Running title: Immune modulation properties of osthole

Osthole Regulates Inflammatory Mediator Expression through Modulating NF-κB, Mitogen-Activated Protein Kinases, Protein Kinase C, and Reactive Oxygen Species

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ABSTRACT

Osthole, a coumarin compound, has been reported to exhibit various biological activities; however the cellular mechanism of its immune modulating activity has not yet been fully addressed. In this study we isolated osthole from the seeds of *Cnidium monnieri* and demonstrated that osthole inhibited TNF-α, NO and COX-2 expression in LPS-stimulated macrophages, without reducing the expression of IL-6. Furthermore, the phosphorylation of p38, JNK1/2, PKC-α and PKC-ε induced by LPS were inhibited by osthole; however, the phosphorylation of ERK1/2 and PKC-δ were not reduced by osthole. Osthole also inhibited NF-κB activation and ROS release in LPS-stimulated macrophages. Our current results indicated that osthole is the major anti-inflammatory ingredient of *Cnidium monnieri* seeds ethanol extract.

KEYWORDS: Osthole; LPS; inflammation; signaling

ABBREVIATIONS: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; NO, nitric oxide; COX-2, cyclooxygenase-2; IL-6, interleukin-6; TLR, Toll-like receptor; NF-κB, nuclear transcription factor kappa-B; MAPKs, mitogen-activated protein kinases; PKC, protein kinase C

INTRODUCTION

Cnidium monnieri Cuss. is not only a traditional Chinese herb but also an economically important agricultural product via artificial planting, especially in China in recent years. Osthole (7-methoxy-8-isopentenoxycoumarin), a coumarin compound isolated from the seeds of *C. monnieri*, exhibits significant bioactivities including: induction of apoptosis in HER2-overexpressing breast cancer cells *(1)*; inhibition of voltage-gated Na^+ channels in mouse neuroblastoma N2A cells *(2)*; inhibition of rat vascular smooth muscle cell proliferation *(3)*; suppression of the secretion of hepatitis B virus through increasing the glycosylation of hepatitis B surface antigen which are important steps for the viral particle maturation *(4)*; inhibition of contact dermatitis in experimental animals *(5)*; and inhibition of cytokine expression in rat peritoneal cells and human peripheral blood mononuclear cells *(6)*. However, the molecular mechanism of osthole-mediated downregulation of tumor necrosis factor-alpha (TNF-α), nitric oxide (NO) and cyclooxygenase-2 (COX-2) expression in macrophages is unclear.

The innate immunity of mammals is triggered by pathogen-associated molecular patterns that are shared by groups of different microbial pathogens; these are recognized by Toll-like receptors (TLRs) expressed on the cell surface of macrophages *(7)*. Lipopolysaccharide (LPS), one of the most important

58 pathogen-associated molecular patterns, activates macrophages by binding to TLR4,
59 followed by stimulating nuclear transcription factor kappa-B (NF-κB) activation. This
60 leads to the production of pro-inflammatory mediators from macrophages, including
61 TNF-α, interleukin-1β (IL-1β), interleukin-6 (IL-6) and NO *(8)*. Protein kinase C
62 (PKC) is one of the signaling molecules in an LPS-mediated inflammatory response,
63 and regulates a downstream signal transduction cascade via modulation of the
64 mitogen-activated protein kinase (MAPK) pathways, such as extracellular signal
65 regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2) and p38 MAP
66 kinase *(9)*. Recently, the development of potential therapeutic approaches to modulate
67 inflammatory disease has become ever more popular and important. These therapeutic
68 approaches include inhibition of pro-inflammatory mediator production *(10)*.
69 In our previous study we found that ethanol extract of *C. monnieri* seeds
70 inhibited cytokine production in LPS-stimulated macrophages. In addition, Zimecki et
71 al reported that osthole inhibited concanavalin A- and pokeweed mitogen-induced
72 mouse splenocyte proliferation and inhibited TNF-α production in rat peritoneal cells
73 and human peripheral blood mononuclear cells (*6*). This finding promoted us to
74 isolate osthole, the major component of the ethanol extract of *C. monnieri* seeds and
75 dissected its anti-inflammatory mechanisms in macrophages. We demonstrated that
76 osthole inhibited TNF-α, NO and COX-2 expression through inhibiting activation of

NF-κB, p38, JNK1/2, PKC-α, PKC-ε and reactive oxygen species (ROS) in LPS-activated macrophages. Our results provide support for the potential for the future pharmaceutical application of osthole for immune modulation purposes.

