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Effects of *Oenanthe javanica* on Transcriptional Regulation of COX-2 by Inhibiting Translocation of p65 Subunit in LPS-Stimulated Murine Peritoneal Macrophages

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Abstract The extracts of *Oenanthe javanica* were evaluated for their effects on the expression of cyclooxygenase-2 (COX-2), which is mediated by the translocation of the p65 subunit into the nucleus. Fractions of ethyl acetate and chloroform from 80% ethanol extracts of *O. javanica* exhibited inhibitory effects on the secretion of tumor necrosis factor-α (TNF-α) from lipopolysaccharide (LPS)-stimulated peritoneal macrophages; however, the aqueous- and hexane-fractions showed no significant effect. The ethyl acetate- and chloroform-fractions also reduced the COX-2 enzyme levels after 24-hr treatment. RT-PCR showed that the mRNA levels of COX-2 decreased following treatment with these fractions, suggesting that COX-2 expression is transcriptionally regulated by these extracts. We examined the effects of the chloroform- and ethyl acetate-fractions on the cytosolic activation of nuclear factor-κB (NF-κB, p65 subunit) and on the degradation of inhibitor-κBα (I-κBα) in order to determine the mechanism of COX-2 regulation. The LPS-stimulated activation of the p65 subunit was significantly blocked upon the addition of 50 μg/mL of these fractions, and the cytosolic I-κBα degradation process was simultaneously inhibited. These findings suggest that the inhibition of COX-2 expression by the ethyl acetate-and chloroform-fractions may result from the inhibition of p65 translocation by blocking the degradation of I-kBa; this may be the mechanistic basis for the anti-inflammatory effects of *O. javanica*.

Keywords: *Oenanthe javanica*, NF-kB, p65 subunit, COX-2, I-kB, TNF-α

Introduction

The expression of inducible cyclooxygenase-2 (COX-2), a key enzyme in prostaglandin (PG) biosynthesis, is known to be upregulated in acute/chronic inflammatory diseases, transformed cells, and malignant tissues of lung or colorectal cancer (1-3). Aspirin (acetylsalicylic acid), one of the most commonly used non-steroidal anti-inflammatory drugs (NSAIDs), exerts its anti-inflammatory effects by inhibiting COX activity. It has been reported that the significant reductions in the risk of certain cancer development found to be associated with NSAID treatment may be related to decreases in COX-2 expression and subsequent PG production (4). COX, which exists as two isoforms, is the rate-limiting enzyme in PG production. These isoforms, constitutive COX-1 and inducible COX-2, originate from two distinct genes but are structurally conserved (5, 6). COX-1 serves as a constitutive enzyme responsible for PG synthesis and it is essential for maintaining fluid and electrolyte homeostasis, gastric acid secretion, and platelet aggregation. COX-2 activation is induced by several stimuli, including tumor promoters, growth factors, tumor necrosis factor-α (TNF-α), and lipopolysaccharide (LPS) (7).

Nuclear factor-κB (NF-κB), the key molecule in the inflammatory response, is a generic term for a dimeric transcription factor that is formed by the dimerization of proteins in the Rel family (8). NF-κB exerts its activity by regulating the expression of genes that encode inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes, such as COX-2 and inducible nitric oxide synthase (iNOS). The activation of NF-κB is usually inhibited in the cytoplasm via its association with an endogenous inhibitory protein, I-κB (an inhibitor of NF-κB). Upon I-κB kinase (IKK) or mitogen-activated protein kinase (MAPK) activation, I-κB undergoes phosphorylation and degradation in the cytoplasm. This facilitates the release of the p65 subunit from the NF-κB complex, allowing p65 to move to the nucleus of the cell where it binds to the 10-base pair consensus site in DNA promoter regions and subsequently induces transcription (9). In macrophages, NF-κB cooperates with other transcription factors to coordinate the expression of genes related to the inflammatory response such as TNF-α, iNOS, and COX-2 (3).

Currently, there is a strong interest in the development of new anti-inflammatory agents that regulate NF-κB activation and COX-2 expression from plants used in traditional medicine. *Oenanthe javanica* (dropwort) has been widely used as a medicinal food for the treatment of

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jaundice, hypertension, and polydipsia for many years in China, Japan, and Korea. In our previous report, 600 μg/mL of the ethanol extract of *O. javanica* exhibited an anti-inflammatory effect by regulating the activation of NF-kB (10). Therefore, the present study was designed to investigate the anti-inflammatory effects of partially purified fractions from the ethanol extract of *O. javanica* and their putative mechanisms of action based on NF-κB and COX-2 expression in LPS-stimulated murine peritoneal macrophages.

