

Naringin inhibits lipopolysaccharide-induced activation of microglia cells

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Abstract: The purpose of this study was to investigate the effect of naringin on lipopolysaccharide (LPS)-induced activation of BV2 microglia and inflammatory factor release, and the mechanism involved. Different concentrations of naringin were used to pretreat BV2 cells for 30 min, after which they were stimulated with 100 ng/mL LPS for different durations. The levels of NO, IL-1 β and TNF- α in the cell culture medium was determined with ELISA and Griess method. The mRNA expressions of IL-1 β and TNF- α was determined with RT-PCR. Changes in ERK and p65/NF- κ B signaling pathway proteins were assayed with Western blotting. After 12 h stimulation of BV2 cells with LPS, the levels of IL-1 β and TNF- α in the cell culture medium were significantly increased, but naringin had no significant effect on these inflammatory factors. In the cells pretreated with naringin, LPS stimulated the activation of microglia to produce IL-1 β and TNF- α in a dose-dependent manner. Naringin inhibited LPS-induced release of IL-1 β , to a certain extent. The TNF- α gene was overexpressed. In addition, LPS stimulated a dose-dependent decrease in NO production by BV2 after pretreatment with different concentrations of naringin. Naringin pretreatment of cells significantly inhibited the activation of p65/NF- κ B in a concentration-dependent manner. In BV2 microglia, naringin inhibits LPS-induced production of NO and inflammatory factors, through a mechanism involving inhibition of activation of the p65/NF- κ B signaling pathway.

Key words: Naringin; Lipopolysaccharide; Inflammatory factors; BV-2 cells.

Introduction

In the 1930s, DelRio-Hortega pointed out that microglia were few in number, accounting for only about 5 % of all brain cells. They were considered as resident phagocytes with phagocytic and antigen-presenting functions in the brain (1). In the physiological state, microglia are involved in weak immune surveillance which is called "resting state" (2). In a manner similar to that of surrounding macrophages, microglia induce classic inflammatory responses, and also have anti-inflammatory effects (3). Microglia play a decisive role in balancing neuroprotective and inflammatory responses in diseases and injuries (4). Activated microglia release cellular inflammatory proteases and pro-inflammatory factors such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) and other cytokines through nuclear factor- κ B (NF- κ B), an inflammatory signaling protein (5-7). These factors are believed to cause neuronal damage, especially in diseases such as Alzheimer's, Parkinson's and multiple sclerosis (8, 9). In recent years, studies have found that flavonoids in ginkgo biloba extract scavenge free radicals, function as anti-oxidants, and inhibit central nervous system cells (10). Flavonoids exert other pharmacological effects such as anti-aging and neuroprotective effects (11, 12).

Naringin (Nar, Figure 1) is a dihydroflavonoid compound which has a variety of pharmacological effects such as blood pressure-lowering, lipid-regulating, anti-inflammatory, antioxidant, anti-apoptosis, anti-insulin

resistance, as well as regulatory effects on carbohydrate and fat metabolism (13-15). However, the role of naringin in microglia has not been systematically studied.

In the current study, an *in vitro* inflammatory model was established using lipopolysaccharide (LPS) to stimulate microglia cell line BV-2 cells, and reverse transcription-PCR (RT-PCR) was used to determine the changes in expressions of related inflammatory factors. Western blot was used to assess changes in ERK and p65/NF- κ B signaling pathways, and to investigate the inhibitory effect of naringin on LPS-induced BV-2 cell activation and release of inflammatory factors.

Materials and Methods

Materials

The BV-2 cell line was purchased from Shanghai

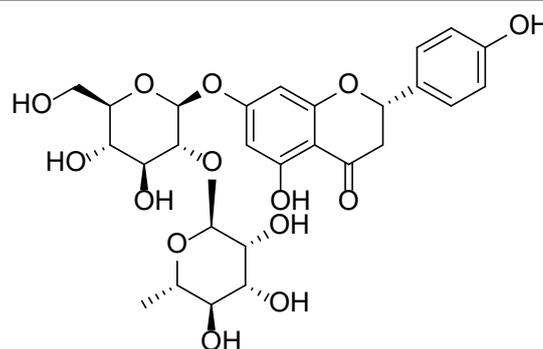


Figure 1. Naringin molecular structure.

