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Biochemical Pharmacology 68 (2004) 2327–2336
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Anti-inflammatory effect of caffeic acid methyl ester and its mode of action through the inhibition of prostaglandin E_2, nitric oxide and tumor necrosis factor-α production

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Received 18 June 2004; accepted 4 August 2004

Abstract

The anti-inflammatory effects of caffeic acid (CA), caffeic acid methyl ester (CM) and di-*O*-acetylcaffeic acid (DAC) were investigated in rats using the carrageenin-induced edema model and the antinociceptive effects of these compounds were also assessed in mice by means of the acetic acid-induced abdominal constriction test and hot plate test. CM (10 mg/kg, p.o.) showed the most potent anti-inflammatory and antinociceptive effects in these animal models. To investigate the mechanism of the anti-inflammatory action, we examined the effects of these compounds on the lipopolysaccaride (LPS)-induced NO and PGE_2 responses in the murine macrophage cell line, RAW 264.7. Our data indicate that CM is the most potent inhibitor of NO and PGE_2 production and it also significantly decreased tumor necrosis factor-alpha (TNF-α) release. Consistent with these observations, the protein and mRNA expression levels of iNOS and COX-2 were found to be inhibited by CM in a dose-dependent manner. Furthermore, CM inhibited the nuclear factor-κB (NF-κB) activation induced by LPS, which was associated with the prevention of the degradation of the inhibitor κB, and subsequently with decreased p65 protein levels in the nucleus. Taken together, our data indicate that the anti-inflammatory properties of CM might result from the inhibition of iNOS, COX-2 and TNF-α expression through the down-regulation of NF-κB binding activity.

Keywords: Caffeic acid methyl ester; Anti-inflammation; Nitric oxide; Prostaglandin E_2; TNF-α; NF-κB

1. Introduction

Chronic inflammations and infections lead to the up-regulation of a series of enzymes and signaling proteins in affected tissues and cells. Among these pro-inflammatory enzymes, the inducible forms of nitric oxide synthase (NOS) and cyclooxygenase (COX), which are responsible for increasing the levels of NO and prostaglandins (PGs), respectively, are known to be involved in the pathogenesis of many chronic diseases including multiple sclerosis, Parkinson's and Alzheimer's diseases, and colon cancer [1]. NO is produced by iNOS in macrophages, hepatocytes and renal cells, under the stimulation of lipopolysaccharide (LPS), tumor necrosis factor-alpha (TNF-α), interleukin-1 or interferon-γ [2]. It can also react with superoxide anion radicals to form the even stronger oxidant, peroxinitrite (ONOO) [3,4]. Many studies have shown that the chronic phase of inflammation is closely associated with an increase in iNOS activity [5]. The most conclusive evidence for NO acting as a mediator of tissue injury has been obtained in the case of arthritis, which is based on studies in an animal model, human osteoarthritis and rheumatoid arthritis [6]. However, the constitutive epithelial and

Abbreviations: LPS, lipopolysacchride; EMSA, electrophoretic mobility shift assay; iNOS, inducible nitric oxide synthase; FBS, fetal bovine serum; TNF, tumor necrosis factor; NO, nitric oxide; MTT, 3-(4,5-dimethylthiazoyl-2-yl) 2,5 diphenyl tetrazolium bromide; ECL, enhanced chemical luminoscence

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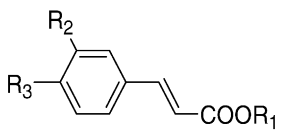
doi:10.1016/j.bcp.2004.08.002

neuronal forms of NOS contribute relatively little to inflammation and carcinogenesis. COX is the enzyme that converts arachidonic acid to PGs. Like NOS, COX has been found to exist in two isoforms and one of these, COX-2, is an inducible form which is responsible for the production of large amounts of pro-inflammatory PGs at the inflammatory site [7]. Furthermore, TNF-α is one of the most important pro-inflammatory cytokines and is mainly produced by monocytes and macrophages [8]. It is secreted during the early phase of acute and chronic inflammatory diseases such as asthma, rheumatoid arthritis, septic shock and other allergic diseases, as well as the activation of T cells [8].

The expression of these pro-inflammatory enzymes and cytokines is regulated at multiple levels, including during the transcriptional, post-transcriptional, translational and post-translational steps [9,10]. One of the most ubiquitous transcription factors that regulate the gene expressions involved in cellular proliferation, inflammatory responses and cell adhesion is NF-κB. The functionally active NF-κB exists mainly as a heterodimer, consisting of subunits of the Rel family, which is normally sequestered in the cytosol as an inactive complex, by binding to IκBs in unstimulated cells [11]. The activation mechanism of NF-κB involves the phosphorylation of IκBs in two critical serine residues (Ser^{32} and Ser^{36}) via the IκB kinase (IKK) signalosome complex. Once the IκBs are phosphorylated, they are targeted for ubiquitination and subsequent degradation by 26S proteosome [12–14]. The resulting free NF-κB is translocated to the nucleus, where it binds to the κB binding sites in the promoter regions of the target genes, thereby controlling their expression [15].

Caffeic acid and its derivatives are nonflavonoid catecholic compounds, which are distributed in many medicinal plants [16]. It has been reported that caffeic acid has anti-inflammatory, anti-mutagenic and anti-carcinogenic activities [17–20], and some of its derivatives show antioxidant and anti-inflammatory activities [21–23]. Although the anti-inflammatory activities of caffeic acid derivatives have already been studied, their mechanisms of action are not fully understood. In this study, we investigated the anti-inflammatory and antinociceptive activities of caffeic acid, caffeic acid methyl ester and di-*O*-acetylcaffeic acid in animal models (Fig. 1). Furthermore, the effects of caffeic acid derivatives on the lipopolysaccharide (LPS)-induced NO, PGE_2 and TNF-α release via NF-κB binding were also investigated, in order to gain a better insight into the mechanism of the observed anti-inflammatory action.