Materials and Methods

Sample and extraction *O. javanica* was obtained from Hanwoomool Co. (Jeonnam, Korea) and authenticated by Dr. Woojin Jun at Chonnam National University; its voucher specimen was deposited at the same Institute. Dried *O. javanica* was extracted with 20 volumes of 80% ethanol and filtered, followed by the evaporation of filtrate under vacuum conditions. The crude extract was then resuspended in distilled water and partitioned in the following order: hexane, chloroform, ethyl acetate, and water to yield fractions soluble in these respective solvents (Fig. 1). The fractions were evaporated under vacuum conditions, dried, and stored at -20°C until use.

Preparation of peritoneal macrophage ICR mice were given an intraperitoneal (i.p.) injection of 2 mL of 3% thioglycollate 4 days prior to sacrifice. Peritoneal macrophages were collected by peritoneal lavage using 7 mL of Hank's balanced salt solution (HBSS, Sigma, St. Louis, MO, USA). The cells were centrifuged, washed, and then suspended in Dulbecco's modified Eagle's medium (DMEM, BioWhitaker, Walkersville, MD, USA) supplemented with 10% heat-inactivated (55°C, 30 min) fetal bovine serum (FBS, BioWhitaker), 2 mM L-glutamine (Sigma), 100 U/mL penicillin and 100 mg/mL streptomycin (BioWhitaker) at 37°C in an atmosphere of 5% CO_2-95% air. The cells were purified by adherence to tissue culture plates for 2 hr.

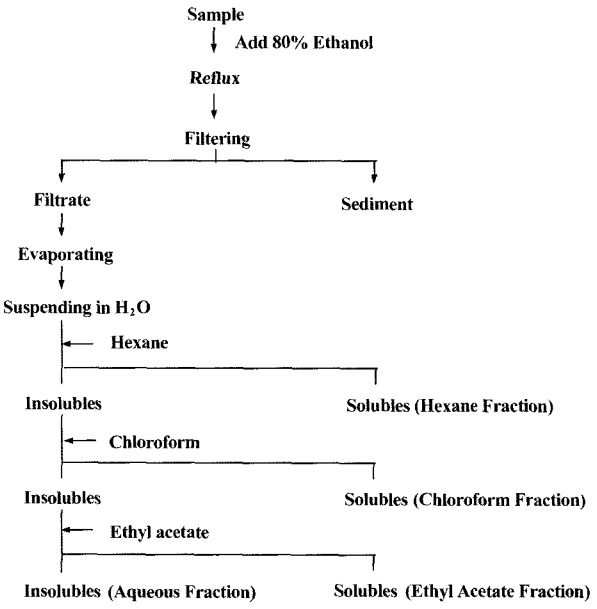

Fig. 1. Extraction scheme of *Oenanthe javanica*.

Cell viability assay The number of viable cells was determined by the ability of mitochondria to convert MTT (3-[4,5-2-yl]-2,5-diphenyl tetrazolium bromide) to formazan dye. The macrophages were cultured in a 96-well plate at 1×10^4 cells/well. After 24-hr incubation with various concentrations of each fraction, the medium was removed and 50 mg/mL of MTT in Hank's balanced salt solution (Sigma) was added to each well. Following further incubation of the cells for 4 hr at 37°C under a humidified atmosphere of 5% CO_2-95% air, the supernatants were removed and the resulting formazans were dissolved in 100 mL of DMSO. The absorbance was then measured at 570 nm on a microplate reader (BioTek, Winooski, VT, USA) using a reference wavelength of 665 nm.

Measurement of TNF-α levels The macrophages were plated at 2×10^5 cells in a 24-well plate and stimulated with 1 μg/mL LPS in the presence or absence of each fraction, which was dissolved in DMSO. The level of TNF-α in the cultured media of the macrophages was determined by an enzyme-linked immunosorbent assay (ELISA) as described by Chouaib *et al.* (11). The anti-TNF-α monoclonal antibody, biotinylated polyclonal antibody, and recombinant human TNF-α were obtained from R&D Systems (Minneapolis, MN, USA).

