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Ethanol Extract of *Oenanthe javanica* Modulates Inflammatory Response by Inhibiting NF-κB Mediated Cyclooxygenase-2 Expression in RAW 264.7 Macrophage

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Abstract Effect of *Oenanthe javanica* ethanol extract (OJE) on nuclear factor-κB (NF-κB)-mediated inflammatory reaction in RAW 264.7 macrophage cells was investigated. The OJE dose-dependently inhibited secretions of tumor necrosis factor-α (TNF-α) and prostaglandins E_2 (PGE_2) from lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and blocked LPS-induced expression of cyclooxygenase-2. To clarify mechanistic basis for its inhibitions of NF-κB and activator protein-1 (AP-1) activations, effects of OJE on activations of NF-κB and AP-1 genes by luciferase reporter activity were examined. The LPS-stimulated activations of NF-κB and AP-1 were significantly blocked by 400 and 600 μg/mL of OJE, implicating that OJE might regulate gene expression through more than one signaling pathway. Cytosolic degradation of I-κBα was inhibited by OJE dose-dependently, indicating that the nuclear translocation of p65 was inhibited by OJE. These findings suggest that the inhibition of LPS-stimulated COX-2 expression by OJE is due to its inhibition of NF-κB activation by blocking I-κBα degradation, which may be mechanistic basis of anti-inflammatory effects of OJE.

Keywords: *Oenanthe javanica*, PGE_2, NF-κB, COX-2, inflammation, I-κBα

Introduction

Prostaglandins E_2 (PGE_2) generated from arachidonic acid through cyclooxygenase (COX) pathway is a major mediator in the regulation of inflammation and immune function (1). The interplay between PGE_2 and other local factors, including inflammatory cytokines, is likely to influence the outcome of inflammatory and immune responses in many cellular systems. The COX, which exists as two isoforms, is the rate-limiting enzyme in prostaglandin production. These isoforms, namely constitutive COX-1 and inducible COX-2, originate from two distinct genes, but are structurally conserved (2, 3). The COX-1 serves as a constitutive enzyme responsible for prostaglandin synthesis that is essential for fluid and electrolyte homeostasis, gastric acid secretion, and platelet aggregation, while COX-2 is induced by inflammatory stimuli such as tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) (4).

Extensive research during the last few years has shown that most inflammatory agents mediate their effects through the activation of nuclear factor κB (NF-κB) and that most anti-inflammatory agents suppress NF-κB activation. Activation of NF-κB has been recently reported to actually participate in the transcriptional activation of COX-2 gene induced by interleukin (IL)-1, TNF-α, and LPS (5-7). Furthermore, the LPS-induced activation of the COX-2 gene has been shown to be mediated by the inhibitor κB (I-κB) kinase (IKK), which specifically catalyzes I-κB phosphorylation, followed by its degradation and the subsequent NF-κB nuclear translocation, leading to the stimulation of the *cis*-acting κB element-mediated transcription (8). In macrophages, NF-κB, in cooperation with other transcriptional factors, coordinates the expression of genes related to inflammation process such as TNF-α, inducible nitric oxide synthase (iNOS), and COX-2 (9).

Oenanthe javanica, umbelliferae, has been widely used as a medicinal food in China, Japan, and Korea for treatments of jaundice, hypertension, and polydipsia diseases for many years. Although its effect on anti-peroxidation in hepatic tissue was very recently reported, little information is available about the effect of *O. javanica* extract on inflammatory process or how the putative mechanism might involve the inactivation of NF-κB. The significance of these possibilities led us to explore the effect of ethanol extract of *O. javanica* (OJE) on inflammatory indices such as NF-κB inactivation, I-κBα degradation, and COX-2 induction in LPS-stimulated RAW 264.7 macrophage cell line.

Materials and Methods

Sample and extraction *O. javanica* obtained from Hanwoomool Co. (Jeollanamdo, Korea) was authenticated by Dr. Woojin Jun at Chonnam National University, and its voucher specimen was deposited at the same institute. Dried *O. javanica* was extracted with 20 volumes of 80% ethanol and filtered. The filtrate was evaporated under

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vacuum conditions and freeze-dried to yield OJE.

Macrophage cell culture RAW 264.7 cell line (ATCC TIB-71; American Type Culture Collection, Rockville, MD, USA) was cultured 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, St. Louis, MO, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (BioWhitaker) at 37°C in an atmosphere of 5% CO_2. For all experiments, cells were grown to 80-90% confluency and subjected to no more than 20 cell passages.

