Anti-inflammatory activity of *Camellia japonica* oil

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***Camellia japonica* oil** (CJ oil) **has been used traditionally in East Asia to nourish and soothe the skin as well as help restore the elasticity of skin. CJ oil has also been used on all types of bleeding instances. However, little is known about its anti-inflammatory effects. Therefore, the anti-inflammatory effects of CJ oil and its mechanisms of action were investigated. CJ oil inhibited LPS-induced production of NO, PGE_2, and TNF-α in RAW264.7 cells. In addition, expression of COX-2 and iNOS genes was reduced. To evaluate the mechanism of the anti-inflammatory activity of CJ oil, LPS-induced activation of AP-1 and NF-κB promoters was found to be significantly reduced by CJ oil. LPS-induced phosphorylation of IκBα, ERK, p38, and JNK was also attenuated. Our results indicate that CJ oil exerts anti-inflammatory effects by downregulating the expression of iNOS and COX-2 genes through inhibition of NF-κB and AP-1 signaling. [BMB reports 2012; 45(3): 177-182]**

INTRODUCTION

Inflammation is the body's response to cellular injury. It is a diverse process that is mediated by inflammatory or immune cells. Among these cells, macrophages play a central role in managing the overproduction of pro-inflammatory cytokines and inflammatory mediators. Among its mediators, inducible nitiric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) have been shown to be important enzymes that regulate inflammatory responses. Both iNOS and COX-2 are inducible enzymes that mediate similar pathological processes (1). NO is produced by nitric oxide synthase (NOS), which converts L-arginine to L-citrulline (2). Once expressed, iNOS synthesizes large amounts of NO, which has both regulatory and detrimental effects (3, 4). During the inflammation response, NO overproduction may become cytotoxic (5).

COX converts arachidonic acid to prostaglandins (PGs), and like NOS, COX exists in two isoforms, COX-1 and COX-2. COX-1 and COX-2 catalyze the rate-limiting step in the production of PGs, which are bioactive compounds involved in processes such as fever and sensitivity to pain (6). COX-1 is ubiquitously and constitutively expressed, whereas COX-2 is highly inducible and generally present at very low levels, unless increased by one of many types of stimuli. COX-2 is also regulated at the post-transcriptional and enzymatic levels.

The transcriptional mediator nuclear factor-kappaB (NF-κB) plays a major role in regulating inflammatory responses by increasing the level of cytokines, chemokines, growth factors, and cell adhesion molecules (7). The activation of NF-κB results in the expression of these pro-inflammatory genes (8). These include the transcription of various inflammatory cytokines and TNF-α (9), as well as genes encoding COX-2 and iNOS (10). In addition, the transcription factor activator protein-1 (AP-1) regulates transcriptional genes of inflammatory responses (11). Mitogen-activated protein kinase (MAPKs) can phosphorylate transcription factors such as NF-κB and AP-1, which leads to the expression of pro-inflammatory mediators and cytokines of extracellular stimuli (12).

Camellia japonica is a native plant grown in Jeju Island which is called the island of camellia trees. CJ oil has a long history of use as a cosmetic protectant to keep skin and hair healthy and as a soothing agent. It has been reported that *Camellia japonica* possesses a variety of biological activities, including antibacterial activity (13), inhibitor of human immunodeficiency virus type 1 protease (14), Epstein-Barr virus inhibitor (15), anti-metastasis activity (16), antioxidant activity (17, 18), inhibitor of human type I pro-collagen production (19), and anti-allergic responses (20). Despite its wide spread use, there have been no studies that have examined the effects of CJ oil on inflammation-associated gene expression.