Western blot analysis Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analyses were performed according to the method described by Lee *et al.* (10) with a slight modification. Briefly, the macrophages were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 0.2% SDS, 1% NP-40, 5 mM sodium fluoride, and a protein inhibitor cocktail (Roche, Penzberg, Germany). The cell lysates were centrifuged at 12,000×*g* for 30 min to remove debris, fractionated by 12% gel electrophoresis, and electrophoretically transferred onto nitrocellulose paper, which was subsequently incubated with a polyclonal anti-COX-2 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), a monoclonal anti-I-κBα antibody (1:200, Cell Signaling Technology, Danvers, MA, USA), or a monoclonal anti-actin antibody (1:100, Santa Cruz Biotechnology). Following incubation with a primary antibody, the papers were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000, Santa Cruz Biotechnology). Finally, the papers were developed using an electrogenerated chemiluminescence (ECL) detection kit (Amersham, Piscataway, NJ, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was isolated from cells using a total RNA isolation kit (Qiagen, Valencia, CA, USA). One mg of total RNA obtained from the macrophages was reverse-transcribed using an oligo(dT) 18mer as a primer and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in order to produce the cDNAs. PCR was performed using the selective primers for the mouse COX-2 (sense primer: 5'-CAGAACCGCATTGCCTCTG-3'; antisense primer: 5'-CAGTTCCATGACATCGAT-3'; 389 bp) and GAPDH genes (sense primer: 5'-ACAGCCGCAT

CTTCTTGTGCAGTG-3'; antisense primer: 5'-GGCCTT GACTGTGCCGTTGAATTT-3'; 225 bp) under the following conditions for 35 cycles: denaturation at 97°C for 10 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min. The band intensities of the amplified DNAs were compared after visualization on a UV transilluminator.

Statistical analysis All data are expressed as means ± standard deviation (SD). The paired Student's *t*-test was used to assess significant differences among the treatment groups. The statistical significance was set at $p<0.05$.

Results and Discussion

Cell viability and TNF-α secretion The cytotoxic effects of *O. javanica* fractions on macrophages were initially evaluated. The cells were treated for 24 hr with various concentrations, ranging from 1 to 1,000 μg/mL, of each *O. javanica* fraction and were then subjected to the MTT assay. As shown in Table 1, the aqueous- (1,000 μg/mL) and hexane- (200 μg/mL) fractions had no effect on cell survival. On the other hand, the cell viabilities were markedly reduced to 63 and 67% compared to that of the control group when the cells were treated with 100 μg/mL of the ethyl acetate- or chloroform-fraction, respectively. Thus, the concentration of the ethyl acetate- or chloroform-fraction applied to the cells did not exceed 50 μg/mL in all subsequent experiment. Co-incubation of cells with *O. javanica* fractions and 1 μg/mL LPS did not alter the cell growth compared to the control group or to cells treated with LPS alone (data not shown). These results assured us that the effects observed with the *O. javanica* fractions in other experiments were not caused by a reduction in cell viability under each condition. Wang *et al.* (12) recently reported that 1 mg/mL of *O. javanica* flavones, subsequently fractionated with 80% ethanol and ethyl acetate, did not influence the cell viability of HepG2.2.15 cells. According to the cell viability assays used in our study, the ethyl acetate-fraction was toxic at a lower concentration than that reported by Wang *et al.* (12). This difference may result from the fact that our study employed peritoneal macrophages rather than a cell line that is better adjusted to harsh environmental conditions. Therefore, peritoneal macrophages may be more sensitive in their response to stimuli and reveal the cytotoxicity at a lower concentration.

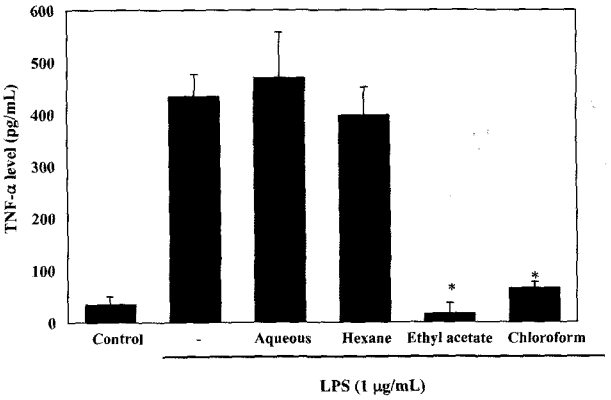

Fig. 2. Effect of four fractions extracted from *Oenanthe javanica* on TNF-α secretion in LPS-stimulated peritoneal macrophages. 2×10^5 cells/well in a 24-well plate were stimulated with 1 μg/mL LPS and further incubated with aqueous- (1,000 μg/mL), hexane- (200 μg/mL), ethyl acetate- (50 μg/mL), and chloroform (50 μg/mL)-fractions for 24 hr. The data is represented as the mean±SD of triplicate experiments. *indicates a significant difference from the LPS-stimulated group at $p<0.05$.