Cell viability An MTT (3-[4,5-2-yl]-2,5-diphenyl tetrazolium bromide) assay was performed to measure the viability of RAW 264.7 cells. The MTT (Sigma) is a pale yellow substrate that produces a dark blue formazan product when incubated with living cells. An MTT ring is cleaved in the active mitochondria, and the process occurs only in living cells. The cells were incubated at 37°C with MTT (50 μg/mL) for 4 hr, and the absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

Measurement of PGE_2 and TNF-α by RAW 264.7 cells Cells were plated at 5×10^5 cells in a 24-well plate and stimulated with 1 μg/mL of LPS with or without OJE. After 24-hr incubation, the level of TNF-α in cultured medium of RAW 264.7 cells was determined by sandwich ELISA as described by Chouaib *et al.* (10). An anti-TNF-α monoclonal antibody, biotinylated polyclonal antibody, and recombinant murine TNF-α were obtained from R&D Systems (Minneapolis, MN, USA). The level of PGE_2 production from endogenous arachidonic acid in the cell culture supernatants was measured by ELISA (Assay Designs, Ann Arbor, MI, USA).

Western blot analysis Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analyses were performed according to the method of Kang *et al.* (11) with a slight modification. Briefly, the cells were lysed in buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM sodium chloride, 0.2% SDS, 1% nonidet P-40 (NP-40), 5 mM sodium fluoride, and a protein inhibitor cocktail (Roche, Penzberg, Germany). Cell lysates were centrifuged at 12,000×g for 30 min to remove debris, fractionated by 12% gel electrophoresis, electrophoretically transferred on to the nitrocellulose paper, which was been sequentially incubated with polyclonal goat anti-COX-2 antibody (1:200, Santa Cruz, CA, USA), monoclonal mouse anti-actin antibody (1:100, Santa Cruz), and horseradish peroxidase-conjugated secondary antibody (1:5.000, Santa Cruz). Finally, the papers were developed using an electro-generated chemiluminescence (ECL) detection kit (Amersham, Piscataway, NJ, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) One microgram of total RNA isolated from the cells using total RNA isolation kit (Qiagen, Valencia, CA, USA) was reverse-transcribed using an oligo (dT) 18mer as a primer and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to produce the cDNAs. Thirty-five cycles of PCR was performed using the selective primers for the mouse COX-2 (sense primer: 5'-CAGAACCGCA TTGCCTCTG-3'; antisense primer; 5'-CAGTTCCATGA CATCGAT-3'; 389 bp) and GAPDH genes (sense primer: 5'-ACAGCCGCATCTTCTTGTGCAGTG-3'; antisense primer: 5'-GGCCTTGACTGTGCCGTTGAATTT-3'; 225 bp) under the following conditions: 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.

NF-κB and activator protein-1 (AP-1) luciferase reporter gene assays Cells were plated at 2×10^4 cells/well in a 24-well plate and transfected on the following day. A dual-luciferase reporter assay system (Promega, Madison, WI, USA) was used to determine the promoter activity. Briefly, the cells were transiently transfected with 1 mg/mL of pNF-κB-luciferase or pAP-1-luciferase plasmid and 20 ng of pRL-SV plasmid (Promega) using the Lipofectamine™ 2000 reagent (Invitrogen), and exposed to LPS for 16 hr. Firefly and *Renilla* luciferase activities in the cell lysates were measured using a luminometer (Labsystems, Fluoroskan Ascent, FL, USA). Relative luciferase activity was calculated by normalizing NF-κB or AP-1 promoter-driven firefly luciferase activity versus that of *Renilla* luciferase.

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. Statistical significance was set at $p<0.05$.

Results and Discussion

Inhibition of TNF-α induction by OJE The MTT assay was performed to measure the cytotoxicity of OJE to RAW 264.7 cells. Since cell viability was not significantly affected by OJE up to 800 μg/mL (Fig. 1), we chose 100-600 μg/mL of OJE for the subsequent experiments.

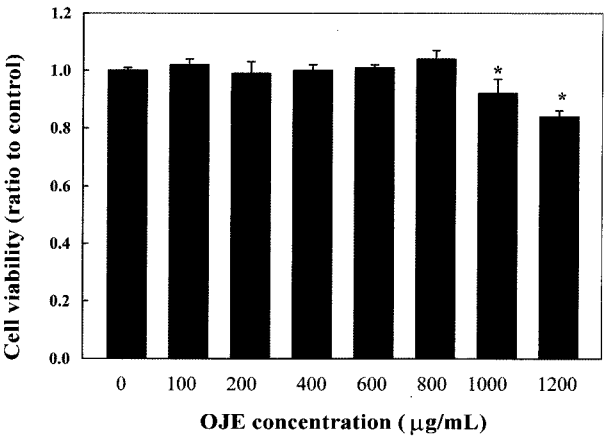

Fig. 1. Effect of ethanol extract (OJE) from *Oenanthe javanica* on RAW 264.7 cell viability. Data represent the mean ± SD in triplicate experiments. *Indicates the significant difference from other groups at $p<0.05$.