Compound	R_1	R_2	R_3
Caffeic acid	H	OH	OH
Caffeic acid methyl ester	CH_3	OH	OH
Di-*O*-acetylcaffeic acid	H	$OCOCH_3$	$OCOCH_3$

Fig. 1. Chemical structures of caffeic acid, caffeic acid methyl ester and di-*O*-acetylcaffeic acid.

2. Materials and methods

2.1. Materials

Caffeic acid methyl ester and di-*O*-acetylcaffeic acid were prepared from caffeic acid as follows: caffeic acid methyl ester (CM): a solution of caffeic acid (2 g, 11.1 mmol) in methanol (50 mL) was treated with a catalytic amount of c-H_2SO_4 and heated at reflux for 10 h. The reaction mixture was cooled at room temperature and concentrated. The residue was dissolved in EtOAc and washed successively with water and brine. The ethylacetate (EtOAc) layer was dried over anhydrous $MgSO_4$ and purified by flash column chromatography on silica gel (EtOAc:*n*-hexane = 1:2) to give caffeic acid methyl ester (1.53 g, 71%) as a white solid. ^{1}H NMR (500 MHz, pyridin-d_5) δ: 3.71 (3H, s, $COOC\underline{H}_3$), 6.56 (1H, d, J = 15.9 Hz, H-8), 7.16 (1H, dd, J = 1.9 and 8.1 Hz, H-6), 7.19 (1H, d, J = 8.1 Hz, H-5), 7.57 (1H, d, J = 1.9 Hz, H-2), 7.92 (1H, d, J = 15.9 Hz, H-7); ^{13}C NMR (125.5 MHz, pyridine-d_5) δ: 51.2 ($COO\underline{C}H_3$), 114.6 (C-8), 115.7 (C-2), 116.7 (C-5), 122.0 (C-6), 126.8 (C-1), 146.0 (C-7), 147.7 (C-3), 150.5 (C-4), 167.8 (C-9). Di-*O*-acetylcaffeic acid (DAC): a solution of caffeic acid (2 g, 11.10 mmol) in pyridine (5.0 mL) was treated with acetic anhydride (3 mL) and stirred overnight. The reaction mixture was poured into ice-containing water and the resulting solid was filtered and washed with water. The solid was further purified by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 10:1) to give di-*O*-acetylcaffeic acid (1.23 g, 42%) as a white solid. ^{1}H NMR (500 MHz, pyridine-d_5) δ: 2.24, 2.27 (each 3H, s, acetyl), 6.84 (1H, d, J = 16.0 Hz, H-8), 7.35 (1H, d, J = 8.3 Hz, H-5), 7.47 (1H, dd, J = 1.9 and 8.3 Hz), 7.67 (1H, d, J = 1.9 Hz, H-2), 7.93 (1H, d, J = 16.0 Hz, H-7); ^{13}C NMR (125.5 MHz, pyridine-d_5) δ: 20.79 ($\underline{C}H_3CO$) and 20.81 ($\underline{C}H_3CO$), 122.4 (C-8), 123.8 (C-2), 124.9 (C-5), 127.1 (C-6), 134.6 (C-1), 142.7 (C-7), 143.8 (C-3), 144.6 (C-4), 168.7, 168.8 ($CH_3\underline{C}O$), 169.3 (C-9). The RAW 264.7 mouse macrophage-like cell line was obtained from the Korean Cell Line Bank. DMEM, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Life Technologies. COX-2, iNOS, p65 and IκB-α monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology. The enzyme immunoassay (EIA) kit used for the determination of prostaglandin E_2 and TNF-α was obtained from R&D Systems. NS-398, a COX-2 enzyme inhibitor was obtained from Calbiochem. The RNA extraction kit was purchased from Intron

Biotechnology. The iNOS, COX-2, TNF-α and β-actin oligonucleotide primers were purchased from Bioneer. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tertazolium bromide (MTT), sulfanilamide, aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), L-N^6-(1-iminoethyl) lysine (L-NIL), *Escherichia coli* LPS and all other chemicals were purchased from Sigma.

2.2. Animals

ICR male mice weighing 20–25 g and Sprague–Dawley male rats weighing 100–120 g were purchased from the Daehan Biolink and maintained under constant conditions (temperature: 20 ± 2 °C, humidity: 40–60%, 12 h light/dark cycle) and acclimatized for 1 week or more. For 24 h period before the experiment, only water was offered to the animals. To minimize the effect of their daily enzyme activity variations, the animals were all sacrificed at 10:00–12:00 a.m. All animal experiments were approved by the University of Kyungsung Animal Care and Use Committee, and all procedures were conducted in accordance with the ''Guide for the Care and Use of Laboratory Animals'' published by the Korea National Institute of Health.

2.3. Carrageenin-induced paw edema

The initial hind paw volume of the Sprague–Dawley strain rats was determined volumetrically. A 1% solution of carrageenin in saline (0.1 mL/rat) was injected subcutaneously into the right hind paw 1 h after the test substance had been administered orally. The test samples were dissolved in 10% Tween 80 and diluted with saline. The same volume of pure solvent was used for the control group. The test solution (10 mg/kg) was administered orally on the seven consecutive days prior to the injection of the carrageenin. The paw volumes were measured upto 5 h after the injection at intervals of 60 min and the volume of the edema was measured with a plethysmometer [24]. Ibuprofen, an anti-inflammatory drug, was used as a standard [25].

