 was determined by RT-PCR. Quantification of HO-1 mRNA was performed by densitometric analysis. GAPDH was used as an internal control. The total cell lysates obtained 24h after OGA treatment was subject to Western blot to assess the levels of HO-1 expression (upper). α-Tubulin was used as an internal control. Quantification of expression was performed by densitometric analysis (lower). (C) Nuclear and cytoplasmic protein were subjected to Western blot to assess the levels of NRF2 in nuclear or cytoplasm (upper). Lamin B (nuclear) and α-tubulin (cytoplasm) were used as an internal control. Quantification of expression was performed by densitometric analysis (lower). (D) The total cell lysates obtained 24h after OGA or U0126, PDTC treatment was subject to Western blot to assess the levels of HO-1 expression (upper). α-Tubulin was used as an internal control. Quantification of expression was performed by densitometric analysis (lower). The data were expressed as the mean±S.D. (n=3), and are representative of results obtained from three independent experiments. *p<0.05; **p<0.01 as compared with LPS alone treatment.
OGA (40 µM)  
LPS (200 ng/mL)  
ZnPP (2 µM)

OGA-pretreatment significantly improved the cell viability (Fig. 11).

**DISCUSSION**

In the present study, we compared the effects of three flavone C-glycosides on the NO production in LPS-stimulated microglia cells, found that that OGA is the most potent inhibitor on LPS-induced NO production among three compounds tested. OGA attenuated LPS-induced the production of proinflammatory mediators in microglia cells. Inhibition of NF-κB and ERK signaling pathways appeared to be involved in the anti-inflammatory mechanisms of OGA in BV-2 microglia cells. Moreover, OGA showed neuroprotective effects by attenuating microglial neurotoxicity in a microglia/neuron coculture system.

OGA Inhibited Microglial Neurotoxicity in Microglia/Neuron Co-culture Model As various neuron-inflammatory mediators released by activated microglial cells could lead to neuronal cell death and contribute to progression of neuronal degeneration, inhibition of microglia activation may be neuroprotective. In order to investigate whether OGA has a neuroprotective effect in vitro, a microglia/neuron coculture system was employed. The neuronal toxicity of conditioned media (CM) from LPS-stimulated BV-2 microglia cells cultured in absence or presence of OGA was tested in HT-22 hippocampal cells. However, treatment of HT22 cells with CM collected from LPS-stimulated BV-2 microglia cells with OGA-pretreatment significantly improved the cell viability.

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Nobiletin, another flavone C-glycosides compound, markedly inhibited microglia activation by suppressing activation of NF-κB and phosphorylation of MAPKs in LPS-stimulated microglia cells. In the present study, we found that OGA inhibited IκB-α phosphorylation, degradation and nuclear translocation of p65 in BV-2 microglia cells. Additionally, it is well-known that ERK is closely associated with the induction of proinflammatory gene expression in glia cells or macrophages. We found that OGA inhibited LPS-induced activation of ERK in BV-2 cells, suggesting that inhibition of NF-κB and ERK pathways might be involved in the anti-inflammatory mechanisms of OGA. The relation between NF-κB and ERK pathways are not consistent. In the previous study, it was reported that NF-κB is a downstream signal molecule of ERK in peripheral cells. Other studies demonstrated that NF-κB and ERK is two independent signal pathways. In the present study, we demonstrated that ERK specific inhibitor did not inhibited NF-κB activation indicating that NF-κB is not downstream molecule of ERK. We also found that NF-κB specific inhibitor suppressed ERK activation indicating that ERK appeared to be a downstream molecule of NF-κB. However, we did not exclude possibility that inhibition of ERK activation was due to inhibition of TNF-α production by NF-κB inhibitor with an autocrine/paracrine fashion. Thus, relation between NF-κB and ERK in the regulation of gene expression is likely dependant on cell type and nature of stimulus.

