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# Chebulagic acid (CA) attenuates LPS-induced inflammation by suppressing NF-κB and MAPK activation in RAW 264.7 macrophages

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ABSTRACT

Chebulagic acid (CA), a natural anti-oxidant, showed potent anti-inflammatory effects in LPS-stimulated RAW 264.7, a mouse macrophage cell line. These effects were exerted via inhibition of NO and $PGE_2$ production and down-regulation of iNOS, COX-2, 5-LOX, TNF-α and IL-6. CA inhibited NF-κB activation by LPS, and this was associated with the abrogation of IκB-α phosphorylation and subsequent decreases in nuclear p50 and p65 protein levels. Further, the phosphorylation of p38, ERK 1/2 and JNK in LPS-stimulated RAW 264.7 cells was suppressed by CA in a concentration-dependent manner. LPS-induced generation of reactive oxygen species (ROS) was also effectively inhibited by CA. These results suggest that CA exerts anti-inflammatory effects in LPS-stimulated RAW 264.7 macrophages by inhibition of NF-κB activation and MAP kinase phosphorylation.



Inflammation is a complex process regulated by a cascade of cytokines, growth factors, NO and prostaglandins produced by activated macrophages. Macrophages are key players in the immune response to foreign invaders such as infectious microorganisms. Macrophages are activated by IFN-γ, pro-inflammatory cytokines [1], bacterial lipopolysaccharide (LPS) [2], GM-CSF and phorbol esters. Activated macrophages play an important role in inflammatory diseases via production of cytokines, interleukin-1 beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α) and other inflammatory mediators such as nitric oxide (NO) and prostaglandins ($PGE_2$) [3]. Excess production of inflammatory mediators is involved in many diseases including rheumatoid arthritis [4], atherosclerosis [5], asthma [6] and pulmonary fibrosis [7].

Nuclear transcription factor kappa-B (NF-κB) regulates various genes involved in immune and acute phase inflammatory responses, and in cell survival [8]. NF-κB activation in response to pro-inflammatory stimuli involves the rapid phosphorylation of IκBs by the IKK signalosome complex [9]. The resulting free NF-κB then translocates to the nucleus, where it binds to κB-binding sites in the promoter regions of target genes and induces the transcription of pro-inflammatory mediators such as, iNOS, COX-2, TNF-α, IL-1β, IL-6 and IL-8 [10].

The mitogen-activated protein kinases (MAPKs) react to extracellular stimuli (mitogens) and control various cellular activities including gene expression, mitosis, differentiation and cell survival/apoptosis [11]. MAP kinases (ERK, p38 and JNK) regulate inflammatory and immune responses [12] and their signaling pathways are involved in LPS-induced COX-2 and iNOS expression in macrophages [13].

The overproduction of reactive oxygen species (ROS) by macrophages causes oxidative damage to membrane lipids, DNA, proteins and lipoproteins. ROS also participate in the modulation of NF-κB activation [14]. Therefore, inhibition of ROS production is a popular therapeutic target for many inflammatory diseases [15].

Fruits of *Terminalia chebula* have been used in various Ayurvedic preparations for the treatment of a variety of disorders. Their fruit powder is one of the main constituents of *Triphala*, a well known Ayurvedic medicine used to treat allergies and common health disorders. CA is one of the main bio-active constituents of *Terminalia chebula* fruit powder. CA has been shown to inhibit α-glucosidase activity [16], ROS generation from PMA-stimulated leukocytes [17] and CTL-mediated cytotoxicity [18]. In addition, it has been reported to suppress arthritis in mice [19] and LPS-induced nitric oxide (NO) generation in RAW 264.7 mouse macrophage cells [20]. However the detailed molecular anti-inflammatory mechanism has not yet been studied. In the present study, we show for the first time that CA inhibits NF-κB activity and phosphorylation of MAP kinases in LPS-stimulated RAW 264.7 macrophages.