MATERIALS AND METHODS

Materials. Seeds of *C. monnieri* were purchased from a traditional Chinese medicine dispensary in Taiwan. LPS (from *Escherichia coli* 0111:B4), anti-phospho-ERK1/2 antibody, anti-phospho-JNK1/2 antibody, anti-phospho-p38 antibody and anti-actin antibody were purchased from Sigma (St. Louis, MO). Anti-phospho-IKK-α antibody, anti-IKK-α antibody, anti-phospho-IκB-α antibody, anti-IκB-α antibody, anti-COX-2 antibody, anti-phospho-PKC-α antibody, anti-phospho-PKC-δ antibody, anti-phospho-PKC-ε antibody, anti-ERK1/2 antibody, anti-JNK1/2 antibody and anti-p38 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). TNF-α and IL-6 ELISA kits were purchased from R&D Systems (Minneapolis, MN).

Extraction and Purification. *C. monnieri* seeds (580 g dry weight) were treated with 15 L of ethanol (95% v/v, 10 days repeat 3 times) at room temperature. The extracts were then concentrated to produce alcoholic extracts (AE) ca. 42.54 g (yield % = 7.33%). AE (240 mg) was dissolved in 2 ml ethanol and then purified by

96 semi-preparative high-performance liquid chromatography (HPLC) (Knauer 100
97 pump with a Knauer RI 2400 refractive index detector) with a Phenomenex Luna
98 Silica (2) column (250 mm length, 10 mm i.d., particle shape/size 5.0 µm). Five pure
99 compounds were obtained. The separation conditions were as follows: 500 µl was
100 injected for each separation; flow rate, 4 ml/min; mobile phase, acetone/hexane = 1/6.
101 The structures of the five pure compounds were identified by physical and spectral
102 data (mp, EIMS, ^{1}H-NMR, UV, IR) compared with previous research values. UV and
103 IR spectra were recorded on Jasco V-550 and Bio-Rad FTS-40 spectrophotometers,
104 respectively. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker
105 Avance 400 MHz FT-NMR spectrometer. Mass spectra (MS) were obtained using a
106 Finnigan MAT-95S mass spectrometer.
107 Five pure compounds isolated from *C. monnieri* seeds ethanol extract whose
108 characteristics are described as follows:
109 Osthole (**1**) – Colorless prisms from ether; mp 79-81 °C; EI-MS *m/z* (%): 244
110 (M^{+}, 100), 229 (38), 213 (23), 201 (28), 189 (38), 131 (25); UV MeOH λ_{max}: 249, 258,
111 322; IR KBr, λ_{max}, cm^{-1}: 1720, 1610; ^{1}H-NMR δ: 7.61 (1H, d, *J* = 9.4 Hz), 7.29 (1H, d,
112 *J* = 8.7 Hz), 6.83 (1H, d, *J* = 8.7 Hz), 6.23 (1H, d, *J* = 9.4 Hz), 5.22 (1H, t, *J* = 7.4 Hz),
113 3.92 (3H, s), 3.52 (2H, d, *J* = 7.4 Hz), 1.84 (3H, s), 1.67 (3H, s).
114 Imperatorin (**2**) – mp 97.5-99 °C; UV MeOH λ_{max}: 301, 249, 218; IR KBr, λ_{max},

115 cm^{-1}: 1722, 1707 1587, 1150, 838; ^{1}H-NMR ($CDCl_3$) δ: 7.76 (1H, d, *J* = 9.3 Hz),

