

Protective effects of osthole against inflammation induced by lipopolysaccharide in BV2 cells

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Abstract. Inflammation and oxidative stress are implicated in the development of neurodegenerative diseases. Osthole is a compound that is extracted from *She Chuang Zi*, which is a type of traditional Chinese medicine. Osthole has previously been demonstrated to exhibit anticancer activities and has a low toxicity. However, to the best of our knowledge, the anti-inflammatory effects of osthole in microglial cells have not been investigated extensively. The aim of the present study was to investigate the potential protective effects of osthole against inflammation induced by lipopolysaccharide (LPS) in microglial cells. The present study employed LPS-stimulated BV2 mouse microglia to establish an inflammatory cell model and to investigate the anti-inflammatory effects of osthole. Cells were pretreated with osthole for 1 h prior to LPS (10 μ g/ml) stimulation. At 6 h after the addition of LPS, alterations in the levels of inflammatory factors, including tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-1 β , were determined by ELISA. Furthermore, at 24 h after the addition of LPS, western blot analysis was performed to analyze the alterations in the protein expression of nuclear factor- κ B (NF- κ B) p65, phosphorylated-NF- κ B p65, nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase (HO)-1. The results demonstrated that the secretion of the inflammatory cytokines TNF- α , IL-6 and IL-1 β by LPS-stimulated BV2 cells was significantly reduced by osthole treatment. Simultaneously, osthole treatment inhibited the LPS-induced activation of the NF- κ B signaling pathway. In addition, osthole upregulated the expression of Nrf2 and HO-1 in a dose-dependent manner.

Based on these results, osthole may exhibit anti-inflammatory effects via the NF- κ B and Nrf2 pathways, indicating that osthole has the potential to be developed into an effective anti-inflammatory drug.

Introduction

Osthole (C₁₅H₁₆O₃), or 7-methoxy-8-isopentenoxycoumarin, is also termed 'She Chuang Zi Su' in China. As a traditional Chinese medicine, it has been widely used in the treatment of various diseases. According to reports, osthole exerts numerous positive effects, including antioxidant (1), anti-hypertension (2), anti-arrhythmia (3), anticancer (4) and antitumor (5) activities. Lipopolysaccharide (LPS), which is also termed bacterial endotoxin (6), is an important component of the cell wall of Gram-negative bacterial cells. Previous studies have demonstrated that LPS induces inflammatory responses in cells (7-11). Therefore, the present study employed LPS to establish an inflammatory model for the investigation of the potential anti-inflammatory properties of osthole. Inflammation of the central nervous system may lead to the development of various diseases, including Alzheimer's disease, multiple sclerosis and Parkinson's disease (12). Nerve cells are fragile cell types and their regulatory ability is weak during inflammation and oxidative stress. However, research concerning nerve inflammation and their anti-inflammatory mechanisms is lacking.

Nuclear factor- κ B (NF- κ B) is an early-stage nuclear transcription factor. It participates in the early stage of the immune response and in each stage of inflammation, as NF- κ B regulates numerous factors that are associated with inflammation (13). At rest, a complex between NF- κ B and NF- κ B inhibitor (I κ B) forms in the cytoplasm. If stimulated, I κ B becomes activated, which frees NF- κ B and allows NF- κ B to transfer from the cytoplasm to the nucleus (14) to induce an inflammatory response in the body. It has been previously verified that the NF- κ B pathway is closely associated with numerous diseases. For example, Brambilla *et al* (15) demonstrated that spinal cord injuries may be treated with drugs that inhibit the NF- κ B pathway, while Noort *et al* (16) reported that the activation of NF- κ B may be one cause of rheumatoid arthritis. Additionally, Duh *et al* (17) confirmed that human immunodeficiency virus type 1, which causes the pathogenesis

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of acquired immune deficiency syndrome, is also associated with NF- κ B. These diseases are all associated with inflammation. Therefore, we hypothesized that the activation of the NF- κ B signaling pathway may also be a major factor in the development of neurodegenerative disease.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an antioxidant response element. In normal conditions, Nrf2 combines with kelch-like ECH-associated protein 1 (Keap-1), and this combined form of Nrf2 is degraded. Degradation effectively controls the level of Nrf2 in cells (18). Heme oxygenase-1 (HO-1) is located downstream of Nrf2 (19); therefore, alterations in the levels of Nrf2 subsequently affect the expression of HO-1. In the present study, we hypothesized that osthole may protect cells against inflammation through the Nrf2 and NF- κ B pathways. The aim of the present study was to determine the potential protective effect of osthole against inflammation in cells and to investigate the underlying mechanisms.