## Materials and methods

*Chemicals.* Phosphate-buffered saline (PBS), RPMI-1640, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (California, USA). *Escherichia coli* lipopolysaccharide

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(LPS), trypsin-EDTA, protease inhibitor cocktail and β-actin antibodies were purchased from Sigma Chemical Company (St. Louis, USA). iNOS, COX-1, COX-2, 5-LOX, p65, p50, p-IκB-α, IκB-α, p-ERK, ERK, p-p38, p38, p-JNK, JNK and PARP monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc., (CA, USA). Enzyme immunoassay kits for the measurement of $PGE_2$ and NF-κB were purchased from Cayman Chemical (Ann Arbor, MI, USA) and Invitrogen Inc., (CA, USA) respectively.

*Plant material and the isolation of CA.* CA was isolated from the ethanolic extract of *Terminalia chebula* fruits by RP-HPLC (see Supplementary data). The purity of the obtained CA was greater than 98.0%.

*Cell culture.* RAW 264.7 (murine macrophage) cells were obtained from NCCS, Pune, India. Cells were maintained in a humidified atmosphere with 5% $CO_2$ at 37 °C. The medium for cell lines was RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

*Nitrite measurement.* RAW 264.7 cells were plated at $5 \times 10^5$ cells/well in 24-well plates and then incubated with or without LPS (1 μg/ml) in the absence or presence of various concentrations (5, 10, 15, 20 and 25 μM) of CA for 16 h. Nitrite levels in the culture media were determined using the Griess reaction. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl] and incubated at room temperature for 10 min. Absorbance was then measured at 540 nm using a microtiter plate reader from Quant Bio-Tek Instruments, VT. Fresh culture media were used as blanks for all experiments. Nitrite levels in samples were determined using a standard sodium nitrite curve.

*Determination of $PGE_2$ and NF-κB levels.* $PGE_2$ (in culture medium supernatants) and NF-κB (in nuclear extracts) concentrations were determined by using EIA kits according to the manufacturer's instructions.

*Preparation of cytosolic and nuclear extracts.* RAW cells were cultured in 6-well plates ($4 \times 10^6$ cells/well) with or without LPS (1 μg/ml) and in the presence or absence of CA. The cytoplasmic and nuclear extracts were prepared for protein level measurement by Western blotting and enzyme immuno assay (EIA). Briefly, cells were collected and washed twice with cold PBS, lysed in 400 μl of cold buffer A [HEPES 10 mmol/L, pH 7.9, KCl 10 mmol/L, 1 mM EDTA, phenylmethanesulphonylfluoride (PMSF) 1 mmol/L, 1 mM EGTA, dithiothreitol (DTT) 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L and pepstatin A 1 mg/L]. After a 15 min incubation on ice, 0.1% NP-40 was added to the homogenates and the tubes were vigorously rocked for 1 min. Then, the homogenates were centrifuged (20,800*g*, 5 min) at 4 °C. The supernatant (cytosolic extracts) was collected and stored in aliquots at −70 °C. The nuclear pellets were washed once with cold buffer A, then suspended in 50 μl of cold buffer B (Hepes 20 mmol/L, pH 7.9, NaCl 420 mmol/L, edetic acid