116 7.69 (1H, d, *J* = 2.4 Hz), 7.36 (1H, s), 6.81 (1H, d, *J* = 2.4 Hz), 6.37 (1H, d, *J* = 9.3

117 Hz), 5.61 (1H, t-like, *J* = 7.3 Hz), 5.01 (2H, d, *J* = 7.3 Hz), 1.74 (3H, s), 1.72 (3H, s).

118 Xanthotoxin (**3**) – mp 146-148 °C; UV MeOH λ_{max}: 299, 248, 218; IR KBr, λ_{max},

119 cm^{-1}: 1710, 1620, 1586, 1155, 821; ^{1}H-NMR ($CDC1_3$) δ: 7.77 (1H, d, *J* = 9.3 Hz),

120 7.70 (1H, d, *J* = 2.4 Hz), 7.36 (1H, s), 6.83 (1H, d, *J* = 2.4 Hz), 6.37 (1H, d, *J* = 9.3

121 Hz), 4.30 (3H, s).

122 Isopimpinellin (**4**) – Colorless prism; mp 150 °C; EI-MS *m/z* (%): 246 (M^+, 95),

123 231 (100), 203 (15), 188 (25), 175 (22), 160 (20), 147 (14); IR KBr, λ_{max}, cm^{-1}: 1720,

124 1590, 1478, 1352; ^{1}H-NMR ($CDCl_3$) δ: 8.12 (1H, d, *J* = 9.7 Hz), 7.63 (1H, d, *J* = 2.3

125 Hz), 7.00 (1H, d, *J* = 2.3 Hz), 6.29 (1H, d, *J* = 9.7 Hz), 4.17 (3H, s), 4.16 (3H, s).

126 Peroxyauraptenol (**5**) – Colorless prisms from ether; mp 114-116 °C; EI-MS *m/z*

127 (%): 259, 244, 243, 190 (67), 189 (100), 175 (16), 159, 146, 131 (84), 118, 103; UV

128 MeOH λ_{max}: 248, 257, 322; IR KBr, λ_{max}, cm^{-1}: 3500, 1730, 1610; ^{1}H-NMR δ: 8.46

129 (1H, br s), 7.64 (1H, d, *J* = 9.4 Hz), 7.36 (1H, d, *J* = 8.7 Hz), 6.87 (1H, d, *J* = 8.7 Hz),

130 6.26 (1H, d, *J* = 9.4 Hz), 4.94 (1H, s), 4.88 (1H, s), 4.60 (1H, dd, *J* = 5.4, 7.7 Hz),

131 3.95 (3H, s), 3.27 (1H, dd, *J* = 7.7, 13.8 Hz), 3.16 (1H, dd, *J* = 5.4, 13.8 Hz), 1.91 (3H,

132 s).

133 **Cell Cultures.** Murine macrophages J774A.1 and RAW 264.7 were obtained

from the American Type Culture Collection (Rockville, MD). RAW 264.7 macrophages stably transfected with the NF-κB reporter gene (RAW-Blue™ cells) were purchased from InvivoGen (San Diego, CA). J774A.1 and RAW 264.7 cells were propagated in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine (Life Technologies, Carlsbad, CA), and cultured at 37 °C in a 5% CO_2 incubator (RAW-Blue™ cells cultured in the presence of Zeocin™).

Microculture Tetrazolium (MTT) Assay for Cell Viability. Cells were seeded in 96-well plates at a density of 5×10^3 cells/well. Cells were incubated with or without osthole in the absence or presence of LPS for 24 h. Cell viability was determined using colorimetric MTT assays, as described in a previous report *(11)*.