Materials and methods

Reagents. Osthole (98%) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and was dissolved in 0.1% dimethyl sulfoxide (DMSO) to reach a concentration of 0.7 mg/ml and stored at -20°C. The final concentration for DMSO used to dissolve the drug was 0.1%; DMSO exhibited no effect on cell viability, as demonstrated in a previous study (20). Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). LPS was purchased from Sigma-Aldrich (Merck KGaA). The components of the cell lysis buffer were obtained from Beyotime Institute of Biotechnology (Haimen, China). NF- κ B p65, phosphorylated (p)-NF- κ B p65, Nrf2, HO-1, IkB α , p-IkB α , lamin B and β -actin primary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). MTT reagent and DMSO were provided by Wuhan Boster Biological Technology, Co., Ltd. (Wuhan, China). Fetal bovine serum (FBS) was obtained from Zhejiang Tianhang Biotechnology Co., Ltd. (Huzhou, China). ELISA kits were purchased from Cell Signaling Technology, Inc.

Cell culture. BV2 mouse microglial cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM with 10% FBS and 1% antibiotics (100 U/ml penicillin and streptomycin) (5). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. The medium was changed once a day and passage occurred when the cells grew on a logarithmic scale.

Cell viability assay. BV2 cells in the logarithmic growth phase were added to 96-well plates (7x10³ cells/well) and cultured in a cell culture box at 37°C for 12 h until the cells completely attached to the wall. At 1 h prior to LPS (1 μ g/ml) stimulation, different concentrations of osthole (4, 7 and 10 μ g/ml) were added to each well at 37°C, and the activity of cells was detected at 24 h after stimulation with LPS. For the control group, an equivalent volume of empty media was added. MTT reagent was added to all cell groups and were subsequently cultivated in the incubator for 2-4 h at 37°C, leading to the

development of purple crystals. DMSO (150 μ l/well) was added and shaken until all of the crystals disappeared. The optical density of the 96-well plate was measured at 570 nm.

Western blotting. BV2 cells were stimulated with LPS (1 μ g/ml) at 1 h after treatment with osthole (4, 7 and 10 μ g/ml) in 6-well plates (1x10⁶ cells/well), and total protein samples were collected at 24 h after the addition of LPS. Protein extraction was performed in strict accordance with the specifications of the Protein Extraction kit (cat. no. AR0103; Boster Biological Technology, Pleasanton, CA, USA). Following removal of the medium, the cells were washed with 4°C pre-cooled PBS three times. Subsequently, 1 ml lysate was added, including 10 μ l protease inhibitors and 10 μ l phosphorylase inhibitors. After 30 min, cells from the 6-well plates were scraped, collected in a 1.5-ml centrifuge tube and centrifuged for 5 min at 4°C and 1,000 x g. The supernatant obtained following centrifugation was the protein, and the protein concentration was detected with a BCA protein assay kit. The protein (15 μ g/lane) was concentrated in 10% of the concentrated gel at 80 V for 30 min and separated by 12% SDS-PAGE at 120 V for 70 min. After cutting the gel, the target protein was transferred to polyvinylidene difluoride membranes. Membranes were subsequently incubated with 5% bovine serum albumin for 2 h, followed by incubation with primary antibodies, including NF- κ B p65, p-NF- κ B p65, IkB α , p-IkB α (NF- κ B pathway sampler kit; cat. no. 9936), Nrf2 (cat. no. 12721), HO-1 (cat. no. 5853), lamin B (cat. no. 12255) and β -actin (cat. no. 3700) overnight at 4°C. All antibodies were purchased from CST Biological Reagents Co., Ltd. (Shanghai, China) and were added at 1:1,000 dilution. Prior to incubation with secondary antibodies, the membrane was washed three times with TBS-Tween-20 (0.1% Tween-20) for 10 min each time. After 45 min of incubation with anti-mouse IgG and anti-rabbit IgG secondary antibodies (1:2,000; cat. nos. 7076 and 7074; CST Biological Reagents Co., Ltd.) at room temperature, the washing procedure was repeated. Protein expression was visualized using an enhanced chemiluminescent reagent (Beyotime Institute of Biotechnology) with ImageJ software (version 1.51k; National Institutes of Health, Bethesda, MD, USA), and the membrane was exposed to an X-ray film. Protein levels were detected in blots according to β -actin or lamin B.