Therefore, in this study we characterized the inhibitory effects and mechanisms of CJ oil against inflammatory signals and demonstrated that CJ oil inhibits LPS-induced inflammatory reactions through inactivation of AP-1 and NF-κB pathway in RAW264.7 cells.
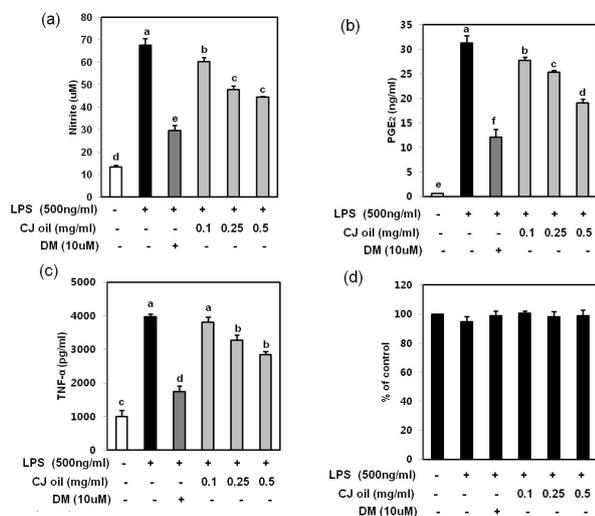
Fig. 1. CJ oil inhibited NO, PGE$_2$, and TNF-α production in RAW264.7 cells. (a) NO production, (b) PGE$_2$ production, (c) TNF-α production, (d) Cell viability. Data are expressed as means ± S.D. Data points with different letters represent values that are significantly different from each other at the P = 0.05 level. DM: Dexamethasone.

RESULTS

CJ oil reduced LPS-induced production of NO, PGE$_2$, and TNF-α in RAW264.7 cells

Pro-inflammatory mediators such as NO, PGE$_2$, and TNF-α play very important roles in the inflammatory response. To investigate the effect of CJ oil on the production of these mediators, the levels of secreted NO, PGE$_2$, and TNF-α were measured. As shown in Fig 1a, b, and c, LPS-induced production of NO, PGE$_2$, and TNF-α was significantly reduced by CJ oil in a concentration-dependent manner. Dexamethasone was used as a positive control. To exclude the possibility that this effect was due to the cytotoxic effect of CJ oil, an MTT assay was performed. Cytotoxic effects of CJ oil were not observed at the tested concentrations (Fig. 1d). These results indicate that CJ oil produces anti-inflammatory effects.

LPS-induced expression of iNOS and COX-2 genes was attenuated by CJ oil in RAW264.7 cells

NO and iNOS mediate inflammatory responses (4). COX-2 is also a key enzyme that regulates the production of prostaglandins, which are central mediators of inflammation (21). Therefore, to investigate the effects of CJ oil on the expression of iNOS and COX-2 genes, a luciferase reporter and Western blot assays for iNOS and COX-2 were performed. As shown in Fig. 2a and b, LPS-induced activation of iNOS and COX-2 promoters was significantly inhibited by CJ oil. Similarly, CJ oil reduced LPS-induced expression of iNOS and COX-2 proteins (Fig. 2c and d), suggesting that CJ oil exerts anti-inflammatory effects by down-regulating the expression of iNOS and COX-2 genes.
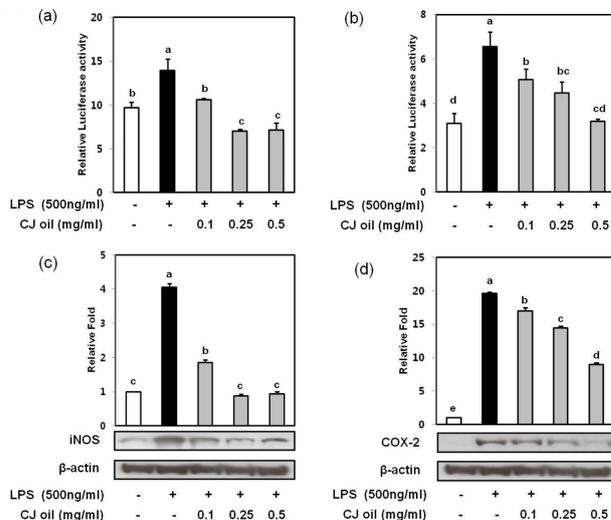