Fuxiang Biotechnology Co., Ltd.; high glucose DMEM, penicillin-streptomycin double antibody, and fetal bovine serum were purchased from Gibco, USA, while LPS was obtained from Sigma. The TNF- α , IL-1 β and NF- κ B antibodies; and TRITC-conjugated fluorescent secondary antibody were purchased from Santa Cruz, USA. Primers for TNF- α , IL-1 β and NF- κ B genes; Trizol•RNA extraction kit, and reverse transcription kit were purchased from Invitrogen, USA. Naringin was product of Shanghai Jizhi Biochemical Technology Co., Ltd.

Methods

Cell culture

The BV-2 cells were cultured in high glucose DMEM containing 10 % fetal bovine serum, 10^5 U/L penicillin and 100 mg/L streptomycin in a cell culture incubator at 37 °C in a 5 % CO₂, saturated humidity incubator. The medium was changed once every 2 to 3 days, and the cells were passaged once every 3 to 4 days. Cells in the logarithmic growth phase were used for experiments.

Determination of cell viability

The BV-2 cell suspension (100 μ L) was inoculated into a 96-well plate at a density of 1×10^5 cells/mL, and incubated at 37 °C in a 5% CO₂ incubator for 24 h. When the cells were well adherent, they were randomly divided into blank control group and treatment group, with 3 duplicate wells in each group. The treatment group was administered different concentrations of naringin (2.5, 5, 10 and 20 μ M) for 1 h, followed by incubation with 10 μ L of CCK-8 solution for 2 h. The absorbance of each well was read at 450 nm in a microplate reader. The cell viability was determined in terms of difference in absorbance between the treated groups and blank control group. Cells to which only 100 μ L of DMEM medium was added were the zero-adjustment group. The cell viability of the blank control group and the treated group was obtained by subtracting the absorbance value of the zero-adjustment group.

Determination of cytokines using ELISA

This was done using double antibody sandwich ELISA. A known antibody was bound to the surface of the solid phase carrier, and the antigen in the sample to be tested was bound to the corresponding antibody, and was also immobilized on the surface of the solid phase carrier. Unbound components were washed away. Then, an antibody containing a horseradish peroxidase-labeled antibody was added to bind to the antigen bound to the surface of the solid phase carrier, thereby forming an immune complex. Unbound components were washed away as before. Then, the chromogenic substrate was added for color development. The absorbance of the color was measured at 450 nm and the corrected wavelength was set at 540 nm or 570 nm. A standard curve

was prepared based on the absorbance of the standard well, and the concentration values of IL-1 β and TNF- α in each well were determined, that is, the concentrations of IL-1 β and TNF- α in the cell culture supernatant.

The experiment was performed in strict compliance with the instructions provided by the ELISA kit.

Griess assay for NO content

Nitric oxide (NO) is easily oxidized in aqueous solution to form NO²⁻. Under acidic conditions, NO²⁻ reacts with Griess reagent to form a diazo compound which concentration is linearly related to the concentration of NO²⁻. Therefore, the Griess method can indirectly reflect the amount of NO released by the cells in the medium. In this study, NO contents of the different groups of BV-2 cells were determined at room temperature in 96-well plates. The absorbance of each well was read at 540 nm in a microplate reader, and the concentration of NO was determined from an NO standard curve.

RT-PCR

Total RNA was extracted from the different groups of BV2 cells with TRNzol•RNA extraction kit. The extracted RNA was reverse-transcribed to cDNA with reverse transcription kit, and the cDNA was used as a template for PCR amplification using 2 \times TaqPCR MasterMix reagent.