2.4. Acetic acid-induced abdominal constriction and hot plate method

The acetic acid-induced abdominal constriction test was performed as described by Whittle [26]. The vehicle, aminopyrine (100 mg/kg) and test solution (10 mg/kg) were administered orally 30 min before the experiment and 0.1 mL/10 g of 0.7% acetic acid–saline was then injected i.p. 10 min after the injection. Subsequently, the frequency of abdominal constriction in the mice was counted for the next 10 min. The hot plate test was used to measure the response latencies according to the method described previously by Eddy and Leimback [27], with minor modifications. In these experiments, the hot plate (Ugo Basile, model-DS 37) was maintained at 56 ± 1 °C. The reaction time was noted by observing either the licking of the hind paws or the jumping movements before and after drug administration. The cut-off time was 10 s and 10.0 mg/kg of morphine sulphate (Kuju Pharmaceutical, CO), administered intraperitoneally, was used as the reference drug [28].

2.5. Cell culture and sample treatment

The RAW 264.7 cells were cultured in DMEM containing 10% heat-inactivated FBS, penicillin (100 units/mL) and streptomycin sulfate (100 μg/mL), and maintained in a humidified atmosphere of 5% CO_2. The cells were incubated with the compounds being tested at various concentrations or with the positive chemicals and stimulated with LPS (1 μg/mL) for the period indicated.

2.6. MTT assay for cell viability

RAW 264.7 viability after 24 h of continuous exposure to the tested compounds were measured with a colorimetric assay based on the ability of mitochondria in viable cells to reduce MTT as described previously [21].

2.7. Nitrite determination

The nitrite, which accumulated in the culture medium, was measured as an indicator of NO production by means of the Griess reaction. Briefly, 100 μL of cell culture medium (without phenol red) was mixed with an equal volume of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine–HCl), incubated at room temperature for 10 min, and then the absorbance was measured at 550 nm using a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was obtained by means of the $NaNO_2$ serial dilution standard curve and the nitrite production was measured.

2.8. PGE_2 and TNF-α assay

The PGE_2 and TNF-α levels in the macrophage culture medium were quantified using EIA kits according to the manufacturer's instructions.

2.9. Western blot analysis

Cellular proteins were extracted from both the control and CM-treated RAW 264.7 cells. The cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM PMSF, 0.5 mM DTT, 5 mM NaF, 0.5 mM Na_3VO_4), containing 5 μg/mL each of leupeptin and aprotinin, and incubated for 30 min at 4 °C. Fifty micrograms of cellular

protein from both the treated and untreated cell extracts was electroblotted onto a nitrocellulose membrane following separation using 8–12% SDS-polyacrylamide gel electrophoresis. The immunoblots were blocked overnight in Tween 20/Tris-buffered saline (TTBS) containing 5% (w/v) non-fat milk at 4 °C, followed by incubation for 4 h with a 1:1000 dilution of monoclonal anti-iNOS, COX-2, NF-κB (p65), IκB-α and β-actin antibodies. The blots were washed twice with TTBS and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody for 1 h at room temperature. The blots were washed again three times with TTBS and then developed by enhanced chemiluminescence (ECL).

2.10. RNA preparation and polymerase chain reaction

Total cellular RNA was isolated using an Easy Blue® kit according to the manufacturer's instructions. From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM dNTP and 0.5 μg/μL of oligo ($dT_{12–18}$). Then, PCR analysis was performed on aliquots of the cDNA preparations, in order to detect iNOS, COX-2, TNF-α and β-actin (as an internal standard) gene expression using a thermal cycler. The reactions were carried out in a volume of 25 μL containing 1 unit of Taq DNA polymerase, 0.2 mM dNTP, 10× reaction buffer and 100 pmol of the 5′ and 3′ primers (final concentration). After initial denaturation for 2 min at 95 °C, 30 amplification cycles were performed for iNOS, COX-2 and TNF-α (1 min of 95 °C denaturation, 1 min of 55 °C annealing and 1 min of 72 °C extension). The PCR primers used in this study are listed below and were purchased from Bioneer; sense strand iNOS primer, 5′-AATGGCAACATCAGGTCGGCCATCACT-3′; anti-sense strand iNOS primer, 5′-GCTGTGTGTCACAGAAGTCTCGAACTC-3′; sense strand COX-2 primer, 5′-GGAGAGACTATCAAGATAGT-3′; anti-sense strand COX-2 primer, 5′-ATGGTCAGTAGA CTTTTACA-3′; sense strand TNF-α primer, 5′-ATGAGCACAGAAAGCATGATC-3′; anti-sense strand TNF-α primer, 5′-TACAGGCTTGTCACTCGAATT-3′; sense strand β-actin primer, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′; anti-sense strand β-actin primer, 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

2.11. Preparation of nuclear extraction and EMSA

RAW 264.7 macrophages were plated in 100-mm dishes (5×10^6 cells). The cells were treated with various CM concentrations (25, 50 and 100 μM) or CM 100 μM for the periods indicated, stimulated with LPS for the periods indicated, washed twice with PBS, scraped into 1 mL of cold PBS and pelleted by centrifugation. Nuclear extracts were prepared according to a previously described procedure with a slight modification [28]. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μg/mL aprotinin) and incubated on ice for 15 min. Then the cells were lysed by the addition of 0.1% Nonidet P-40 and vigorous vortexing for 10 s. The nuclei were pelleted by centrifuging them at 12,000 × *g* for 1 min at 4 °C and resuspended in high-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM Na_3VO_4). A 10 μg sample of the nuclear extract was mixed with the double-stranded NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) end-labeled by [γ^{-32}P] dATP (underlining indicates a κB consensus sequence or a binding site for the NF-κB/cRel homodimeric and heterodimeric complexes). The binding reactions were performed at 37 °C for 30 min in 30 μL of reaction buffer at pH 7.5 containing 10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly(dI-dC) and 1 mM DTT. The specificity of binding was examined by competition with the 80-fold unlabeled oligonucleotide. DNA–protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5× TBE buffer. The gels were vacuum-dried for 1 h at 80 °C and exposed to X-ray film at −70 °C for 24 h.