Fig. 2. CJ oil inhibited LPS-induced expression of iNOS and COX-2 genes in RAW264.7 cells. To determine the effect of CJ oil on LPS-induced activation of (a) iNOS and (b) COX-2 promoters, RAW264.7 cells were transiently co-transfected with 2 ug of the iNOS or COX-2 firefly luciferase reporter vector, and 0.2 ug of the Renilla luciferase vector using the Superfect™ reagent (Qiagen). After 24 h, cells were stimulated with 500 ng/ml LPS in the presence or absence of CJ oil. Luciferase activity is expressed as a ratio of iNOS or COX-2-dependent firefly luciferase activity divided by control thymidine kinase *Renilla* luciferase activity (relative luciferase units). Data are expressed as means ± S.D. Data points with different letters represent values that are significantly different from each other at the P = 0.05 level. Results were confirmed by three independent experiments. Cells were treated for 2 h with the indicated concentrations of CJ oil. LPS (500 ng/ml) was then added and cells were further incubated for 24 h. (c) iNOS and (d) COX-2 protein levels were determined via Western blotting as described in the Materials and Methods. Data are expressed as means ± S.D. Data points with different letters represent values that are significantly different from one another at the P = 0.05 level. Results were confirmed by three independent experiments.

CJ oil effects were mediated by inhibition of NF-κB and AP-1 (ERK, p38, and JNK) activation

NF-κB and AP-1 are important transcriptional factors that regulate the expression of the iNOS (22) and COX-2 (23) gene. Thus, we investigated the role of these transcription factors in the CJ oil-induced expression of iNOS and COX-2 genes. To this end, luciferase reporter assays for AP-1 and NF-κB promoters were performed in RAW264.7 cells. In this study, CJ oil suppressed LPS-induced activation of AP-1 and NF-κB promoters (Fig. 3a and b), suggesting that the effects of the CJ oil are dependent on AP-1 and NF-κB signaling. These results were further confirmed by Western blot for the phosphorylated forms of MAPKs and IκBα. As shown in Fig. 4, CJ oil suppressed LPS-induced phosphorylation of ERK, p38, and JNK. In addition, LPS-induced phosphorylation of IκBα was reduced by CJ oil. These results indicate that CJ oil reduces expression of iNOS and COX-2 genes via inhibition of NF-κB and AP-1
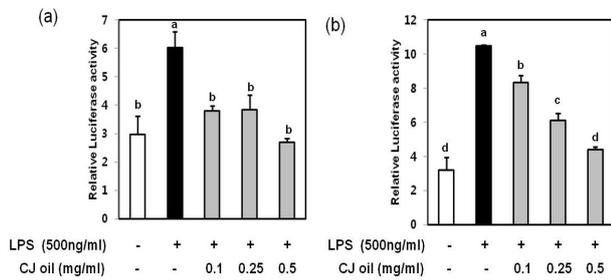
Fig. 3. CJ oil inhibited LPS-induced activation of AP-1 and NF-κB promoters in transfected RAW264.7 cells. To determine the effect of CJ oil on LPS-induced activation of (a) AP-1 and (b) NF-κB promoters, RAW264.7 cells were transiently co-transfected with 2 ug of the AP-1 or NF-κB luciferase vector, and 0.2 ug of the *Renilla* luciferase vector using the Superfect™ reagent (Qiagen). After 24 h, cells were stimulated with 500 ng/ml LPS in the presence or absence of CJ oil. Luciferase activity is expressed as a ratio of AP-1 or NF-κB-dependent firefly luciferase activity divided by control thymidine kinase *Renilla* luciferase activity (relative luciferase units). Data are expressed as means ± S.D. Data points with different letters represent values that are significantly different from each other at the P = 0.05 level. Results were confirmed by three independent experiments.