Enzyme-Linked Immunosorbent Assay (ELISA). J774A.1 macrophages were seeded in 6-well plates at a density of 1×10^6 cells/ml, and then incubated with or without LPS (1 μg/ml) in the absence or presence of osthole for 24 h. The effects of osthole on TNF-α and IL-6 production were measured by ELISA according to the manufacturer's protocol. In brief, 50 μl of biotinylated antibody reagent and 50 μl of supernatant were added to an anti-mouse TNF-α and IL-6 precoated stripwell plate, and incubated at room temperature for 2 h. After washing the plate three times with washing buffer, 100 μl of diluted streptavidin-HRP (horseradish peroxidase)

concentrate was added to each well and incubated at room temperature for 30 min. The washing process was repeated; then 100 µl of a premixed tetramethylbenzidine substrate solution was added to each well and developed at room temperature in the dark for 30 min. Following the addition of 100 µl of stop solution to each well to stop the reaction, the absorbance of the plate was measured by a microplate reader at a 450 nm wavelength.

NO Inhibitory Assay. RAW 264.7 cells were seeded in 24-well plates at a density of 2×10^5 cells/ml, and then incubated with or without LPS (1 µg/ml) in the absence or presence of osthole for 24 h. The effects of osthole on NO production were measured indirectly by analysis of nitrite levels using the Griess reaction *(11).*

NF-κB Reporter Assay. RAW-Blue™ cells (InvivoGen) – RAW 264.7 macrophages which stably express a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-κB, and are resistant to the selectable marker Zeocin – were seeded in 60 mm dishes at a density of 4×10^5 cells/ml, and grown overnight in a 5% CO_2 incubator at 37 °C. After pretreatment with osthole, followed by LPS stimulation for 24 h, the medium was harvested. Medium samples (20 µl) were then mixed with QUANTI-Blue™ (InvivoGen) medium (200 µl) in 96-well plates at 37 °C for 15 min. The results of SEAP activity were assessed by measuring the optical density at 655 nm using an ELISA reader *(11).*

Western Blot Assay. Whole cell lysates were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membranes were incubated in blocking solution – 5% nonfat milk in phosphate buffered saline (PBS) with 0.1% Tween 20 – at room temperature for 1 h. Each membrane was incubated with specific primary antibody at room temperature for 2 h. After washing three times in PBS with 0.1% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody directed against the primary antibody. The membrane was developed by an enhanced chemiluminescence Western blot detection system.

Measurement of Intracellular ROS Production. Intracellular ROS stimulated by LPS was measured by detecting the fluorescence intensity of 2',7'-dichlorofluorescein diacetate (H_2DCFDA) oxidized product (DCF) (Molecular Probes, Eugene, OR). Briefly, J774A.1 macrophages (1×10^6/ml) grown in a phenol red-free RPMI medium for 24 h were then preincubated with 2 μM H_2DCFDA, osthole (10 μg/ml), or NAC (10 mM) at 37 °C for 30 min in the dark; this was followed by adding medium containing LPS for an additional incubation period of 0–180 min. The relative fluorescence intensity of fluorophore DCF, formed by peroxide oxidation of the non-fluorescent precursor, was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm with a CytoFluor 2300 fluorometer (Millipore, Bedford, MA).

Statistical Analysis. All values are given as mean ± SE. Data analysis involved one-way ANOVA with a subsequent Scheffé test.

RESULTS

Effect of Osthole on the Proliferation of Macrophages. Five compounds were isolated from the ethanol extract of seeds of *C. monnieri*, including: (**1**) osthole (yield rate: 43.1%); (**2**) imperatorin (yield rate: 28.4%); (**3**) xanthotoxin (yield rate: 3.3%); (**4**) isopimpinellin (yield rate: 4.2%); and (**5**) peroxyauraptenol (yield rate: 2.5%). Since osthole is the major compound of the ethanol extracts of seeds of *C. monnieri*, the goal of this study was to investigate the effect of osthole on the immune-modulating functions of macrophages. Considering that J774A.1 macrophages are normal immune cells, the dosage of osthole used in this study should not affect their survival. To examine the toxicity of osthole, J774A.1 macrophages were treated with osthole at various concentrations (0, 1, 3 and 10 μg/ml) in the absence or presence of LPS (1 μg/ml) for 24 h. Cell viability was analyzed by MTT assay. The results revealed that the cell survival rate did not differ obviously when J774A.1 macrophages were treated with ≤ 10 μg/ml osthole (data not shown). At these osthole concentrations, even the presence of LPS at 1 μg/ml did not affect the cell survival rate (data not shown). Cinnamaldehyde was used as a positive control for

reducing cell viability *(12)*. In addition, osthole was found to be not toxic to another murine macrophage cell line, RAW 264.7, at concentrations ≤ 10 μg/ml (data not shown).