The 25 μ L reaction system contained 1 μ L of upstream and downstream primers, 2 μ L of cDNA, 12.5 μ L of 2 \times TaqPCR Master Mix, and 8.5 μ L of RNase-free ultra-pure water. Primer design software (Primer3 software version 1.0) was used for TNF- α , IL-1 β and NF- κ B and β -actin gene primers. The PCR amplification conditions were as follows: pre-denaturation of TNF- α , IL-1 β and NF- κ B/p65 and β -actin at 95 °C for 15 min, denaturation at 95 °C for 15 sec, annealing at 60 °C for 25 sec, and extension at 2 °C for 20 sec. The reaction was carried out in 45 cycles. The PCR reaction product was electrophoresed on a 1% agarose gel containing 1 mg/mL ethidium bromide for 45 min at a voltage of 90 V. After electrophoresis, the agarose gel was placed in a Bio-Rad Chemi-DocXRS gel image analysis system to capture images. Quantityone 4.4.0 was used to calculate the fluorescence intensity values of each band and its ratio to β -actin. The primer sequences and their product lengths are shown in Table 1.

Western blot

The steps used in the western blot analysis were: preparation of protein samples, determination of protein concentration using bicinchoninic (BCA) acid method, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transfer of separated proteins to polyvinylidene membrane, blocking of non-specific binding sites, incubation with primary and secondary antibodies, and color analysis.

Table 1. Primer sequences and product lengths.

Gene	Forward primer	Reverse primer	Product length/bp
IL-1 β	5'-GCCCATCTCTGTGACTCAT-3'	5'-AGGCCACAGGTATTTGTCTG-3'	229
TNF- α	5'-CGTCAGCCGATTTGCTATCT-3'	5'-CGGACTCCGCAAAGTCTAAG-3'	205
β -actin	5'-AGCCATGTACGTAGCCATCC-3'	5'-GCTGTGGTGGTGAAGCTGTA-3'	222

Statistical analyses

Results are expressed as mean \pm standard deviation ($\bar{x} \pm s$). One-way analysis of variance (ANOVA) was used for statistical comparison between multiple groups. Data were processed using SPSS13.0 statistical software package. Values of $p < 0.05$ were considered statistically significant.

Results

Effect of naringin on BV-2 cell activity

In order to rule out any toxic effect of naringin on BV2 cells, the effect of naringin on the viability of BV2 cells was determined using MTT assay. The results showed that naringin at concentrations of 2.5, 5, 10 and 20 μM had no significant toxicity on BV2 cells, when compared with the control untreated group (Figure 2). Therefore, naringin concentration of 20 μM was used for subsequent experiments.

Naringin reduced the pro-inflammatory factors produced by LPS-stimulated BV2 cells

The levels of IL-1 β and TNF- α in the cell culture medium increased significantly when BV-2 cells were stimulated with LPS for 24 h. Naringin had no obvious effect on these inflammatory factors (Figure 3). After pretreatment with different concentrations of naringin (2.5, 5, 10 and 20 μM), there were dose-dependent reductions in IL-1 β and TNF- α in the LPS-stimulated BV2 cells (Figure 3).

Naringin reduced NO production in LPS-stimulated BV2 cells

Griess method was used to determine the NO levels in the cell culture supernatant of BV-2 cells stimulated with LPS after pre-treatment with naringin. The results showed that treatment of cells with naringin alone (20 μM) did not cause any changes in NO content of the cell supernatant, but the amount of NO produced by BV2 was significantly increased after LPS stimulation for 24 h (Figure 4). After pretreatment with different concentrations of naringin (2.5, 5, 10 and 20 μM), there were dose-dependent decreases in NO production by LPS-stimulated BV2 cells (11.66, 20.55, 34.99 and 43.72 % decreases, respectively; $p < 0.05$).

Naringin inhibited LPS-induced expressions of cellular inflammatory factors at the transcriptional level

The mRNA expressions of IL-1 β and TNF- α in BV-2 cells stimulated with LPS after naringin pre-treatment were determined with RT-PCR. The mRNA expression levels of IL-1 β and TNF- α were significantly up-regulated in BV2 cells after 6 h of LPS stimulation. After pretreatment with naringin (20 μM), the upregulations of IL-1 β and TNF- α mRNA by LPS stimulation were significantly inhibited (Figure 5).