(ERK, p38, and JNK).

DISCUSSION

Although CJ oil has been used as a soothing agent in traditional therapy and cosmetic formulations, no studies have systematically examined the effects of CJ oil on inflammation. In this study, the anti-inflammatory effects of CJ oil were demonstrated and its mechanisms of action were characterized. Specifically, CJ oil exerted anti-inflammatory effects by downregulating the expression of pro-inflammatory mediators such as iNOS and COX-2 via inhibition of AP-1 and NF-κB signaling.

It is well known that NO and PGE_2 are the main macrophage-derived inflammatory mediators (24). Furthermore, TNF-α is an important pro-inflammatory immune modulator that leads to increased cytokine production and secretion (25). Aberrant production of NO, PGE_2, and TNF-α induces an inflammatory response that causes damage to neighboring cells of the host. NO is produced by a specific enzyme called NOS from L-arginine. Massive amounts of NO produced by iNOS under diseased conditions are potentially harmful. Thus, inhibiting NO production in response to inflammatory stimuli may be a useful therapeutic strategy for the treatment of inflammatory diseases (26, 27). In addition, prostanoids and arachidonic acid metabolites have been shown to play pivotal functions in inflammation. The rate-limiting step for prostanoid synthesis is the release of arachidonic acid in response to various physiological and pathological stimuli. Arachidonic acid is released from membrane phospholipids by phospholipase A_2 and converted to PGH_2 by COX. PGH_2, is the common substrate for a number of different synthases that produce the ma-
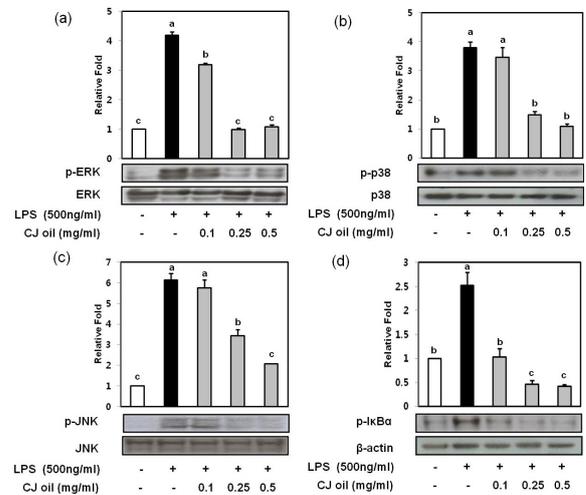

Fig. 4. Effects of CJ oil on LPS-induced phosphorylation of MAPKs and IκBα in RAW264.7 cells. RAW264.7 cells were pretreated with the indicated concentrations of CJ oil for 2 h. Cells were then stimulated with 500 ng/ml LPS for another 30 min. Western blot of whole cell lysates was performed as described in Materials and Methods. (a) Changes in ERK phosphorylation. (b) Changes in p38 phosphorylation. (c) Changes in JNK phosphorylation. (d) Changes in IκBα phosphorylation. Data are expressed as means ± S.D. Data points with different letters represent values that are significantly different from each other at the P = 0.05 level. Results were confirmed by three independent experiments.

jor prostanoids including PGD_2, PGE_2, PGI_2, and thromboxane A_2 (TXA_2). Among these prostanoids, PGE_2 is a central mediator of febrile responses triggered by the inflammatory process and intradermal PGE_2 is hyperalgesic in the peripheral nervous system (28). TNF-α is produced mainly by monocytes and macrophages. Newly synthesized TNF-α is inserted into the cell membrane and subsequently released through the action of the TNF-α converting enzyme, to become biologically active. Also, TNF-α, which activates NF-κB, is a key transcription factor for activating genes involved in inflammation. TNF-α triggers production of other cytokines, induces endothelial adhesion molecules, stimulates collagenase, and stimulates osteoclast differentiation. Hence, the blockade of TNF-α has a greater effect on inflammation than the blockade of other cytokines. Taken together, these data indicate that NO, PGE_2, and TNF-α are involved in the regulation of various processes of acute and chronic inflammation. Therefore, in this study, we examined the effects of CJ oil on the production of NO, PGE_2, and TNF-α. In this analysis, we found that CJ oil inhibited LPS-induced production of NO, PGE_2, and TNF-α in RAW264.7 cells. In addition, expression of iNOS and COX-2 genes was inhibited by CJ oil. These results indicate that CJ oil exerts anti-inflammatory effects by downregulating expression of iNOS and COX-2 and suggests the possibility that CJ oil can act as an anti-inflammatory agent.