2.12. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons, and the Student's *t*-test for single comparisons. The data are reported as means ± S.E.M. The numbers of independent experiments assessed are given in the figure legends.

3. Results

3.1. In vivo anti-inflammatory and antinociceptive activity of caffeic acid, caffeic acid methyl ester and di-O-acetyl caffeic acid

We examined the anti-inflammatory effect of CA, CM and DAC using the carrageenin-induced edema model and maximal edema inhibition was observed at 3 h after the edema induction. In particular, treatment with CM (10 mg/kg, p.o.) reduced the edema rate by 23.6% at 3 h and ibuprofen (100 mg/kg, p.o.) treatment decreased the edema rate by 42.2% at 3 h (Table 1). This reduction remained statistically significant for more than 5 h after the edema induction. In addition, the antinociceptive effect of the test samples was assayed using two different models, i.e., by the acetic acid-induced abdominal constriction test and the hot plate test in mice. In the case of the former test, CM and

Table 1
Effect of caffeic acid, caffeic acid methyl ester and di-*O*-acetylcaffeic acid on carrageenan-induced paw edema in rat

Treatment	Dose (mg/kg)	Swelling volume (mL)				
		1 h	2 h	3 h	4 h	5 h
Control		1.27 ± 0.02^{m}	$2.18 \pm 0.02^{d,e,f,g}$	3.01 ± 0.02^{a}	$2.52 \pm 0.02^{b,c}$	1.86 ± 0.02^{h}
CA	10	1.25 ± 0.03^{m}	$2.16 \pm 0.01^{e,f,g}$	2.65 ± 0.01^{b}	$2.33 \pm 0.02^{c,d,e}$	1.91 ± 0.01^{h}
DAC	10	1.22 ± 0.02^{m}	$2.18 \pm 0.02^{d,e,f}$	$2.77 \pm 0.02^{a,b}$	$2.50 \pm 0.02^{b,c}$	1.91 ± 0.02^{h}
CM	10	1.25 ± 0.20^{m}	$2.11 \pm 0.02^{f,g}$	$2.30 \pm 0.02^{d,e,f}$	2.08 ± 0.02^{g}	$1.57 \pm 0.03^{j,k}$
Ibuprofen	100	0.86 ± 0.02^{n}	$1.47 \pm 0.03^{k,l}$	$1.74 \pm 0.02^{h,I,j}$	$1.59 \pm 0.05^{i,j,k}$	1.20 ± 0.01^{m}

Samples were orally administered for 1 week after carrageenin-induced paw edema. The rats were tested 24 h for last treated materials; values represent means ± S.E.M. ($n = 6$); values sharing the same superscript letter are not significantly different from each other ($p < 0.05$) by Duncan's multiple range test.

CA were found to significantly reduce the number of abdominal constrictions induced by 0.7% acetic acid solution. The percentages of protection afforded by CM and CA after their oral administration were 67.8 and 55.6%, respectively (Table 2). Although this test constitutes a non-specific model (e.g., anti-cholinergic, anti-histaminic and other agents also show activity in this test), it is widely used for analgesic screening and involves local peritoneal receptors (cholinergic and histamine receptors) and the mediators of acetylcholine and histamine. The results of the hot plate test in mice (Table 2) shows that CM and CA at a dose of 10 mg/kg significantly increased the latency of the jumping response without affecting the animal's ability to detect pain. Morphine (10 mg/kg, i.p.) was used as a positive control in the hot plate test. Among the tested compounds, caffeic acid methyl ester showed the most significant anti-inflammatory activity in the carrageenin-induced paw edema assay and it also exhibited the most significant antinociceptive activities in the abdominal constriction and hot plate assays. The potencies of these three compounds were found to be in the following order: CM > CA > DAC.

3.2. Effect of caffeic acid, caffeic acid methyl ester and di-O-acetyl caffeic acid on NO and PGE_2 production

To assess the effects of CA, CM and DAC on the LPS-induced NO production in RAW 264.7 cells, cell culture medium was harvested and the production of nitrite was measured using the Griess reaction. CA, CM and DAC reduced the NO production in a dose-dependent manner with IC_{50} values of 330, 45.8 and 294.5 μM, respectively (Fig. 2A). L-NIL (10 μM) was used as a positive inhibitor. To examine whether the tested compounds could inhibit PGE_2 production in the same manipulation, the cells were pre-incubated with the CA, CM and DAC for 1 h and then activated with 1 μg/mL LPS for 24 h. As shown in Fig. 2B, CM significantly inhibited the production of PGE_2 in a dose-dependent manner. The cytotoxic effects of CA, CM and DAC were evaluated in the presence or absence of LPS using the MTT assay and these compounds did not affect the cell viability of RAW 264.7 cells in either the presence or absence of LPS even at a dose of 400 μM after a period of 24 h (data not shown).

3.3. Inhibition of LPS-induced TNF-α production by CM

Since CM was the most potent inhibitor of the pro-inflammatory mediators among the tested compounds, we further investigated the effect of CM on the LPS-induced TNF-α release using an enzyme immunoassay and RT-PCR. Pre-treatment of the cells with CM for 1 h decreased both the TNF-α production and mRNA expression in a dose-dependent manner (Fig. 3A and B).