Naringin inhibited the activation of p65/NF- κB signaling pathway in LPS-stimulated BV2 cells

The effect of naringin on the activation of the p65/NF- κB pathway in LPS-stimulated BV2 cells was investigated. Western blot results showed that treatment with naringin (20 μM) for 12 h after LPS stimulation of BV2 cells significantly inhibited the activation of p65/

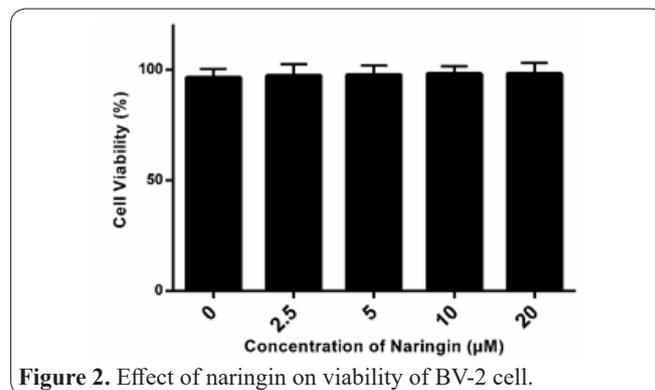


Figure 2. Effect of naringin on viability of BV-2 cell.

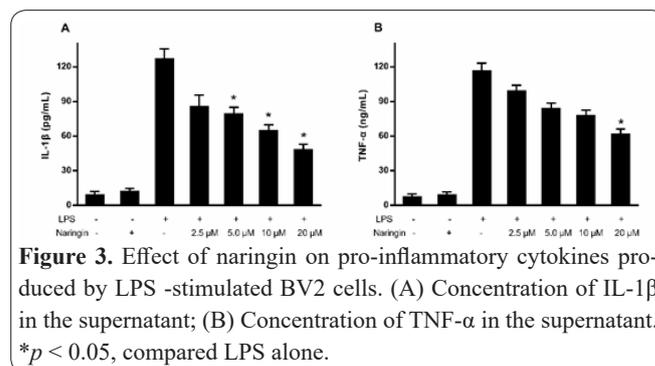


Figure 3. Effect of naringin on pro-inflammatory cytokines produced by LPS-stimulated BV2 cells. (A) Concentration of IL-1 β in the supernatant; (B) Concentration of TNF- α in the supernatant. * $p < 0.05$, compared LPS alone.

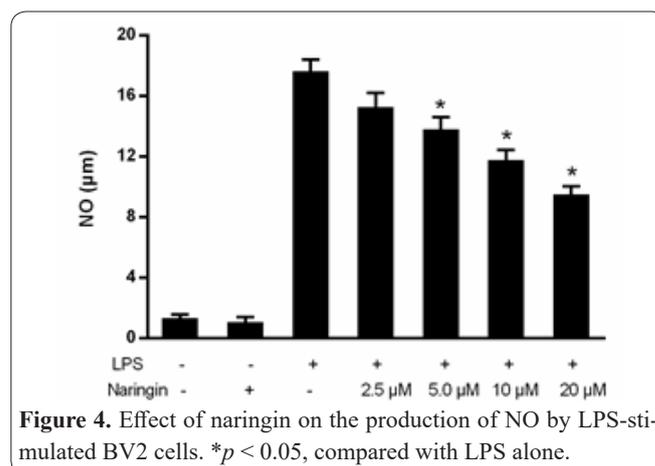


Figure 4. Effect of naringin on the production of NO by LPS-stimulated BV2 cells. * $p < 0.05$, compared with LPS alone.

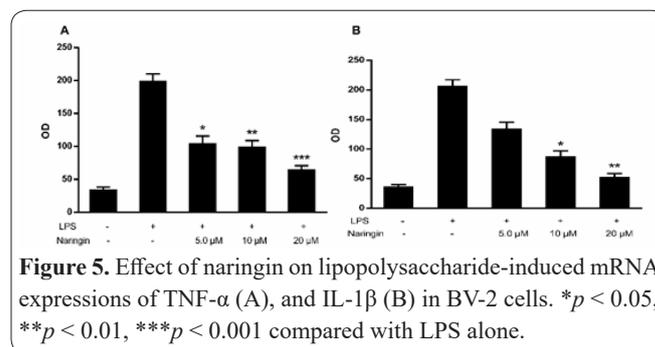


Figure 5. Effect of naringin on lipopolysaccharide-induced mRNA expressions of TNF- α (A), and IL-1 β (B) in BV-2 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with LPS alone.