Activated microglia cells consistently produce ROS which actively involved in neuronal degeneration. It was reported that intracellular ROS can modulates the expression of proinflammatory genes.
flamatory genes via activation of diverse downstream signaling molecules including NF-κB, MAPK and protein kinase C. In the present study, we also demonstrated that ROS inhibitor suppressed activation of NF-κB and ERK and subsequent NO production in activated microglia cells, suggesting that inhibition of ROS/NF-κB and ROS/ERK pathways are, at least in partly involved in anti-inflammatory mechanisms of OGA.

HO-1 is an inducible rate-limiting enzyme which facilitates the degradation of heme into carbon monoxide (CO), biliverdin and free iron. The final products of heme catabolism exert certain antioxidative effects by neutralizing intracellular ROS. There is growing evidence that induction of HO-1 expression can inhibits pro-inflammatory gene expression in activated microglia cells via negativa regulation of NF-κB and MAPK signaling pathway. Previous studies have reported that HO-1 inhibitor blocked anti-inflammatory effect of compounds on LPS-stimulated macrophages cells. In the present study, we found that OGA induced HO-1 expression in parallel to suppression of activation of NF-κB and ERK pathway and HO-1 inhibitor markedly reversed anti-inflammatory property of OGA in microglia cells in microglia cells, suggesting that induction of HO-1 expression contributed to anti-inflammatory activity of OGA. HO-1 expression is induced by diversity of stimuli in macrophage/monocyte, suggesting that molecular mechanism of HO-1 expression are rather complex. Several studies have reported that the expression of HO-1 is controlled by NRF2-ARE signaling pathways. We found that OGA significantly induced NRF2 activation in microglia cells suggesting that OGA induced HO-1 expression was mediated by NRF2 activation. It was reported that MAPK, ROS, PKC and NF-κB are involved in the regulation of HO-1 expression under oxidative stress. However, in the present study, OGA alone did not activate MAPK (p38, JNK, ERK), indicating that activation of MAPKs are not involved at least in OGA induced HO-1 expression in microglia cells. The contradictory results of the regulatory role of MAPK pathways on HO-1 expression were observed under different conditions. It was demonstrated that JNK specific inhibitor, SP600125 induced HO-1 expression in BV-2 microglia cells and which was related with its anti-inflammatory effects. PDTC, a NF-κB inhibitor, can induce HO-1 expression in rat aortic vascular smooth muscle (aVSM) cells. In the present study, both ERK inhibitor and NF-κB inhibitor did not induced HO-1 expression in microglia cells, suggesting that these two pathways are not involved in HO-1 expression at least in our experimental condition. Further studies are required to understand the detailed mechanism of HO-1 induction by OGA in microglia cells. Taken together, at least two pathways are possibly involved in the anti-inflammatory activity of OGA: 1) HO-1 induction mediated inhibition of NF-κB and ERK activation; 2) HO-1 induction mediated inhibition of ROS production and subsequent inhibition of NF-κB and ERK activation. Clearly, further studies are necessary to elucidate the precise molecular mechanisms underlying the anti-inflammatory effect of OGA.

Excessive activation of microglia contributes to progression of neurodegenerative diseases through releasing neurotoxic molecules including free radicals and proinflammatory cytokines. Thus, inhibiting microglial activation may be effective therapeutic approach for neurodegenerative diseases. In the present study, we demonstrated that OGA protected neuronal cells against microglial neurotoxicity. Although the co-culture of the LPS-stimulated microglia with neuroblastaoma cell line may not reflect neurodegenerative conditions, it partially mimics the pathological condition where activated microglia affects the survival of neuronal cells in neurodegenerative diseases. Dietary flavonoid glucosides are hydrolyzed and absorbed in the small intestine, where they are rapidly metabolized to form methylated, glucuronidated or sulfated metabolites. In general, the bioavailability of orally administered flavonoids is relatively low due to limited absorption and rapid elimination. Recently, several studies have demonstrated that some flavonoids and their physiologically relevant metabolites can permeate the blood–brain barrier (BBB) and afford neuroprotection in a wide array of cellular and animal models of neurological diseases. However, there is little information on the bioavailability and metabolisms of OGA in brain tissue. Recent studies suggested that the function of BBB is altered in neurodegenerative diseases and BBB breakdown plays a critical role both in causing and progression of diseases. Although bioavailability of OGA in normal physiological condition is unknown, the BBB permeability of compound might be enhanced in neurodegenerative conditions. The capacity of OGA to achieve effective concentration in the brain of neurodegenerative conditions is also the subject of further studies. Nevertheless, this is the first study for the anti-inflammatory effects of OGA in microglia cells. Further studies are required to evaluate a bioavailability and neuroprotective effect of OGA in the animal models of neuroinflammatory diseases.