The TNF-α secreted from macrophages or immune cells is regarded as a whole marker for the cellular response in the LPS-induced inflammatory process. Therefore, we assessed the effect of *O. javanica* fractions on TNF-α production in LPS-stimulated peritoneal macrophages. LPS treatment significantly increased TNF-α production by 12.4-fold compared to that of the untreated control (Fig. 2). The aqueous- and hexane-fractions had no significant effect on TNF-α production, while both the ethyl acetate- and chloroform-fractions noticeably diminished the levels of LPS-induced TNF-α after 24-hr incubation.

Effect of *O. javanica* fractions on COX-2 protein and mRNA expression COX, the rate-limiting enzyme in the conversion of arachidonic acid to PGE_2, is the primary target of NSAIDs and anti-inflammatory phytochemical agents. Recent reports have also suggested that COX-2 expression may be related to carcinogenesis in certain

Table 1. Effect of *Oenanthe javanica* fractions on cell viability measured by MTT assay in LPS-stimulated peritoneal macrophages

	Concentration (μg/mL)									
	Control	10	20	40	60	80	100	200	500	1000
Aqueous fraction	100	101±8[1)]	103±2	105±4	101±4	98±6	94±1	95±7	97±2	96±5
Hexane fraction	100	99±3	99±1	103±4	101±3	101±8	97±5	99±6	89±2[a,2)]	79±5[a]
Ethyl acetate fraction	100	102±2	105±2	99±5	98±1	89±4[a]	63±4[a]	39±6[a]	0	0
Chloroform fraction	100	97±3	95±5	101±2	97±7	84±2[a]	67±5[a]	43±3[a]	0	0

[1)]Value represents the percentage of control reported as the mean±SD from triplicate experiments.
[2)]Values designated with a letter in the row are significantly different from the control group at $p<0.05$.

tissues or cells. In macrophage cells, it is known that LPS treatment induces expression of the COX-2 protein through up-regulation of transcriptional factors such as NF-κB or its subunit, p65 (13). In the present study, we examined the effect of *O. javanica* fractions on LPS-induced COX-2 protein levels by Western blot analysis. The aqueous- (1,000 μg/mL) and hexane- (200 μg/mL) fractions did not affect COX-2 expression (Fig. 3); whereas, 50 μg/mL of the ethyl acetate- or chloroform-fraction significantly inhibited COX-2 expression. When the mRNA expression level of COX-2 was assessed, both the ethyl acetate- and chloroform-fractions, unlike the aqueous- and hexane-fractions, greatly reduced the mRNA levels of COX-2, which suggests that the inhibited expression of COX-2 by these fractions is regulated at transcriptional level (Fig. 4). Most inflammatory reactions involve the genetic expression of proteins such as COX-2, iNOS, and other proinflammatory cytokines including IL-1β, IL-6, and TNF-α. Further evidence suggests that oxidative stress or the cellular redox state may be associated with COX-2 induction through NF-κB and/or other signaling pathways (14, 15). Since *O. javanica* contains high levels of antioxidative flavonoids and vitamin E, these fractions may inhibit COX-2 induction by scavenging oxidative stress (16, 17). However, in the present study, the antioxidative activities of these fractions and their respective iNOS inductions were not measured. Thus, we cannot comment any further on the involvement of anti-oxidative components in the ethyl acetate- and chloroform-fractions in elucidating the mechanism of action for the inhibition of COX-2 expression.

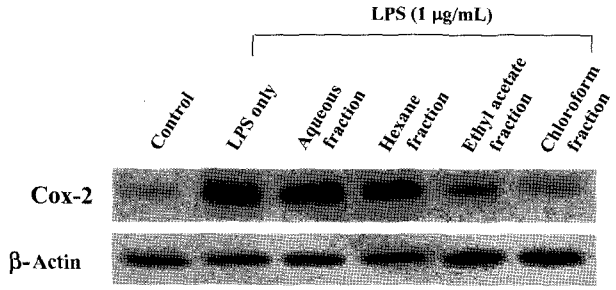

Fig. 3. Effect of four fractions extracted from *Oenanthe javanica* on COX-2 protein expression in LPS-stimulated peritoneal macrophages. COX-2 protein expression in LPS-stimulated peritoneal macrophages was determined by Western immunoblot analysis. Thirty micrograms of total protein was applied to 12% PAGE; β-actin was detected as an internal control.