Osthole Decreases the Production of TNF-α and NO by LPS-Activated Macrophages. TNF-α is one of the important cytokines produced by activated macrophages in response to LPS stimulation *(11, 12)*. In this study, the TNF-α levels in the supernatant of J774A.1 macrophages cultured with osthole at various doses were measured by ELISA. The experimental results indicated that osthole treatment did not alter the background level of TNF-α in J774A.1 macrophages, but it decreased the production of TNF-α by LPS-activated J774A.1 macrophages in a dose-dependent manner (**Figure 1A**). In addition to TNF-α expression, we investigated the inhibitory effects of osthole on the LPS-induced production of NO. The NO levels in the supernatant of RAW 264.7 macrophages cultured with osthole at various doses were measured by Griess reaction. The experimental results indicated that osthole treatment did not alter the background level of NO in RAW 264.7 macrophages, but it decreased the production of NO by LPS-activated RAW 264.7 macrophages in a dose-dependent manner (**Figure 1B**). We next investigated the effect of osthole on the protein expression of COX-2. Treatment with osthole reduced the expression of COX-2 protein obviously when compared with LPS treatment alone (**Figure 1C**). By contrast,

treatment with osthole increased IL-6 expression in LPS-activated J774A.1 macrophages (**Figure 1D**). These results indicated that osthole modulated inflammatory mediator expression in LPS-activated macrophages.

Osthole Inhibits the Activation of MAPK in LPS-Activated Macrophages.

LPS potently induces macrophage activation and the production of pro-inflammatory cytokines by the activation of TLR4 through many signaling pathways, including the MAPK signaling pathways *(11)*. To examine whether the effects of osthole on LPS-induced macrophages are associated with MAPK signaling cascades, J774A.1 macrophages were treated with LPS in the presence or absence of osthole. The phosphorylation levels of MAPK, including p38, JNK1/2 and ERK1/2, were determined by Western blot analysis. The experimental results showed that osthole inhibited the phosphorylation levels of p38 obviously, and JNK1/2 modestly, in LPS-activated macrophages (**Figure 2A** and **2B**). In contrast, osthole increased the phosphorylation levels of ERK1/2 in LPS-activated macrophages (**Figure 2C**). These results indicated that osthole modulated the activation of the MAPK signaling cascades in LPS-activated macrophages.

Osthole Inhibits the Activation of NF-κB in LPS-Activated Macrophages.

In resting macrophages, NF-κB is sequestered in the cytoplasm as an inactive precursor complex by its inhibitory protein, IκB. Upon LPS stimulation, IκB is

248 phosphorylated by IκB kinase (IKK), ubiquitinated, and rapidly degraded via
249 proteasomes to release NF-κB *(13)*. We investigated whether osthole could inhibit
250 LPS-stimulated phosphorylation of IKK-α in macrophages. We found that osthole
251 inhibited the phosphorylation of IKK-α obviously in LPS-activated macrophages
252 (**Figure 3A**). We further investigated whether osthole could inhibit the
253 LPS-stimulated phosphorylation of IκB-α in macrophages. The results showed that
254 the phosphorylation of IκB-α was reduced by osthole in 30-min and 60-min
255 LPS-stimulated macrophages (**Figure 3B**). In addition, LPS induced degradation of
256 IκB-α protein obviously; in contrast, treatment with osthole reversed the degradation
257 of IκB-α protein upon LPS stimulation (**Figure 3B**). We also attempted to test
258 whether osthole could inhibit NF-κB activity in LPS-activated macrophages. Using
259 NF-κB-dependent alkaline phosphatase reporter cells, we demonstrated that NF-κB
260 transcriptional activity in LPS-stimulated macrophages was reduced by osthole
261 (**Figure 3C**). These results indicated that osthole could inhibit the activation of the
262 NF-κB signaling cascades in LPS-activated macrophages.