ELISA. BV2 cells were plated in 6-well plates (1x10⁶ cells/well) and cultured in a cell culture box for 12 h until the cells completely attached to the wall. Subsequently, cells were treated with various concentrations of osthole (4, 7 and 10 μ g/ml) for 1 h (5), followed by stimulation with LPS (1 μ g/ml) for 6 h. ELISA was performed on cell culture medium to determine the levels of tumor necrosis factor (TNF)- α (cat. no. EK0527), interleukin (IL)-6 (cat. no. EK0411) and IL-1 β (cat. no. EK0394), according to the manufacturer's instructions (Boster Biological Technology Co., Ltd.). Absorbance was determined at 450 nm.

Statistical analysis. Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA) with one-way analysis of variance. Data are presented as the mean \pm standard deviation of five independent experiments. Statistically significant differences were detected with one-way analysis of

variance followed by Dunnet's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of osthole on the viability of BV2 cells. The results of the MTT assay, which was used to detect the cell viability following incubation with LPS and/or osthole for 24 h, are presented in Fig. 1. The results demonstrated that the addition of various concentrations of osthole (4, 7 and 10 $\mu\text{g/ml}$) to cells exhibited no cytotoxic effect, compared with the control group. Therefore, these three concentrations were selected for subsequent experiments.

Effects of osthole on inflammation-associated cytokines. To confirm the anti-inflammatory effects of osthole, the present study assessed cytokine production by BV2 cells using ELISA. The results demonstrated that the production of the inflammatory mediators IL-6, TNF- α and IL-1 β in the cells following LPS stimulation was significantly increased, compared with the control group (Fig. 2). However, the LPS-induced increases in IL-6, TNF- α and IL-1 β levels were dose-dependently inhibited by treatment with osthole (Fig. 2).

Effects of osthole on the expression of NF- κB pathway-associated proteins. Members of the NF- κB family of proteins are considered to be key factors in inflammation. To investigate the effects of osthole on NF- κB activation induced by LPS, the present study performed western blotting. As demonstrated in Fig. 3, following LPS treatment, BV2 cells exhibited increased inflammation, as the expression of NF- κB pathway-associated proteins, such as p-NF- κB p65 and p-I $\kappa\text{B}\alpha$, were increased. However, osthole blocked LPS-induced NF- κB activation in a dose-dependent manner (Fig. 3).

Effects of osthole on the protein expression of Nrf2 and HO-1. Previous studies have reported that Nrf2 is involved in the regulation of antioxidant responses and inflammation (21-24). Western blotting demonstrated that the protein expression of Nrf2 and HO-1, which lies downstream of Nrf2, was further upregulated in osthole-treated BV2 microglia, compared with cells exposed to LPS alone (Fig. 4). These results indicated that osthole may induce the activation of the Nrf2 pathway.

Discussion

With increasing research on traditional Chinese medicine and its effective components, an increasing number of traditional Chinese medicines have been recognized worldwide. Traditional Chinese medicine and its effective components are considered to be safe, reliable and inexpensive. The mechanism of emodin has been extensively researched, and osthole, as an emerging Chinese medicine, has been widely investigated in recent years. Hao *et al* (25) demonstrated that osthole exhibited a therapeutic effect on pulmonary fibrosis, while Chen *et al* (26) reported beneficial effects of osthole on cognitive impairment in animals, which may occur via the Nrf2 pathway. These results indicate that osthole may act on the nervous system. Other studies have demonstrated that osthole may also exhibit an active role in non-nervous system diseases.

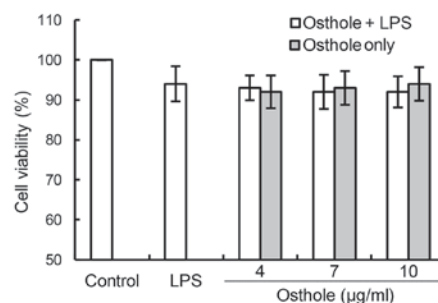


Figure 1. Effects of osthole on the viability of BV2 microglial cells. Cells were treated with osthole (4, 7 and 10 $\mu\text{g/ml}$) for 1 h and LPS (1 $\mu\text{g/ml}$) was subsequently added. Cell viability was determined at 24 h after the addition of LPS using an MTT assay. For groups treated with osthole, white bars indicate those treated with osthole + LPS and gray bars indicate those treated with osthole alone. Data are presented as the mean \pm standard error from five separate experiments. LPS, lipopolysaccharide.