3.4. Effects of CM on LPS-induced iNOS and COX-2 protein and mRNA expressions

Western blot and RT-PCR analyses were performed to determine whether the inhibitory effect of CM on these pro-inflammatory mediators (NO and PGE_2) was related to

Table 2
Antinociceptive effect of caffeic acid, caffeic acid methyl ester and di-*O*-acetylcaffeic acid by acetic acid-induced abdominal constriction and hot plate method in mice

Treatment	Dose (mg/kg)	Frequency (count/10 min)	Inhibition (%)	Action time (s)	Prolongation (%)
Control		40.91 ± 1.43^{a}	0	26.70 ± 0.72^{d}	0
CA	10	18.13 ± 0.46^{b}	55.6	62.95 ± 1.09^{c}	140
DAC	10	38.95 ± 1.03^{a}	4.1	31.35 ± 1.32^{d}	24
CM	10	$13.23 \pm 0.73^{b,c}$	67.8	82.79 ± 2.26^{b}	210.5
Morphine	10			159.22 ± 2.87^{a}	495.9
Aminopyrine	100	9.06 ± 0.45^{c}	78		

Values represent means ± S.E.M. ($n = 10$); values sharing the same superscript letter are not significantly different each other ($p < 0.05$) by Duncan's multiple range test.

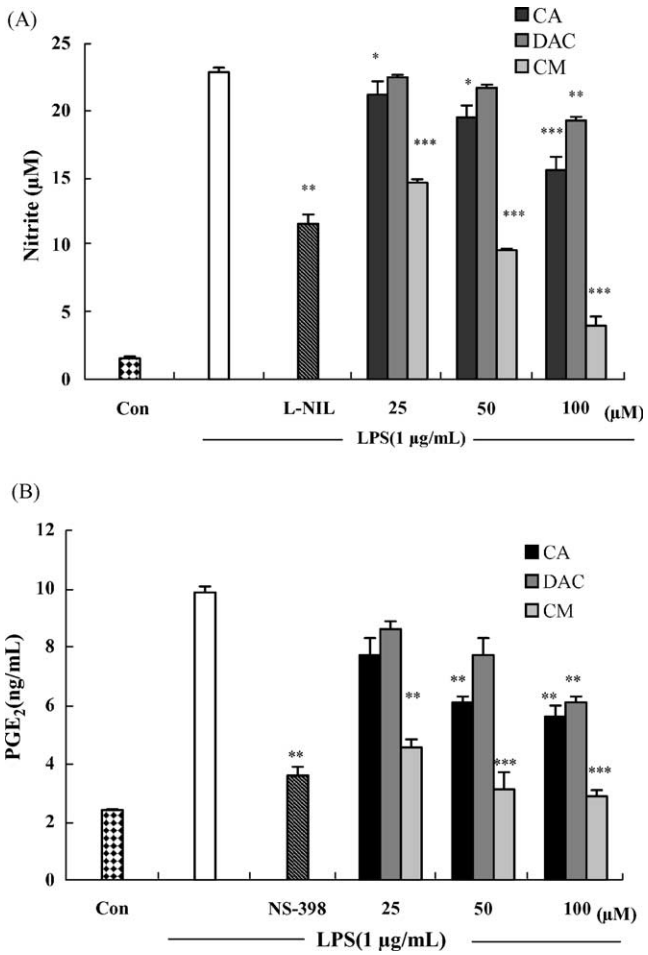


Fig. 2. The effects of caffeic acid, caffeic acid methyl ester and di-*O*-acetylcaffeic acid on NO (A) and PGE_2 (B) production in RAW 264.7 cells. (A) The cells were pretreated for 1 h with different concentrations (25, 50 and 100 μM) of caffeic acid and its derivatives, and then LPS (1 μg/mL) was added and the cells were incubated for 24 h. Control (Con) values were obtained in the absence of LPS or caffeic acid and its derivatives. Ten micromolar of L-N^6-(1-iminoethyl)lysine (L-NIL) was present in the assay as a positive control. The values are the means ± S.E.M. from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. LPS-treated group; the significance of the difference between the treated groups was evaluated using the Student's *t*-test. (B) The conditions of sample treatment were identical to those described for Fig. 2(A). Ten micromolar of NS-398 was used as a positive control in the assay. The values represent the means ± S.E.M. from three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. LPS-treated group; the significance of the difference between the treated groups was evaluated using the Student's *t*-test.

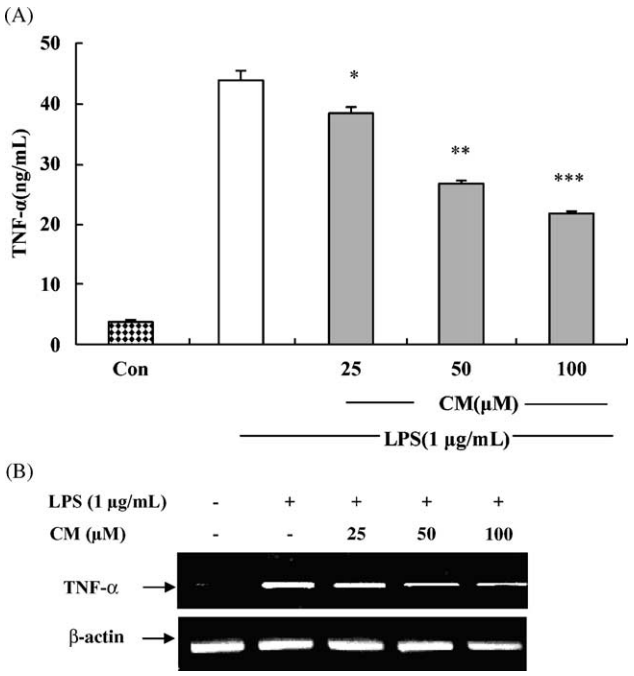


Fig. 3. The effect of caffeic acid methyl ester on LPS-induced TNF-α release in RAW 264.7 cells. (A) The cells were pretreated for 1 h with different concentrations (25, 50 and 100 μM) of caffeic acid methyl ester (CM), and then LPS (1 μg/mL) was added and the cells were incubated for 24 h. Control (Con) values were obtained in the absence of LPS or caffeic acid methyl ester (CM). (B) Total RNA was prepared for the RT-PCR analysis of TNF-α gene expression from RAW 264.7 macrophages that were stimulated with LPS (1 μg/mL) in the absence or presence of various concentrations (25, 50 and 100 μM) of CM for 4 h. The TNF-α-specific sequences (351 bp) were detected by staining the gel (2% agarose) with ethidium bromide. PCR of β-actin was performed to verify that the initial cDNA contents of the samples were similar. The values represent the means ± S.E.M. from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. LPS-treated group; the significance of the difference between the treated groups was evaluated using the Student's *t*-test.