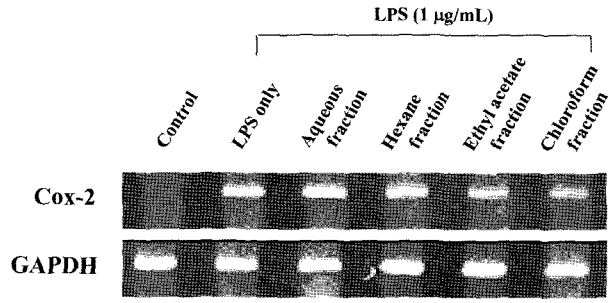

Fig. 4. Effect of four fractions extracted from *Oenanthe javanica* on COX-2 mRNA transcription in LPS-stimulated peritoneal macrophages. COX-2 mRNA expression in LPS-stimulated peritoneal macrophages was determined by RT-PCR analysis. GAPDH was used as an internal control.

Effect of *O. javanica* fractions on p65 expression and I-κBα degradation NF-κB is essential for the expression of COX-2 and other inflammatory cytokines. NF-κB, which consists of a heterotrimer of p50, p65, and I-κBα, is present in its resting state in the cytoplasm of all cells. The phosphorylation, ubiquitination, and degradation of I-κBα lead to the release of the p50/p65 heterodimer, which then translocates into the nucleus, binds to its specific 10-base pair consensus site (9, 18), and regulates the genetic expression of key proinflammatory cytokines and COX-2. In our study, and in studies reported by other authors, LPS treatment alone significantly increased the cytosolic level of p65 and simultaneously caused cytosolic degradation of I-κBα in macrophages (Fig. 5). The increased levels of cytosolic p65 and I-κBα degradation were reversed by treating the macrophages with 50 μg/mL of both the ethyl acetate- and chloroform-fractions, suggesting that these fractions inhibit NF-κB activation by preventing I-κBα degradation and the subsequent nuclear translocation of p65. These results imply that the phosphorylation and subsequent degradation of I-κBα may be the pharmacological target of *O. javanica*. Furthermore, two major kinase-mediated signaling pathway enzyme complexes, MAPK and IKK, are activated by LPS stimulation. These complexes are capable of inducing various upstream signals to activate the activator protein-1 (AP-1) and NF-κB transcription factors (8). The IKK complex can be activated by a variety of upstream kinases such as protein kinase C and tyrosine kinase family members (19, 20). Thus, both the ethyl acetate- and chloroform-fractions may also act on these upstream kinases. Because LPS induces both the NF-κB and MAPK signaling pathways, MAPK is another potential factor that may be affected by exposure to the

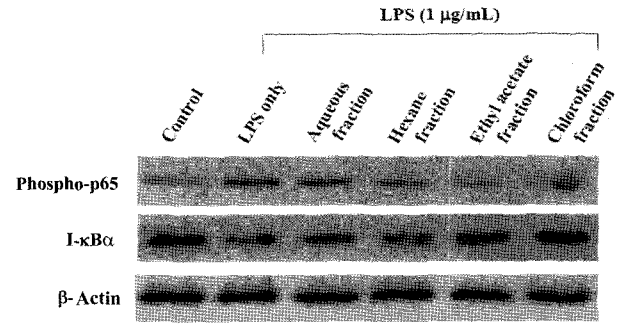

Fig. 5. Effect of four fractions extracted from *Oenanthe javanica* on the translocation of the p65 subunit into the nucleus and on the cytosolic degradation of I-kBa. Peritoneal macrophages were stimulated with 1 μg/mL LPS and incubated for 30 min with each fraction. The cell extract was assayed for the p65 subunit and cytosolic I-κBα using Western blot analysis. Thirty micrograms of total protein was applied to 12% PAGE; β-actin was detected as an internal control.

ethyl acetate- and chloroform-fractions. Further studies are needed to determine which kinase(s) is inhibited by these fractions.

Various active compounds from *O. javanica* have been identified, including isorhamnetin, hyperoside, caffeic acid, coumarin, and persicarin (21-23). Previous reports have demonstrated that caffeic acid and coumarin derivatives exert anti-inflammatory effects on various types of tissues *in vitro* and *in vivo* (24, 25). Further research is currently underway to identify the compounds responsible for the COX-2 inhibition and NF-κB inactivation observed in the present study.

In conclusion, the ethyl acetate- and chloroform-fractions among partially purified extracts from *O. javanica* affected the normalization of TNF-α levels in LPS-stimulated peritoneal macrophages, which suggests that these fractions may be candidates for anti-inflammatory reagents. The present study demonstrated that the ethyl acetate- and chloroform-fractions at 50 μg/mL were capable of regulating the genetic expression of COX-2 at the transcriptional level. This transcriptional regulation may be the result of the inhibition of the nuclear translocation of p65, a potent transcription factor, through the blockade of I-κBα degradation. This is one possible mechanism for the transcriptional regulation of COX-2 by the ethyl acetate- and chloroform-fractions extracted from *O. javanica*.

Acknowledgments

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