Assessment of the effect of OJE on TNF-α production in RAW 264.7, stimulated by 1 μg/mL of LPS, revealed LPS significantly increased TNF-α production from 2 to 12 hr (Fig. 2). OJE at 400 and 600 μg/mL strongly inhibited LPS-induced TNF-α production at 2, 4, 6, 12, and 24 hr, whereas 200 μg/mL of OJE had no significant effect. On the contrary, Wang *et al.* (12) recently reported that 1 mg/mL of *O. javanica* flavone fractionated by 80% ethanol and the resulting ethyl acetate did not influence the cell viability of HepG2.2.15 cells. In our study, OJE, a crude ethanol extract, was toxic to cell viability at a lower concentration than that reported by Wang *et al.* (12). These differences could be due to the different types of cell line used or the fact that crude ethanol extract may contain toxic material that was removed by ethyl acetate fractionation.

Inhibition of LPS-induced COX-2 expression and PGE_2 release by OJE The COX, the rate-limiting enzyme in the conversion of arachidonic acid to PGE_2, is the main target of non-steroid anti-inflammatory drugs (NSAIDs) and anti-inflammatory phytochemical agents. In our study, both 400 and 600 μg/mL of OJE significantly inhibited PGE_2 release by LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Fig. 3). To clarify whether the inhibitory effect of OJE on PGE_2 release could result from a decrease in the COX-2 protein level, we further examined the effect of OJE on LPS-induced COX-2 protein level by western blot analysis. The OJE treatment with 400 and 600 μg/mL significantly reduced the COX-2 expression in a dose-dependent manner (Fig. 4A). The mRNA expression level of COX-2 was also reduced by the increased concentration of OJE up to 600 μg/mL, implicating that the inhibited expression of COX-2 by OJE is regulated at the transcriptional level (Fig. 4B). Most inflammatory reactions involve genetic expressions such as COX-2, iNOS, and other proinflammatory cytokines including IL-1β, IL-6, and TNF-α. Evidences suggest that oxidative stress or cellular redox state may be related to COX-2 induction through NF-κB and/or other signaling pathways (13, 14). Because *O. javanica* contains high level of antioxidative flavonoids and vitamin E, OJE may inhibit COX-2 induction by scavenging oxidative stress (15, 16). However, in the present study, the antioxidative activity of OJE and the iNOS induction were not measured. Thus, we could not conclude the involvement of antioxidative components in OJE to elucidate its action mode on the inhibition of COX-2 induction.

Inhibition of LPS-inducible NF-κB activation by OJE The NF-κB is present in a resting state in the cytoplasms of all cells, and the usual sequence of events is generated only when it is activated and translocated to the nucleus. Under resting conditions, NF-κB consists of a heterotrimer

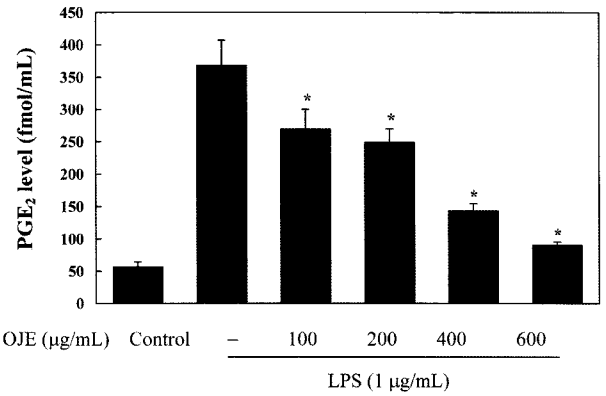

Fig. 3. Effect of ethanol extract (OJE) from *O. javanica* on PGE_2 secretion by LPS-stimulated RAW 264.7 macrophage. Data represent the mean ± SD in triplicate experiments. *Indicates the significant difference from LPS-stimulated group at $p<0.05$.