263 **Osthole Inhibits the Phosphorylation of PKC-α and PKC-ε in**
264 **LPS-Activated Macrophages.** PKC is one of the components of the TLR4 signaling
265 pathway, and thereby plays some roles in macrophage activation in response to LPS
266 *(9)*. We tested the effect of osthole on LPS-induced PKC activation. The

phosphorylation levels of PKC-α, PKC-δ and PKC-ε were increased after LPS stimulation. LPS-mediated phosphorylations of PKC-α and PKC-ε were reduced by osthole; however osthole did not affect the phosphorylation of PKC-δ (**Figure 4**).

Osthole Inhibits ROS Production in LPS-Activated Macrophages. In our previous study, we noted that ROS play important roles in LPS-mediated cytokine expression *(12)*. To test whether osthole has antioxidant activity, intracellular ROS production in LPS-stimulated macrophages was measured. We found that LPS stimulation of cells rapidly induced ROS production when compared with that of control cells. In contrast, pretreatment with *N*-acetyl cysteine (NAC), a potent antioxidant, quickly reduced the production of LPS-induced ROS. Interestingly, we found that cells pretreated with osthole obviously reduced LPS-stimulated ROS production (**Figure 5**). This result indicated that osthole possesses antioxidant activity.

DISCUSSION

Osthole is a coumarin compound present in plant medicines as an active component, and possesses a variety of pharmacological activities including anti-osteoporotic *(14)*, anti-proliferative *(3)*, anti-allergic *(15, 5)*, anti-seizure *(16)*, anti-diabetic *(17)*, and anti-microbial effects *(18, 19)*. The innate immune system plays essential roles in host defense against infections such as bacteria and viruses.

When macrophages are activated by pathogen associated molecular pattern, inflammatory cytokines such as TNF-α and IL-6 are generated as an initial immune response *(8)*. Recent reports have shown that osthole inhibits inflammatory diseases such as arthritis and hepatitis through modulating inflammatory cytokines: TNF-α, interferon-gamma (IFN-γ) and interleukins *(15, 19, 20, 21)*. However no studies have been conducted on the cellular mechanism involved in the osthole-mediated regulation of pro-inflammatory mediators in LPS-stimulated macrophages. Here we provide evidence for the potential role of osthole in reducing LPS-induced inflammation through inactivation of NF-κB, p38, JNK1/2, PKCs and ROS pathways.

A report on the cytotoxicity of osthole in a carcinoma cell line *(22)* indicated that osthole inhibited the growth of a human cervical tumor cell line, HeLa, with IC_{50} values of 19 and 15 μg/ml for 24 and 48 h, respectively. In addition, osthole could induce apoptosis in P-388 D1 cells *in vivo* and prolong the survival days of P-388 D1 tumor-bearing CDF1 mice. Another study demonstrated that osthole prevents anti-fas antibody-induced hepatitis in mice by inhibiting caspase-3 activity *(20)*. Although the cell line and dosage in these studies were different, it appears that osthole exerts certain actions that enhance cell survival. To exclude the possibility that osthole may affect the viability of macrophages, the toxicity of osthole in macrophage cells was investigated in our study. Our results indicate that osthole is not toxic to murine

macrophage cell lines J774A.1 and RAW 264.7 at concentrations ≤ 10 μg/ml.