the modulation of the expression of the iNOS and COX-2 enzymes. In unstimulated RAW 264.7 cells, neither iNOS nor COX-2 protein or mRNA were detectable. In response to LPS, the expression level of iNOS was markedly augmented and CM significantly inhibited iNOS protein induction in a dose-dependent manner (Fig. 4A). RT-PCR analysis showed that the amount of iNOS mRNA was correlated with its protein level (Fig. 4B). A similar pattern was observed when the effect of CM on the LPS-induced COX-2 expression level was examined; the densitometric analysis of three independent experiments demonstrated that the COX-2 protein expression induced by LPS was inhibited by 68.9% in cells treated with CM (100 μM) (Fig. 4A). Under the same conditions, the COX-2 mRNA levels were also significantly decreased in a similar way (Fig. 4B). CM did not affect the expression of the housekeeping gene, β-actin. In general, these results are consistent with the profile of the inhibitory effect of CM on NO and PGE_2 release (Fig. 2).

3.5. Inhibition of LPS-induced NF-κB activation by CM

To further investigate the mechanism of the CM-mediated inhibition of iNOS, COX-2 and TNF-α transcription, we focused on NF-κB, which is known to transactivate iNOS, COX-2, TNF-α and other genes [11]. Electrophoretic mobility shift assay (EMSA) analyses demonstrated that the LPS-induced NF-κB DNA binding activity in RAW 264.7 macrophages was significantly reduced by treatment with CM in a dose-dependent manner (Fig. 5). The extent of this reduction was in a range similar to that of the iNOS and COX-2 proteins and iNOS, COX-2 and TNF-α mRNA expressions.

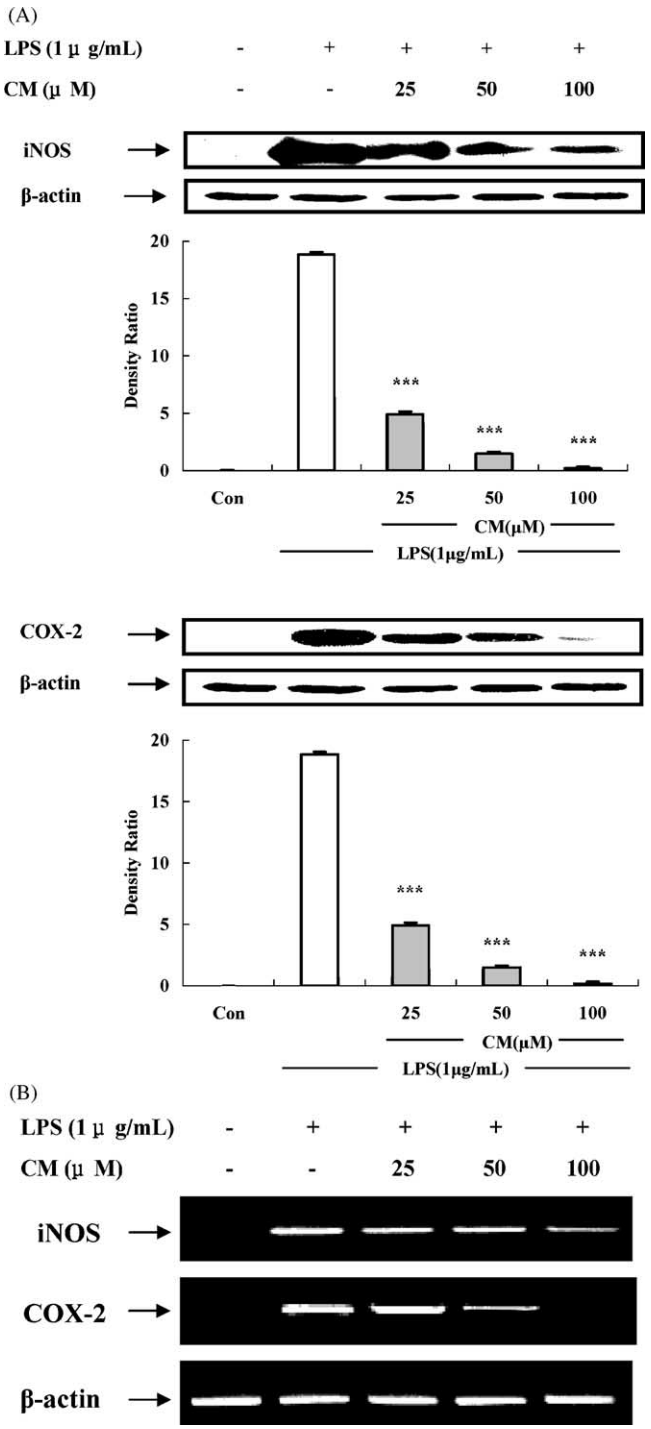


Fig. 4. The effects of caffeic acid methyl ester on LPS-induced iNOS and COX-2 proteins (A) and mRNAs (B) expression in RAW 264.7 cells. (A) Lysates were prepared from control or 24 h LPS (1 μg/mL)-stimulated cells alone or in combination with increasing concentrations (25, 50 and 100 μM) of CM. All lanes contained 50 μg of total proteins. A representative immunoblot from three separate experiments is shown. (B) Total RNA was prepared under conditions identical to those described for Fig. 3B and RT-PCR was performed to compare the mRNA level of iNOS and COX-2. PCR of β-actin was performed to verify that the initial cDNA contents of the samples were similar. The experiment was repeated twice with similar results being obtained in both cases.