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REFERENCES

1) McGeer PL, McGeer EG. Inflammation and the degenerative diseases of aging. Ann. N. Y. Acad. Sci., 1035, 104–116 (2004).
2) Skaper SD, Giusti P, Facetti L. Microglia and mast cells: two tracks on the road to neuroinflammation. FASEB J., 26, 3103–3117 (2012).
3) Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat. Rev. Neurosci., 8, 57–69 (2007).
4) Park SY, Jin ML, Kim YH, Kim Y, Lee SJ. Anti-inflammatory effects of aromatic-turmerone through blocking of NF-kappaB, JNK, and p38 MAPK signaling pathways in amyloid beta-stimulated microglia. Int. Immunopharmacol., 14, 13–20 (2012).
5) Santos RM, Lourenco CF, Ledo A, Barbosa RM, Laranjinha J. Nitric oxide inactivation mechanisms in the brain: role in bioenergetics and neurodegeneration. Int. J. Cell Biol., 2012, 1–13 (2012).
6) Montgomery SL, Bowers WJ. Tumor necrosis factor-alpha and the roles it plays in homeostatic and degenerative processes within the central nervous system. J. Neuroimmune Pharmacol., 7, 42–59 (2012).
7) Oka A, Takashima S. Induction of cyclo-oxygenase 2 in brains of patients with Down’s syndrome and dementia of Alzheimer type.
specific localization in affected neurons and axons. Neuronreport, 8, 1161–1164 (1997).
8) Thameen Al-M, Kaur C, Ling E-A. Microglial activation and its implications in the brain disease. Curr. Med. Chem., 14, 1189–1197 (2010).
9) Cai SQ, Wang R, Yang X, Shang H, Ma C, Shoyama Y. Anti-inflammatory flavonoid-type C-glycosides from the flowers of Trollius chinensis. Chem. Biodivers., 3, 830–840 (2009).
10) Wang RY, Yang XW, Ma CM, Liu HY, Shang MY, Zhang QY, Cai SQ, Park JH. Trolloside, a new compound from the flowers of Trollius chinensis. J. Asian Nat. Prod. Res., 6, 819–824 (2004).
11) Liu SJ, Li SY, Feng JY, Sun Y, Cai JN, Sun XF, Yang SL. Flavone C-glycosides from the flowers of Trollius chinensis and their anti-complementary activity. J. Asian Nat. Prod. Res., 15, 325–331 (2013).
12) Perverse S, El-Shafeae AM, Al-Taweel A, Fawzy GA, Malak A, Atza N, Latif M, Iqbal L. Antioxidant and urease inhibitory C-glycosylflavonoids from Celia africana. J. Asian Nat. Prod. Res., 13, 799–804 (2011).
13) Becker H, Scher JM, Speaker JB, Zapp J. Bioactivity guided isolation of antimicrobial compounds from Lythrum salicaria. Fitoterapia, 76, 580–584 (2005).
14) Lee CY, Chen YS, Chiu TH, Huang GL, Li CC, Chiang JH, Yang JS. Apoptosis triggered by vitexin in U937 human leukemia cells via a mitochondrial signaling pathway. Oncol. Rep., 28, 1883–1888 (2012).
15) Fu XC, Wang X, Zheng H, Ma LP. Protective effects of orientin on myocardial ischemia and hypoxia in animal models. Nan Fang Yi Ke Xue Xue Bao, 27, 1173–1175 (2007).