LPS stimulation of macrophages activates several intra-

cellular signaling pathways, including the NF-κB pathway and three MAPK pathways. The MAPK family of protein kinases comprises ERK, JNK, and p38 MAPKs and the activation and function of these proteins are regulated by upstream kinases and stress-related inducers (29). The MAPK cascade is also involved in the posttranslational control of NF-κB and activation of AP-1 (30, 31), which coordinate the induction of many genes encoding inflammatory mediators. The transcription factor NF-κB, which regulates both iNOS and COX-2 expression, has been implicated in the regulation of many genes that code for mediators of immune responses (32, 33). Transcription factor AP-1 regulates genes that encode for pro-inflammatory mediators and protective antioxidant genes (34). In addition, NF-κB and AP-1 play a critical role in the transcriptional regulation of genes that suppress apoptosis and induce cellular transformation, proliferation, invasion, metastasis, and chemo-resistance. In this study, the inhibitory mechanisms of CJ oil on the expression of iNOS and COX-2 genes was assessed and LPS-induced activation of AP-1 and NF-κB promoters was shown to be significantly reduced by CJ oil. In addition, LPS-induced phosphorylation of ERK, p38, JNK, and IκB was attenuated by CJ oil. These results indicate that CJ oil downregulates the expression of iNOS and COX-2 genes by inhibiting NF-κB and AP-1 (ERK, p38, and JNK). In addition, we found that CJ oil contains palmitic acid, linoleic acid, oleic acid, and stearic acid as major components (data not shown). This suggests that these compounds may contribute to the anti-inflammatory effects of CJ oil.

Taken together, the results of this study demonstrate that CJ oil exerts its anti-inflammatory effects by downregulating the expression of iNOS and COX-2 genes through the inhibition of NF-κB and AP-1 signaling. Additioly, these results show that CJ oil could be used as a potential therapeutic agent for the treatment of inflammatory disorders.

MATERIALS AND METHODS

Materials and cell culture
Bulk wild *Camellia japonica* seeds were obtained from Jeju Island, Korea and voucher specimens were deposited in Jeju Hi-Tech Industry Development Institute, in Jeju Do, Korea. *Camellia japonica* seeds (1 kg) were transferred to the mechanical press (Dongkwang Oil Co, Seoul, Korea) and then pressed at a pressure of 600 kg/cm^2 for 20 min to obtain the CJ oil. The extracted CJ oil was filtered with cheesecloth under vacuum to remove particulate matter. CJ oil had the following characteristics: specific activity 0.9443 (a); acid value 0.99; ester value 35.44; iodine value 80.22; boiling point 164°C; optical rotation +5.045 (at 20°C); reactive index 1.462-1.464 at 20°C; and soluble in 0.1 volume of 90% alcohol. A solution of CJ oil was prepared by dissolving the oil in dimethyl sulfoxide (DMSO). Lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4), β-actin antibody, and dexamethasone were purchased from Sigma (St. Louis, MO, USA). iNOS antibody was obtained