TNF-α and NO are produced by activated macrophages, as well as by many other cell types. In this study, the inhibitory effects of osthole on TNF-α secretion, NO production and COX-2 expression were observed in LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner (**Figure 1A-C**). Our results suggest that the inhibitory capacity of osthole in LPS-induced pro-inflammatory molecule expression may offer an advantage in protecting the host from endotoxic shock. Previous research has shown that osthole, isolated from *Clausena guillauminii* (Rutaceae), had an inhibitory effect on iNOS protein expression at 6 μg/ml in mouse RAW 264.7 macrophages *(23)*. Our study demonstrated that osthole exhibited an obvious inhibiting effect on NO production. This suggests that osthole may reduce NO production in LPS-stimulated RAW 264.7 macrophages through inhibition of iNOS protein expression.

Protein kinase C (PKC) is one of the signaling molecules in an LPS-mediated inflammatory response *(24)*. Previous reports have demonstrated that PKC-α modulates LPS- and IFN-γ-induced COX-2 expression *(24)*. PKC-ε is required for macrophage activation and defense against bacterial infection *(25)*. Classical (cPKC) isoenzymes, especially PKC-β, mediate the upregulation of iNOS expression and NO production in activated macrophages *(26)*. In addition, LPS regulates downstream

MAPKs and NF-κB activation through PKC signaling in macrophages *(27)*. According to our previous study, inhibition of PKC-α by Gö6976 reduced JNK1/2 and NF-κB activation as well as TNF-α and NO expression in LPS-stimulated macrophages *(11)*. Another investigation also demonstrated that PKC-ε is involved in JNK1/2 activation that mediates LPS-induced TNF-α *(27)*. These findings suggest that PKC isoenzymes play an important role in inflammatory response and in the regulation of downstream MAPKs and NF-κB signaling, which are involved in LPS-induced cytokine secretion and pro-inflammatory molecule expression in macrophages. The present study demonstrated that LPS-mediated PKC-α and -ε phosphorylation was reduced by osthole, indicating that osthole inhibited PKC-α and -ε activation upon LPS stimulation. Furthermore, LPS-induced TNF-α secretion, NO production and COX-2 expression were downregulated by osthole. These results suggest that osthole-mediated downregulation of TNF-α, NO, and COX-2 expression may be due, at least in part, to inhibition of PKC-α and PKC-ε activation.

MAPKs regulate inflammatory mediator expression in LPS-activated macrophages (*11*, *12*). In this study we found that osthole inhibited p38 activation obviously, and JNK1/2 modestly, in LPS-activated macrophages. Inhibition of p38 and JNK1/2 reduced NF-κB activation in LPS-activated macrophages (*11*). The inhibitory effects of osthole on LPS-induced NF-κB activation as well as TNF-α, NO,

and COX-2 expression may, at least in part, due to down-regulation of p38 and JNK1/2 activation. Interestingly, osthole increased IL-6 expression in LPS-activated macrophages (**Figure 1D**). We have demonstrated that inhibition of ERK1/2 pathway reduced IL-6 expression in LPS-stimulated macrophages (*12*). This result indicated that LPS induces IL-6 expression through activation of ERK1/2. In this study, we found that osthole increased IL-6 expression and ERK1/2 phosphorylation. According to these results we speculated that osthole-mediated up-regulation of IL-6 expression in LPS-activated macrophages may be due, at least in part, to the increasing of ERK1/2 phosphorylation. ROS played an important role in LPS-mediated signaling and cytokine gene expression in macrophages (*28*). The present study has demonstrated that osthole inhibited LPS-induced ROS release in macrophages; recently, osthole also has been reported to reduce ROS release in 1-methyl-4-phenylpyridinium ion-stimulated rat adrenal pheochromocytoma PC12 cells *(29)*.

In conclusion, our results suggest that osthole inhibits LPS-induced TNF-α, NO and COX-2 expression through, at least in part, inhibition of PKC-α, PKC-ε, JNK1/2, p38, ROS and NF-κB pathways (**Figure 6**). As a powerful anti-inflammatory, osthole could offer an advantage in protecting the host from endotoxic shock. To achieve a more comprehensive understanding of the details of such a mechanism, further *in vivo*

362 investigation is clearly warranted.