16) Yang L, Yang ZM, Zhang N, Tian Z, Liu SB, Zhao MG. Neuroprotective effects of vitexin by inhibition of NMDA receptors in primary cultures of mouse cerebral cortical neurons. Mol. Cell. Biochem., 386, 251–258 (2014).
17) Hassan NF, Rifat S, Campbell DE, McCawley LJ, Douglas SD. Isolation and flow cytometric characterization of newborn mouse brain-derived microglia maintained in vitro. J. Leukoc. Biol., 50, 86–92 (1991).
18) Lee S, Suk K. Heme oxygenase-1 mediates cytoprotective effects of immunostimulation in microglia. Biochem. Pharmacol., 74, 723–729 (2007).
19) Hwang J, Zheng LT, Ock J, Lee MG, Suk K. Anti-inflammatory effects of m-chlorophenylpirazone in brain glia cells. Int. Immunopharmacol., 8, 1686–1694 (2008).
20) Zheng LT, Ryu GM, Kwon BM, Lee WH, Suk K. Anti-inflammatory effects of catechins in lipopolysaccharide-stimulated microglia cells: inhibition of microglial neurotoxicity. Eur. J. Pharmacol., 588, 106–113 (2008).
21) Jung WK, Ahn YW, Lee SH, Choi YH, Kim SK, Yea SS, Choi J, Park SJ, Soo SK, Lee SW, Choi JW. Eclipta alba ethanolic extracts inhibit lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in BV2 microglia via the MAP kinase and NF-kappaB pathways. Food Chem. Toxicol., 47, 401–407 (2009).
22) Kaltischmidt B, Kaltischmidt C. NF-kappaB in the nervous system. Cold Spring Harb. Perspect. Biol., 1, a001271 (2009).
23) Lee TY, Lee KC, Chen SY, Chang HH. 6-Gingerol inhibits ROS and iNOS through the suppression of PKC-alpha and NF-kappaB pathways in lipopolysaccharide-stimulated mouse macrophages. Biochem. Biophys. Res. Commun., 382, 134–139 (2009).
24) Stoll G, Jander S. The role of microglia and macrophages in the pathophysiology of the CNS. Prog. Neurobiol., 58, 233–247 (1999).
25) Streit WJ, Walter SA, Pennell NA. Reactive microgliosis. Prog. Neurobiol., 57, 563–581 (1999).
26) McGeer EG, McGeer PL. The role of anti-inflammatory agents in Parkinson’s disease. CNS Drugs, 21, 789–797 (2007).
27) Schroter ML, Muller S, Lindena J, Wiesner B, Hanisch UK, Wolf G, Blasig IE. Astrocytes induce manganese superoxide dismutase in brain capillary endothelial cells. Neuronreport, 12, 2513–2517 (2001).
28) Rubio-Perez JM, Morillas-Ruiz JM. A review: inflammatory process in Alzheimer’s disease, role of cytokines. The Scientific World Journal, 2012, 1–15 (2012).
29) Hoarau J-J, Krebich-Trott P, Jaffar-Bandjee M-C, Das T, Thon-Hon G-V, Kumar S, Neal JW, Gasque P. Activation and control of CNS innate immune responses in health and diseases: a balancing act finely tuned by neuroimmune regulators (NIReg). CNS Neurol. Drug. Targets, 10, 25–43 (2011).
30) Li YL, Ma SC, Yang YT, Ye SM, But PP. Antiviral activities of flavonoids and organic acid from Trollius chinensis Bunge. J. Ethnopharmacol., 79, 365–368 (2002).
31) Matsuda H, Morikawa T, Ando S, Tokuchida I, Yoshihaka M. Structural requirements of flavonoids for nitric oxide production inhibitory activity and mechanism of action. Bioorg. Med. Chem., 11, 1995–2000 (2003).