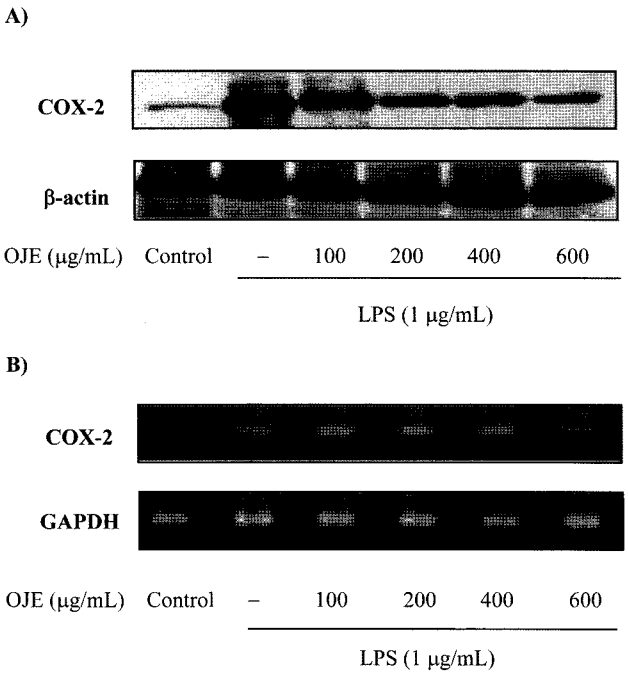

Fig. 4. Effect of ethanol extract (OJE) from *O. javanica* on COX-2 mRNA and protein expression. (A) Effect of OJE on COX-2 protein expression in LPS-stimulated RAW 264.7 cells determined by western immunoblot. (B) Effect of OJE on COX-2 mRNA expression in LPS-stimulated RAW 264.7 cells determined by RT-PCR analysis.

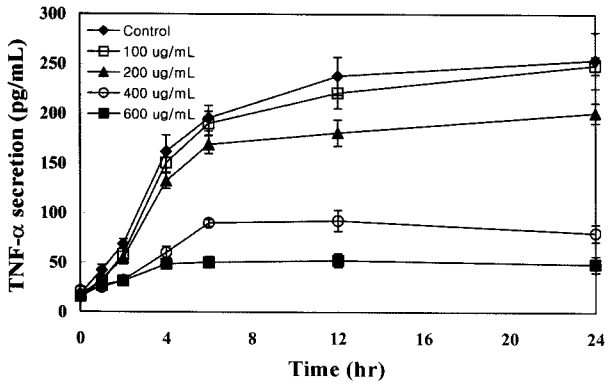

Fig. 2. Effect of ethanol extract (OJE) from *O. javanica* on TNF-α secretion by LPS-stimulated RAW 264.7 macrophage. Data represent the mean±SD in triplicate experiments. *Indicates the significant difference from LPS-stimulated group at $p<0.05$.

of p50, p65, and I-κBα in the cytoplasm. The phosphorylation, ubiquitination and degradation of I-κBα leads to the release of the p50-p65 heterodimer, which then translocates to the nucleus, binds its specific 10-base pair consensus site (8, 17), and regulates the genetic expression of key proinflammatory cytokines and COX-2. To examine whether the transcriptional activation of COX-2 gene is inhibited by OJE, reporter gene assays were performed using RAW 264.7 cells transfected with a mammalian cell expression vector pNF-κB and pAP-1 containing the luciferase structural gene. The LPS treatment for 16 hr caused approximately 2.5-fold increase in NF-κB reporter activity (Fig. 5). In addition, the pre-treatment of cells with 400 and 600 μg/mL of OJE significantly inhibited the elevation of LPS-induced luciferase activity, and the increased NF-κB reporter activity was completely inhibited by 600 μg/mL of OJE. The exposure of cells transiently transfected with AP-1 plasmid to LPS for 16 hr resulted in an 80-fold increase in luciferase activity. The LPS-induced increase in AP-1 reporter activity was dramatically affected by 400 and 600 μg/mL of OJE treatments. These results provide evidence that OJE inhibits both NF-κB and AP-1 activations, indicating that OJE may regulate the gene expression through more than one signaling pathway, and that this may be associated with OJE blocking the COX-2 gene expression.