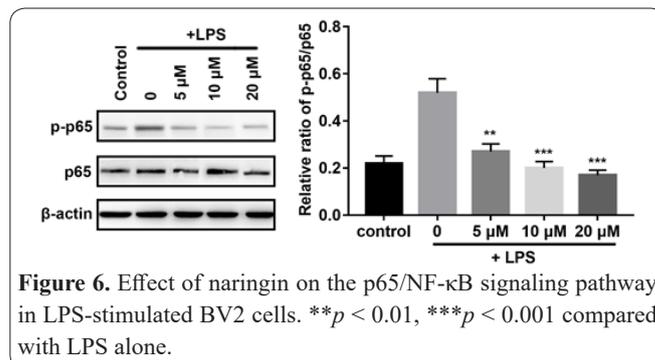


Figure 6. Effect of naringin on the p65/NF- κB signaling pathway in LPS-stimulated BV2 cells. ** $p < 0.01$, *** $p < 0.001$ compared with LPS alone.

NF- κ B signaling pathway in a concentration-dependent manner (Figure 6).

Discussion

Microglia, being important immune cells in the central nervous system, especially in the brain, play the role of macrophages. They account for 20 % of the glial cells in the brain. Damaged nerves and infectious substances in the central nervous system are constantly removed by microglia. Numerous clinical and neuropathological studies have shown that activated microglia play a key role in the pathogenesis of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and multiple sclerosis. Moreover, studies have shown that excessive activation or loss of control of microglia causes neurotoxicity. Activated microglia produce many pro-inflammatory factors such as tumor necrosis factor (TNF), nitric oxide, interleukin (IL) and other neurotoxic substances. Therefore, inhibition of activation of microglia may achieve unexpected effects during the treatment of inflammation-related neurodegenerative diseases. The results obtained in the present study showed that naringin exerted inhibitory effect on LPS-induced activation of microglia. Inhibition of microglia activation can reduce neuronal cell death. In fact, a large number of anti-inflammatory drugs inhibit the activation of microglia or reduce the secretion of pro-inflammatory mediators in neurological diseases, thereby reducing neurodegeneration (15). In BV2 cells, naringin significantly inhibited the production of cellular inflammatory factors and NO. It is well known that L-arginine produces NO under the action of nitric oxide synthase (NOS) which consists of three different subtypes. In the central nervous system, immune cells such as microglia and astrocytes express iNOS mainly when they are stimulated, and these stimuli include IL-1 β , TNF- α and LPS. Microglia activated by stimulation release a certain amount of NO, and the NO released by the cells forms peroxynitrite. The peroxynitrite, in turn reacts with related molecules in the cell, interfering with the mitochondrial respiratory chain, and ultimately leading to cell damage. The results obtained in the current study have confirmed that naringin inhibits the up-regulation of iNOS in LPS-stimulated BV2 microglia, thereby preventing excessive release of NO. In addition, the study has demonstrated that naringin significantly reduces the release of LPS-induced inflammatory factors IL-1 β and TNF- α , and their mRNA overexpression in BV2 cells.

Although it is known that Parkinson's disease is closely related to the inflammatory reaction process, the mechanism involved has not been fully studied. However, the pathological mechanism of inflammation is closely related to cytokines and their mediators. The NF- κ B and MAPK families play key regulatory roles in inflammation. Many studies on BV2 cells have shown that LPS activates p65/NF- κ B by phosphorylation, whereas hyperoside pre-treatment inhibits LPS-induced phosphorylation of p65/NF- κ B. Thus, hyperoside is likely to inhibit microglia activation by inhibiting p65/NF- κ B activation.

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Conflict of Interest

There are no conflicts of interest in this study.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Pengbo Zhang; Jie Bai, Siyuan Li, Gang Wu, Lei Ma, Chen Li, Hongtao Liu and Pengbo Zhang collected and analysed the data; Jie Bai wrote the text and all authors have read and approved the text prior to publication.

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