32) Gonzalez-Ramos R, Defere S, Devoto L. Nuclear factor-kappaB: a main regulator of inflammation and cell survival in endometriosis pathophysiology. Fertil. Steril., 98, 520–528 (2012).
33) Iwai K. Diverse ubiquitin signaling in NF-kappaB activation. Trends Cell Biol., 22, 355–364 (2012).
34) Kameyama N, Ben-Neriah Y. Regulation of NF-kappaB by ubiquitination and degradation of the I kappaBAs. Immunol. Rev., 246, 77–94 (2012).
35) Han S, Lee JH, Kim C, Nam D, Chung WS, Lee SG, Ahn KS, Cho SK, Cho M, Ahn KS. Capillarisin inhibits iNOS, COX-2 expression, and proinflammatory cytokines in LPS-induced RAW 264.7 macrophages via the suppression of ERK, JNK, and NF-kappaB activation. Immunopharmacol. Immunotoxicol., 35, 34–42 (2013).
36) Xie C, Kang J, Li Z, Schauss AG, Badger TM, Nagarajan S, Wu T, Wu X. The acai flavonoid velutin is a potent anti-inflammatory agent: blockade of LPS-mediated TNF-alpha and IL-6 production through inhibiting NF-kappaB activation and MAPK pathway. J. Nutr. Biochem., 23, 1184–1191 (2012).
37) Cui Y, Wu J, Jung S-C, Park D-B, Maeng Y-H, Hong JY, Kim S-J, Lee S-R, Kim S-J, Eun S-Y. Anti-neuroinflammatory activity of nobiletin on suppression of microglial activation. Bio. Pharm. Bull., 33, 1814–1821 (2010).
38) Koistinaho M, Koistinaho J. Role of p8 and p44/p42 mitogen-activated protein kinases in microglia. Glia, 40, 175–183 (2002).
39) Bhat NR, Zhang P, Lee J, Hogan E. Extracellular signal-regulated kinase and p8 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. J. Neurosci., 18, 1633–1641 (1998).
40) Kan H, Xie Z, Finkel MS. TNF-alpha enhances cardiac myocyte NO production through MAP kinase-mediated NF-kappaB activation. Am. J. Physiol., 277, H1641–H1646 (1999).
41) Jiang B, Brecher P, Cohen RA. Persistent activation of nuclear factor-kappaB by interleukin-1beta and subsequent inducible NO synthase expression requires extracellular signal-regulated kinase. Arterioscler. Thromb. Vasc. Biol., 21, 1915–1920 (2001).
42) Dei M, Shichiri M, Katsuyama K, Marumo F, Hirata Y. Cytokine-activated p42/p44 MAP kinase is involved in inducible nitric oxide synthase gene expression independent from NF-kappaB activation in vascular smooth muscle cells. Hypertens. Res., 23, 659–667 (2000).
43) Nishanth RP, Jyotsna RG, Schlager JJ, Hussain SM, Reddanna P. Inflammatory responses of RAW 264.7 macrophages upon exposure to nanoparticles: role of ROS-NFkappaB signaling pathway. Nanotoxicology, 5, 502–516 (2011).
44) Kim SU, Park YH, Min JS, Sun HN, Han YH, Hua JM, Lee TH, Lee SR, Chang KT, Kang SW, Kim JM, Yu DY, Lee SH, Lee DS. Peroxiredoxin I is a ROS/p38 MAP-dependent inducible antioxidant that regulates NF-kappaB-mediated iNOS induction and...
microglial activation. *J. Neuroimmunol.*, 259, 26–36 (2013).