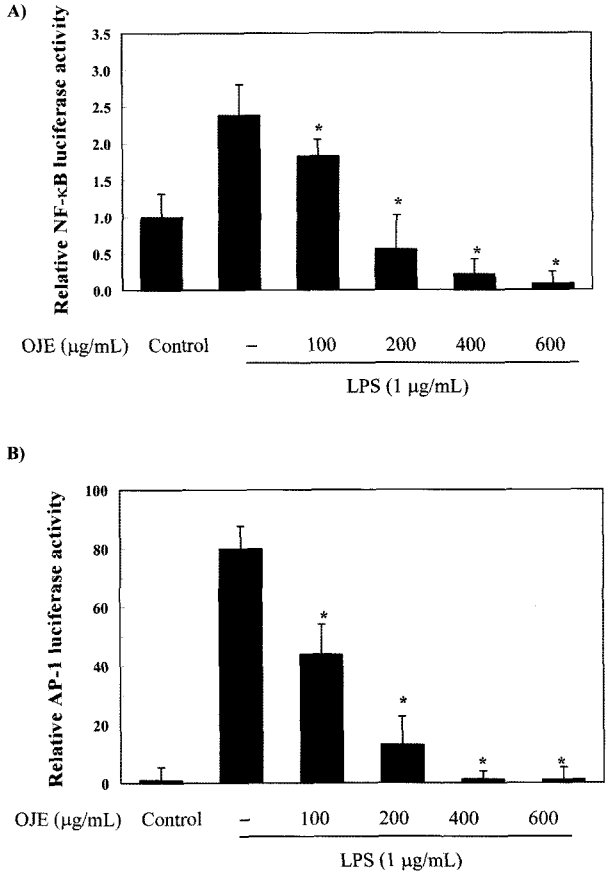

Fig. 5. Effect of ethanol extract (OJE) from *O. javanica* on NF-κB and AP-1 activities. (A) Inhibitory effect of OJE on the transactivation of NF-κB reporter gene. (B) AP-1 reporter gene analysis. Cells were transfected with the pAP-1-Luc plasmid, and reporter gene analysis was performed as described in panel (A). The date shown is representative of the means ± SD of three experiments. *Indicates the significant difference from LPS-stimulated group at $p<0.05$.

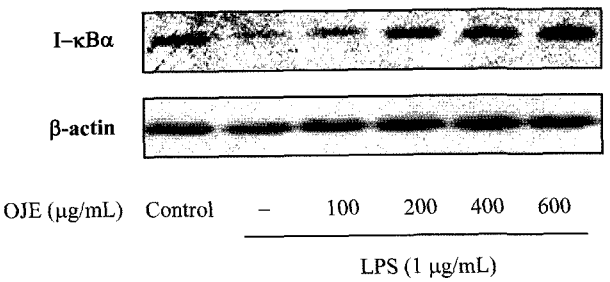

Fig. 6. Effect of ethanol extract (OJE) from *O. javanica* on blocking the cytosolic I-κBα degradation. RAW 264.7 cells were stimulated with LPS and incubated for 16 hr without or with the various concentrations of OJE. Cell extract was assayed for cytosolic I-κBα by western blot analysis. Forty micrograms of total protein was applied on 12% PAGE and the β-actin was detected as an internal control.

Inhibition of I-κBα degradation by OJE The translocation of NF-κB to the nucleus is preceded by the phosphorylation and degradation of the I-κBα subunit. To determine whether the inhibition of NF-κB activation by OJE results from its inhibition of I-κBα degradation, we assessed the protein levels of cytosolic I-κBα in RAW 264.7 cells. Immunoblot analysis using I-κBα antibody showed that the level of I-κBα decreased after 16-hr LPS exposure treatment (Fig. 6). This decrease was reversed by treating the cells with 600 mg/mL of OJE, suggesting that the inhibition of NF-κB activation by OJE is due to the prevention of I-κBα degradation and the subsequent nuclear translocation of p65. These results suggest that the phoshorylation and subsequent degradation step of I-κBα could be the pharmacological targets of OJE. In addition, the phosphorylation of I-κBα bound to NF-κB is considered to be mediated with the I-κBα kinase (IKK) at two conserved serine units within its N-terminal domain (18). The IKK complex can be activated by a variety of upstream kinases such as protein kinase C and tyrosine kinase family (19, 20). Therefore, OJE may also act on these upstream kinases; thus, further study is necessary to determine which kinase(s) is inhibited by OJE.

In summary, OJE normalized the levels of TNF-α and PGE_2 induced by LPS-stimulated RAW 264.7 cell in a dose-dependent manner, which implicated that OJE may be one candidate for anti-inflammatory reagent. The present study demonstrated that OJE at 400-600 μg/mL has the capability of modulating genetic expressions of, among others, NF-κB, AP-1, and COX-2 that were related to inflammatory responses. These findings suggest that the inhibition of LPS-stimulated COX-2 expression by OJE is due to its inhibition of NF-κB activation by blocking I-κBα degradation, which may be the mechanistic basis of the anti-inflammatory effects of OJE.

Acknowledgments

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