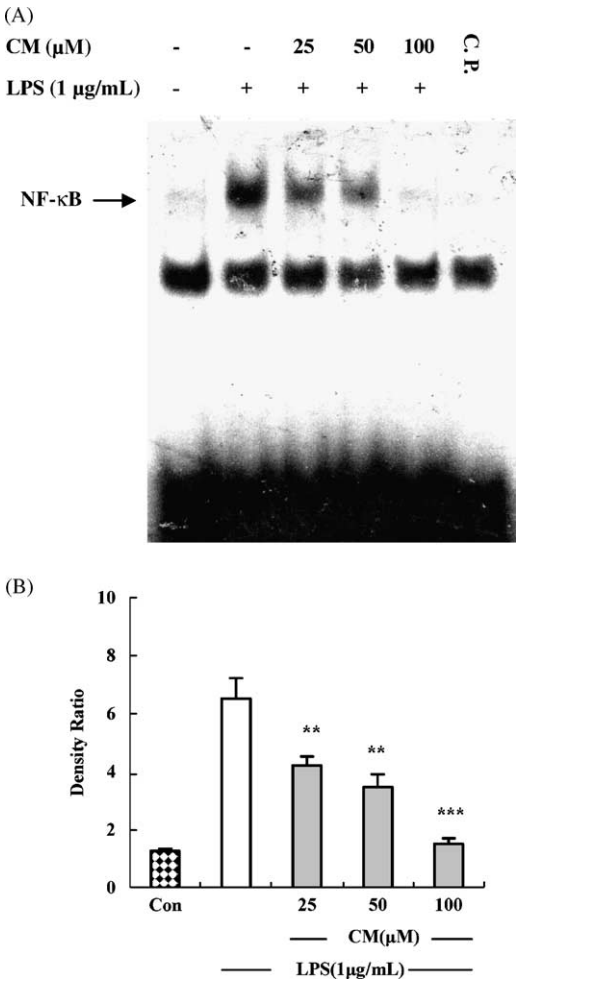


Fig. 5. The inhibition of NF-κB DNA binding by caffeic acid methyl ester (CM) was found to occur in a dose-dependent manner. RAW 264.7 cells were incubated for 60 min with different concentrations of CM. The specificity of binding was examined by competition with a 80-fold cold probe (C.P.). The arrow indicates the position of the NF-κB band. The data shown are representative of three independent experiments.

3.6. Effect of CM on degradation of IκB-α and nuclear translocation of p65

In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitor, IκB, which upon LPS stimulation is phosphorylated by its inhibitor IκB kinases, ubiquitinated and rapidly degraded via the 26S proteosome, thus releasing NF-κB [11]. We further investigated whether CM (100 μM) could inhibit the LPS-stimulated degradation of IκB-α in the cells, by conducting a Western blot assay with the anti-IκB-α antibody. Fig. 6 shows that the LPS induced the degradation of IκB-α after 10–20 min, and that this degradation was significantly blocked by pre-treatment with CM (100 μM). It was investigated as to whether CM prevented the translocation of the subunit of NF-κB, p65 from the cytosol to the nucleus after its release from IκBs. In the case of the treatment with CM, a decrease in the level of p65 in the nuclear fraction was detected by Western blot analysis until 60 min (Fig. 6A). This cascade