45) Kim M, Li YX, Dewapriya P, Ryu B, Kim SK. Floridoside suppresses pro-inflammatory responses by blocking MAPK signaling in activated microglia. *BBM Rep.*, 46, 398–403 (2013).

46) Jazwa A, Cuadrado A. Targeting heme oxygenase-1 for neuroprotection and neuroinflammation in neurodegenerative diseases. *Curr. Drug Targets*, 11, 1517–1531 (2010).

47) Hsu HY, Chu LC, Hua KF, Chao LK. Heme oxygenase-1 mediates the anti-inflammatory effect of curcumin within LPS-stimulated human monocytes. *J. Cell. Physiol.*, 215, 603–612 (2008).

48) Lee IS, Lim J, Gal J, Kang JC, Kim HJ, Kang BY, Choi HJ. Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells. *J. Neurochem.*, 119, 909–919 (2013).

49) Park JS, Jung JS, Jeong YH, Hyun JW, Le TK, Kim DH, Choi EC, Kim HS. Antioxidant mechanism of isoflavone metabolites in hydrogen peroxide-stimulated rat primary astrocytes: critical role of hemeoxygenase-1 and NQO1 expression. *J. Neurochem.*, 119, 909–919 (2011).

50) Chen HG, Xie KL, Han HZ, Wang WN, Liu DQ, Wang GL, Yu YH. Heme oxygenase-1 mediates the anti-inflammatory effect of molecular hydrogen in LPS-stimulated RAW264.7 macrophages. *Int. J. Surg.*, 11, 1060–1066 (2013).

51) Shih RH, Cheng SE, Hsiao LD, Kou YR, Yang CM. Cigarette smoke extract upregulates heme oxygenase-1 via PKC/NADPH oxidase/ROS/PDGF/Pi3K/Akt pathway in mouse brain endothelial cells. *J. Neuroinflammation*, 8, 104–117 (2011).

52) Nguyen CN, Kim HE, Lee SG. Caffeoylserotonin protects human keratinocyte HaCaT cells against H₂O₂-induced oxidative stress and apoptosis through upregulation of HO-1 expression via activation of the PI3K/Akt/Nrf2 pathway. *Phytother. Res.*, 27, 1810–1818 (2013).

53) Jeong YH, Jung JS, Le TK, Kim DH, Kim HS. Lancemaside A inhibits microglial activation via modulation of JNK signaling pathway. *Biochem. Biophys. Res. Commun.*, 431, 369–375 (2013).

54) Hartsfield CL, Alam J, Choi AM. Transcriptional regulation of the heme oxygenase 1 gene by pyrrolidine dithiocarbamate. *FASEB J.*, 12, 1675–1682 (1998).

55) Makino T, Shimizu R, Kanemaru M, Suzuki Y, Moriwaki M, Mizukami H. Enzymatically modified isocoumarin, alpha-oligoglucoyl quercetin 3-O-glucoside, is absorbed more easily than other quercetin glycosides or aglycone after oral administration in rats. *Biol. Pharm. Bull.*, 32, 2034–2040 (2009).

56) Youdim KA, Qaiser MZ, Begley DJ, Rice-Evans CA, Abbott NJ. Flavonoid permeability across an in situ model of the blood-brain barrier. *Free Radic. Biol. Med.*, 36, 592–604 (2004).

57) Spencer B, Marr RA, Gindi R, Potkar R, Michael S, Adame A, Rockenstein E, Verma IM, Masliah E. Peripheral delivery of a CNS targeted, metallo-protease reduces abeta toxicity in a mouse model of Alzheimer’s disease. *PLoS ONE*, 6, e16575 (2011).

58) Ohtsuki S, Yamaguchi H, Kang YS, Hori S, Terasaki T. Reduction of L-type amino acid transporter 1 mRNA expression in brain capillaries in a mouse model of Parkinson’s disease. *Biol. Pharm. Bull.*, 33, 1250–1252 (2010).

59) Jaeger LB, Doehgu S, Sultana R, Lynch JL, Owen JB, Erickson MA, Shah GN, Price TO, Fleegal-Demotta MA, Butterfield DA, Banks WA. Lipopolysaccharide alters the blood–brain barrier transport of amyloid beta protein: a mechanism for inflammation in the progression of Alzheimer’s disease. *Brain Behav. Immun.*, 23, 507–517 (2009).