from Millipore (Bedford, MA, USA). And COX-2, phospho-p38 MAPK (Thr180/Tyr182) (28B10), phospho-SAPK/JNK (Thr183/Tyr185) (G9), phospho-ERK MAPK (Thr202/Tyr204) (E10), p38 MAPK, SAPK/JNK, phospho-IκBα (Ser32), and ERK MAPK antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). RAW264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in Dulbecco's Modified Eagles' Medium (Gibco, MD) containing 10% fetal bovine serum (GibCo, MD) and 1% penicillin-streptomycin at 37°C, in a humidified atmosphere containing 95% air/ 5% CO$_2$. AP-1 Luc and NF-κB-Luc reporter plasmids were purchased from Stratagene (La Jolla, CA, USA). iNOS Luc and COX-2 Luc were a gift from Dr. Yeong-Shik Kim (Seoul National University, Republic of Korea).

Cell viability
Cell viability was assessed by mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to purple formazan. Cells were incubated with MTT (0.5%) for 4 h at 37°C. The medium was removed by aspiration and formazan crystals were dissolved in DMSO. The extent of MTT reduction was quantified by measuring the absorbance at 540 nm.

Measurement of nitrite, PGE$_2$, and TNF-α
The nitrite concentration in the medium was measured according to the Griess reaction and the calculated concentration was used as an indicator of NO production. The supernatant of cell cultures was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). The optical density at 540 nm (A540) was measured and calculated against a sodium nitrite standard curve. PGE$_2$ and TNF-α concentration in the supernatants of the culture medium was determined using an EIA kit (Enzo Life Sciences, NY, USA) according to the manufacturer's instructions.

Luciferase reporter gene activity assay
To assay for NF-κB promoter activities, RAW264.7 cells were transfected with a NF-κB-Luc reporter, or with AP-1, iNOS, and COX-2, which were in a Renilla luciferase expression vector that was driven by a thymidine kinase promoter (Promega, WI) using Superfect^{TM} reagent (Qiagen, USA). After incubation for 24 h, cells that had been pretreated with or without CJ oil for 2 h were stimulated for 24 h by LPS (500 ng/ml). The cells were then harvested and lysed. Supernatants were assayed for luciferase activity. Luciferase activity was determined using a Dual Luciferase Assay system (Promega, WI) and a LB953 luminometer (Berthold, Germany). The activity was expressed as the ratio of the NF-κB-dependent firefly luciferase activity to the control thymidine kinase Renilla luciferase activity.

Western blotting analysis
Twenty-four hours after RAW264.7 cells (1 × 10^6) were seeded,

the cells were pretreated with or without CJ oil (0.1, 0.25, and 0.5 mg/ml) for 2 h and then treated with LPS (500 ng/ml) for another 30 min (phospho-form) or 24 h (iNOS and COX-2). The cell pellets were resuspended in extraction lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 1 mM EDTA, 1 mM phenyl-methylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, 1 uM each of leupeptin, and aprotinin) and incubated for 20 min at 4°C. Cell debris was removed by microcentrifugation at 12,000 rpm for 10 min. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instruction. Lysates (20 ug/lane) were separated by SDS-PAGE on polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% milk or BSA (phospho-form) in a PBS-T solution for 1 h. The membranes were then probed with iNOS (Millipore, MA, USA), COX-2, phospho-p38 MAPK (Thr180/Tyr182) (28B10), phospho-SAPK/JNK (Thr183/Tyr185) (G9), phospho-ERK MAPK (Thr202/Tyr204) (E10), p38 MAPK, SAPK/JNK, phospho-IκBα (Ser32), and ERK MAPK antibodies (Cell Signaling) overnight at 4°C. The blots were washed with PBS-T three times and incubated with horseradish peroxidase-conjugated rabbit anti-mouse or anti-rabbit IgG for 1 h at room temperature. Following three further washings in PBS-T, immunoreactive bands were visualized using the ECL-Plus detection system (Amersham Biosciences).

Statistical analysis

Statistical significance of the data was determined using a one-way ANOVA coupled with a Tukey's post-hoc test. A P value < 0.05 was considered to be significant.

Acknowledgements

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