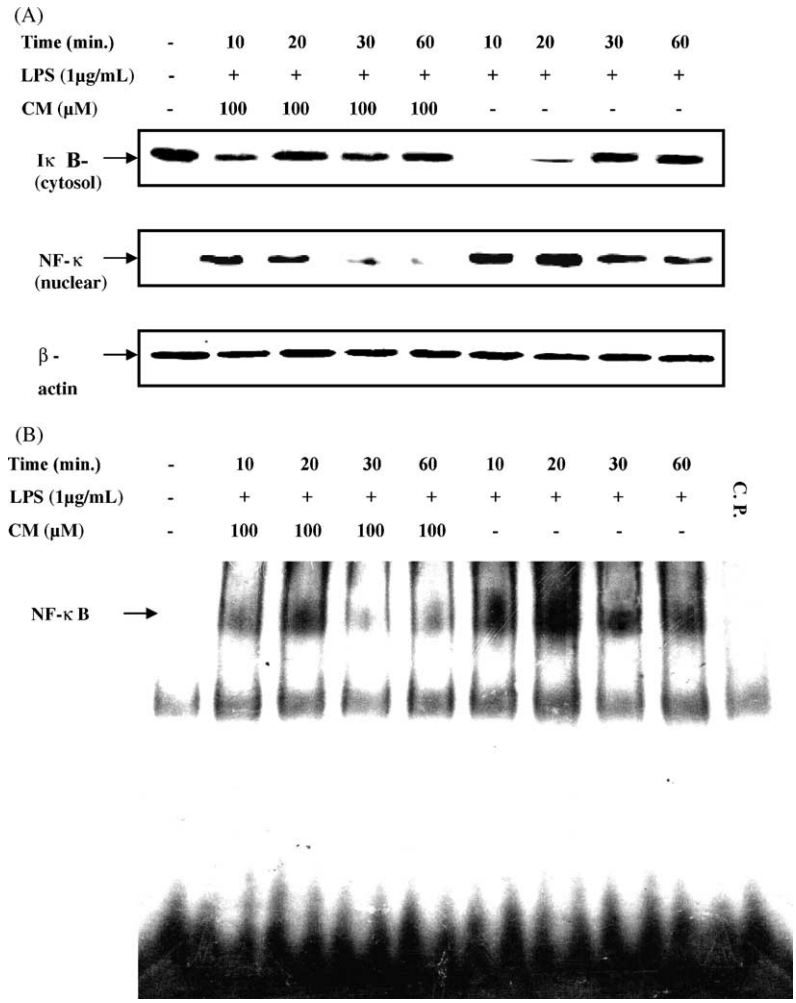


Fig. 6. The inhibition of IκB-α degradation and the nuclear translocation of p65 (A) and NF-κB DNA binding (B) by caffeic acid methyl ester (CM) were found to occur in a time-dependent manner. (A) RAW 264.7 macrophages were treated with LPS (1 μg/mL) and 100 μM of caffeic acid methyl ester (CM) for the indicated periods of time. For each condition, the protein level of IκB-α in the cytosolic fraction and the p65 level in the nuclear proteins were determined by Western blot analysis. (B) The nuclear protein from the LPS-stimulated cells was incubated for the indicated time with 100 μM of CM and the electrophoretic mobility shift assay was performed. The specificity of binding was examined by competition with a 80-fold cold probe (C.P.). The arrow indicates the position of the NF-κB band. The data shown are representative of three independent experiments.

was also confirmed by the inhibition of the NF-κB DNA binding activity between 30 and 60 min after the treatment with CM (100 μM) (Fig. 6B).

4. Discussion

While investigating the anti-inflammatory and antinociceptive effects of CA, CM and DAC in vivo, we found that CM significantly decreased the edema induced by carrageenin, in which the peak edema is characterized by the presence of PGs [29]. The antinociceptive effects of the tested samples were assayed using two different models, i.e., the acetic acid-induced abdominal constriction test and the hot plate test in mice. CM was found to significantly inhibit the acetic acid-induced abdominal constriction response at a dose of 10 mg/kg. In order to distinguish between the peripheral and central analgesic action, the hot plate test in mice was used to examine the antinociceptive effect, since it is well known that centrally acting analgesic drugs elevate the pain threshold of mice towards heat. It was found that CM significantly increased the hot plate reaction time in mice. The antinociceptive activities shown by CM in these models indicate that CM may possess peripherally and centrally mediated antinociceptive properties.

In the present study, we screened the effect of caffeic acid and its derivatives on the LPS-induced pro-inflammatory molecules, including NO and PGE_2. Thus, CM was found to be the most potent inhibitor of these mediators among the tested compounds. To further explore the possible mechanism of these inhibitions by CM, the expression levels of the iNOS and COX-2 proteins and the iNOS, COX-2 and TNF-α mRNA levels were examined. The inhibition by CM of the LPS-stimulated expression of these molecules in RAW 264.7 cells was not

attributable to cytotoxicity as assessed by the MTT assay and the expression of the housekeeping gene β-actin. The inhibition of the iNOS and COX-2 gene expression was evidenced by reductions in the mRNA levels in a parallel concentration-dependent manner with a similar pattern. Thus, the inhibition of NO and PGE_2 release may be attributed to the suppression of iNOS and COX-2 mRNA transcription followed by protein expression.

The expression of iNOS and COX-2 in murine macrophages has been shown to be dependent on NF-κB activation [11]. The possibility that CM might inhibit the activity of NF-κB was examined. The results indicate that the inhibition by CM on the expression of the iNOS and COX-2 proteins and iNOS, COX-2 and TNF-α mRNA was most likely due to the suppression of NF-κB. This is consistent with previous reports that NF-κB response elements are present on the promoters for the iNOS, COX-2 and TNF-α genes [30–34]. NF-κB is primarily composed of two proteins, p50 and p65, which are also referred to as RelA and cRel, respectively [11]. In the resting states, NF-κB is present in the cytosol and is bound to the inhibitory protein, IκB. Following its induction by a variety of agents such as LPS, TNF-α and tissue plasminogen activator, IκB is phosphorylated to trigger proteolytic degradation via the 26S proteosome. NF-κB is then released from IκB and is translocated into the nucleus, where it binds to the κB binding sites in the promoter regions of the target genes. In the present study, we found that CM blocked the LPS-induced activation of NF-κB via the inhibition of the degradation of IκB. These results suggest that the inhibition by CM of the LPS-induced expression of the iNOS and COX-2 genes occurs through the blocking of NF-κB activation, although the inhibition of other factors such as AP-1, the interferon response element and the γ-activated site may also be involved.

Although several studies have shown the anti-oxidative effects of caffeic acid and some of its derivatives [22–24], we go so far as to propose the suppression of the transcriptional activity of NF-κB as a possible mechanism by which CM prevents the inhibition of κB degradation. Caffeic acid phenethyl ester (CAPE), a structural derivative of caffeic acid, has been shown as a potent and specific inhibitor of NF-κB activation [35] and this may provide the molecular basis for its multiple immunomodulatory and anti-inflammatory activities. Moreover, CAPE stimulated Ca^{2+}-activated K^+-current in pituitary GH_3 cells [36] and induced apoptosis by activation of Fas in human breast cancer cells [37] via NF-κB inhibition. These diverse effects of CAPE might be attributed to its ability to pass through the cell membrane more easily than caffeic acid [38]. We also suggest that the more hydrophobic structure of caffeic acid methyl ester compared to the other two compounds may be responsible for its characteristic physicochemical properties for its anti-inflammatory action.

The results of the present study indicate that CM is a potent inhibitor of the LPS-induced NO, PGE_2 and TNF-α production via gene expression and this inhibition was found to be caused by the blocking of NF-κB activation in RAW 264.7 macrophages. This anti-inflammatory effect was also evident in vivo. These findings enhance the importance of caffeic acid methyl ester as a compound for treating LPS-induced sepsis syndrome and rheumatoid arthritis.

Acknowledgments

This research was supported by a Grant (PF002104-07) from the Plant Diversity Research Center of the 21st Frontier Research Program funded by the Ministry of Science and Technology of the Korean government and by the Korea Science & Engineering Foundation (No. R13-2002-020-01002-0).

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