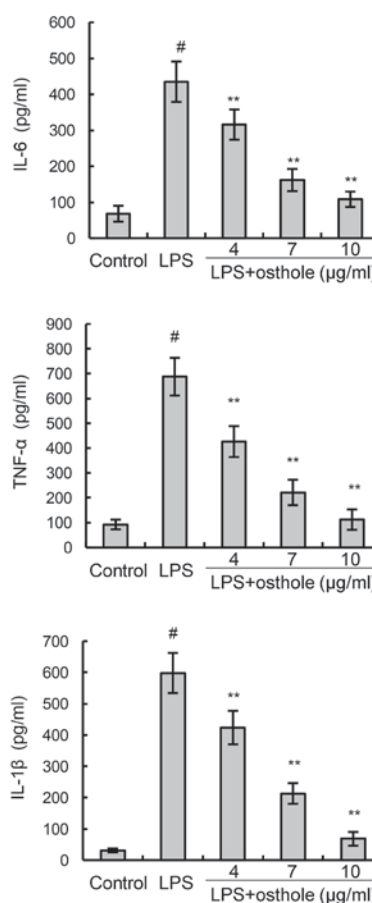


Figure 2. Effect of osthole on the production of inflammatory mediators by BV2 microglial cells. BV2 cells were pretreated with osthole (4, 7 and 10 $\mu\text{g/ml}$) for 1 h, followed by stimulation with LPS (1 $\mu\text{g/ml}$) for 6 h. TNF- α , IL-1 β and IL-6 concentrations in the culture medium were assessed by ELISA. Data are presented as the mean \pm standard error from five separate experiments. # $P < 0.05$ vs. control group; ** $P < 0.01$ vs. LPS treatment group. LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin.

Osthole was previously reported to exhibit anti-inflammatory effects through NF- κB and mitogen-activated protein kinase signaling pathways (27). Furthermore, Liu *et al* (1) demonstrated the effect of osthole in mitochondrial disorders,

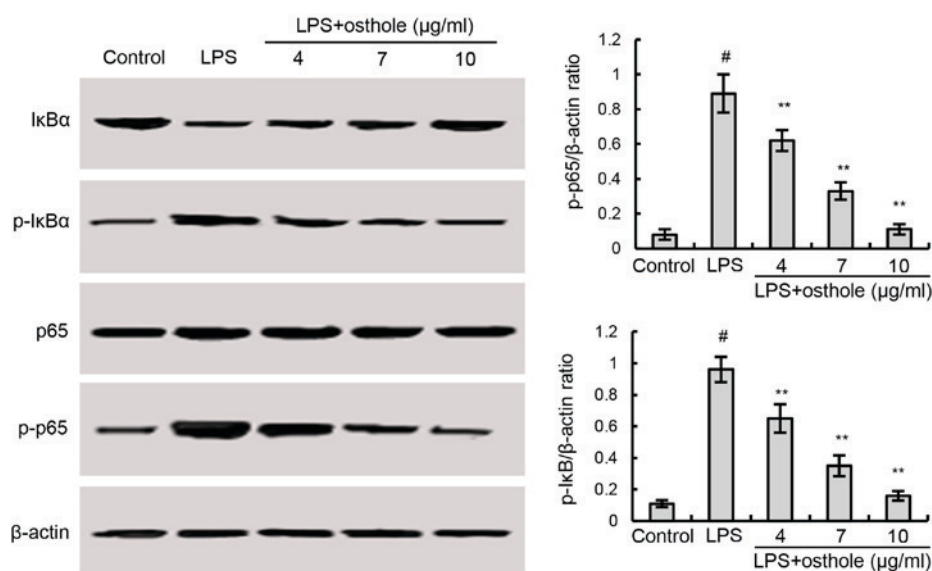


Figure 3. Effects of osthole and LPS on the protein expression of components of the NF-κB pathway in BV2 cells. BV2 cells were treated with osthole (4, 7 and 10 μg/ml) and LPS (1 μg/ml) for 24 h. Western blotting was performed to determine the expression of proteins associated with the NF-κB pathway. Data are presented as the mean ± standard error from five separate experiments. [#]P<0.05 vs. control group; ^{**}P<0.01 vs. LPS treatment group. LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; IκBα, NF-κB inhibitor α; p-, phosphorylated; p65, NF-κB p65.