363

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FIGURE LEGENDS

Figure 1. Effect of osthole on the expression of inflammatory mediators. (**A**) J774A.1 macrophages (1×10^6/ml) were pretreated with osthole or DMSO (vehicle) for 30 min, followed by stimulating with LPS (1 μg/ml) for 6 h. TNF-α concentration in culture medium was assayed by ELISA. Data are expressed as mean ± SE from three separate experiments. *$p < 0.05$; **$p < 0.01$. (**B**) RAW 264.7 macrophages (5×10^5/ml) were pretreated with osthole or DMSO (vehicle) for 30 min, followed by stimulating with LPS (1 μg/ml) for 24 h. Nitric oxide concentration in culture medium was assayed by Griess reaction. Data are expressed as mean ± SE from three separate experiments. *$p < 0.05$; **$p < 0.01$. (**C**) RAW 264.7 macrophages (5×10^5/ml) were pretreated with osthole or DMSO (vehicle) for 30 min, followed by stimulating with LPS (1 μg/ml) for 24 h. COX-2 expression was assayed by Western blot. The result of one of three separate experiments is shown. Quantification of COX-2 protein was normalized to actin using a densitometer. (**D**) J774A.1 macrophages (1×10^6/ml) were pretreated with osthole or DMSO (vehicle) for 30 min, followed by stimulating with LPS (1 μg/ml) for 6 h. IL-6 concentration in culture medium was assayed by ELISA. Data are expressed as mean ± SE from three separate experiments. *$p < 0.05$; **$p < 0.01$.

Figure 2. Effect of osthole on MAPKs phosphorylation. J774A.1 macrophages (1 × 10^6/ml) were pretreated with osthole or DMSO (vehicle) for 30 min, followed by stimulating with LPS (1 μg/ml) for 0–60 min. Phosphorylation levels of (**A**) p38, (**B**) JNK1/2, and (**C**) ERK1/2 were analyzed by Western blot. The result of one of three separate experiments is shown. Quantification of p38, JNK1/2 and ERK1/2 phosphorylation was normalized to p38, JNK1, and ERK1/2, respectively, using a densitometer.

Figure 3. Effect of osthole on NF-κB activation. RAW 264.7 macrophages (1 × 10^6/ml) were pretreated with osthole or DMSO (vehicle) for 30 min, followed by stimulating with LPS (1 μg/ml) for 0–60 min. The phosphorylation level of IKK-α (**A**) and phosphorylation level of IκB-α and protein level of IκB-α (**B**) were analyzed by Western blot. The result of one of three separate experiments is shown. Quantification of IKK-α and IκB-α phosphorylation was normalized to IKK-α and actin, respectively, using a densitometer. (**C**) RAW- Blue™ cells were pretreated with osthole or DMSO (vehicle) for 30 min, followed by stimulating with LPS (1 μg/ml) for 24 h. SEAP activity was measured by QUANTI-Blue™. Data are expressed as mean ± SE from three separate experiments. *$p < 0.05$; **$p < 0.01$.

Figure 4. Effect of osthole on PKC phosphorylation. J774A.1 macrophages (1×10^6/ml) were pretreated with osthole or DMSO (vehicle) for 30 min, followed by stimulating with LPS (1 μg/ml) for 0–60 min. The phosphorylation levels of PKC-α, PKC-δ, and PKC-ε were analyzed by Western blot. The result of one of three separate experiments is shown.

Figure 5. Effect of osthole on ROS production. J774A.1 macrophages were pretreated with CM-DCFDA (2 μM) for 30 min, followed by substitution with fresh medium. Cells were treated with osthole (10 μg/ml), NAC (10 mM) or DMSO (vehicle) for 30 min, followed by incubating with LPS (1 μg/ml) for 0–180 min. The fluorescence intensity of fluorophore CM-DCF was detected as described in "Materials and Methods." Data are expressed as relative mean fluorescence intensity (MFI) ± SE from three independent experiments.

Figure 6. Proposed diagram of signaling pathways regulating anti-inflammatory effect of osthole.