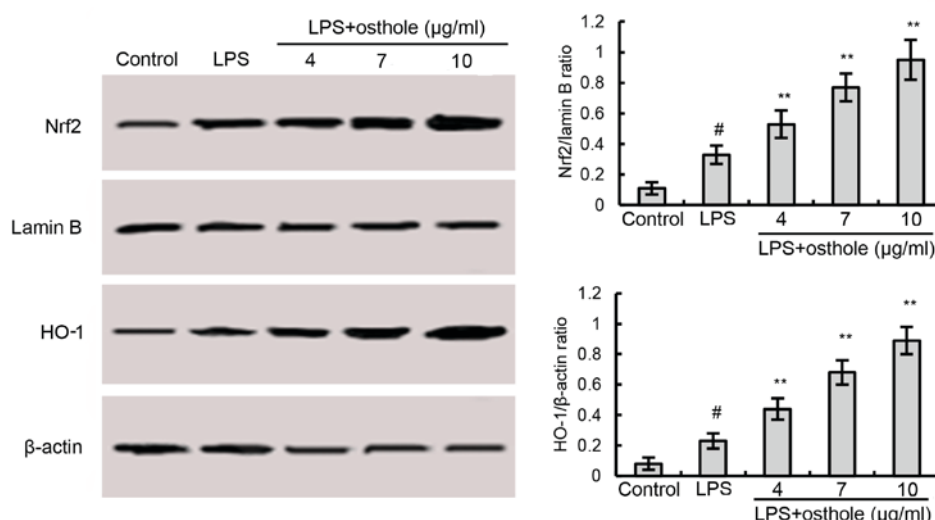


Figure 4. Effects of osthole and LPS on the protein expression of HO-1 and Nrf2. BV2 cells were treated with osthole (4, 7 and 10 μg/ml) and LPS (1 μg/ml) for 24 h. Western blotting was performed to determine the protein expression of HO-1 and Nrf2. Data are presented as the mean ± standard error from five separate experiments. [#]P<0.05 vs. control group; ^{**}P<0.01 vs. LPS treatment group. LPS, lipopolysaccharide; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2.

Wang *et al* (28) reported that osthole inhibited inflammatory cytokine release in adipocytes, Huang and Dong (29) indicated that osthole exhibited anti-inflammatory effects in chronic kidney failure through the NF-κB pathway, and Hao and Liu (30) concluded that osthole reduced inflammatory responses in rats with pulmonary fibrosis. Although previous reports have investigated the role of osthole in the process of inflammation, the present study focused on inflammation in neurodegenerative diseases by using BV2 microglial cells. In addition to investigating conventional inflammatory factors, the present study also investigated the activation of the NF-κB and Nrf2 signaling pathways following osthole treatment. According to the results of western blotting and ELISA, osthole

reduced the expression of proteins associated with NF-κB and Nrf2 pathways. These results highlight the potential medicinal value of *Fructus cnicidii*, which is another term for the traditional Chinese herb *She Chuang Zi*. With the discovery of the mechanism of action of this traditional Chinese medicine, osthole may have potential as an anti-inflammatory drug option.

Inflammation is a common pathological phenomenon that occurs in various disease states (31). BV2 cells are a type of mouse microglial cell, which are present in the brain and spinal cord (32). They are considered to be the first and most important line of defense in the central nervous system. A number of studies have demonstrated that macrophages

stimulated by external antigens, such as LPS, release IL-1 β , TNF- α and other important inflammatory factors (33-37). These inflammatory cytokines promote an inflammatory reaction and lead to tissue damage. The results of the present study also demonstrated that stimulation of BV2 cells with LPS also led to the production of inflammatory factors, including TNF- α , IL-6 and IL-1 β . It was also observed in the present study that the traditional Chinese medicine osthole affected this process and reduced the inflammatory response of BV2 cells following LPS stimulation.

In conclusion, the results of the present study demonstrated that osthole may protect inflammatory BV2 cells against inflammation induced by LPS stimulation, and this may occur through inhibition of the NF- κ B and Nrf2 signaling pathways. However, a previous report by Chen *et al* (38) indicated that osthole was not able to inhibit the Nrf2 pathway. As a result, it may be concluded that, in the present study, the alterations in the expression of proteins associated with the Nrf2 pathway may occur as a result of the anti-inflammatory effects of osthole; it was previously reported that LPS induced Nrf2 and HO-1 activation (39). In the present study, osthole also reduced the release of inflammatory factors such as IL-1 β and TNF- α . These results may provide the scientific foundation for the effect of osthole in the prevention of nerve inflammation. Therefore, further investigation of this traditional Chinese is required to determine whether it may be employed for the treatment of various types of inflammation in the future.

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