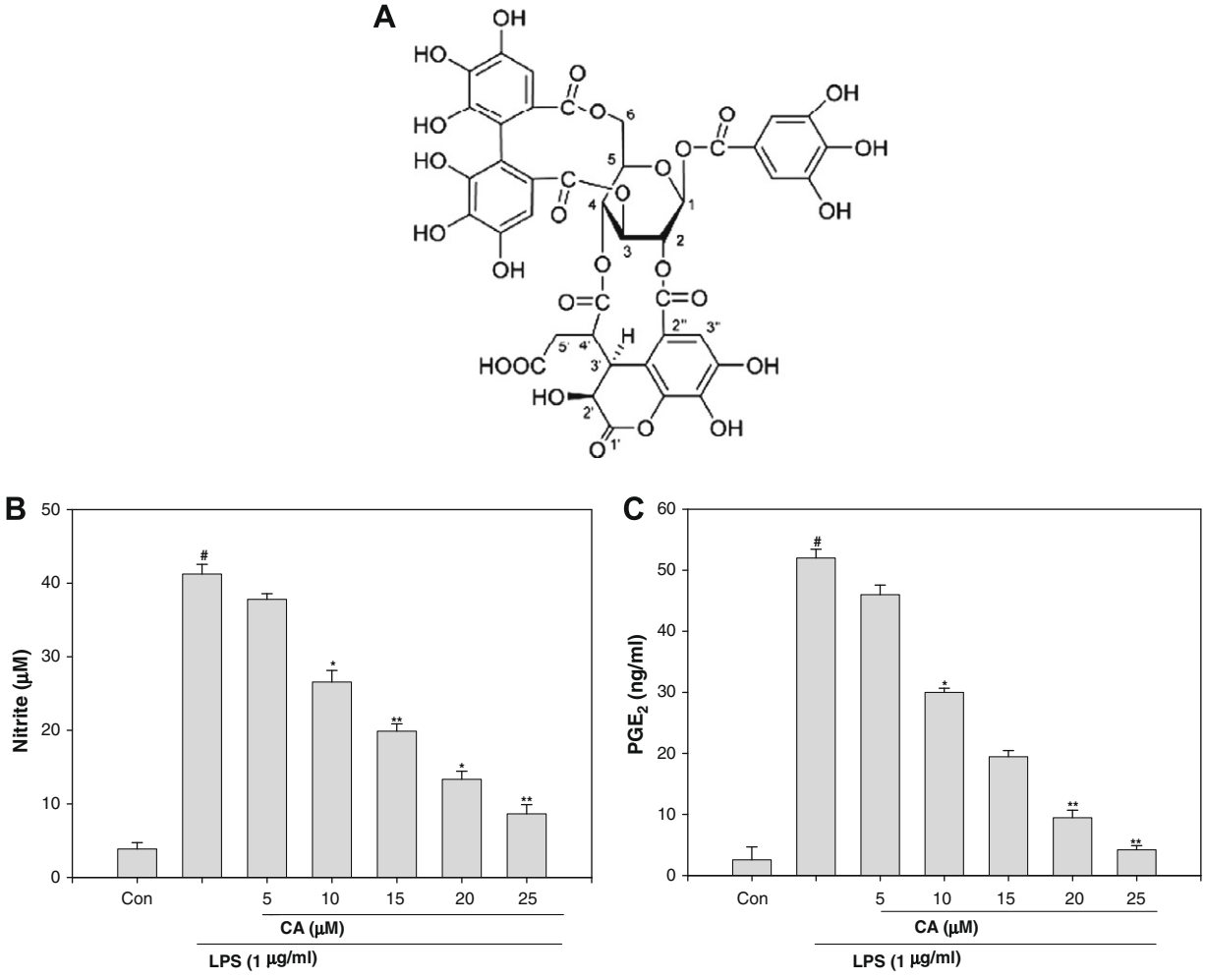


**Fig. 1.** (A) Chemical structure of CA. (B,C) Effect of CA on LPS-induced NO and $PGE_2$ production in RAW 264.7 cells. The culture supernatants were isolated and analyzed for nitrite (B) and $PGE_2$ (C) levels. Control (Con) values were obtained in the absence of LPS and of CA. Data represent the mean ± SEM of three independent experiments ($N = 3$). $^{\#}P < 0.05$ compared with control group (Con), $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with the groups treated with LPS (1 μg/ml) alone.

0.1 mmol/L, egtazic acid 0.1 mmol/L, PMSF 1 mmol/L, DTT 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L and pepstatin A 1 mg/L) and vigorously rocked at maximum speed and 4 °C for 30 min. The solution was clarified by centrifugation at 20,800*g* for 5 min, and the supernatant fluid (nuclear extract) was stored in aliquots at −70 °C. The protein concentration was determined according to the Bradford method [21].

*Western blotting.* Equal quantities of cytosolic/nuclear proteins from each treatment (70 μg of total protein/lane) were resolved on 8–12% SDS–PAGE and then transferred to nitrocellulose membranes. The membranes were blocked with 5% w/v non-fat dry milk and then incubated with the primary antibodies in 10 ml of buffer (Tris-buffered saline and 0.05% Tween 20 with 5% milk) with gentle shaking at 4 °C for 8–12 h and then incubated with the respective conjugated secondary antibodies. Signals were detected using Western blot detection reagents.

*RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR).* Total cellular RNA was extracted from both the controlled and treated cells in 6-well plates using TRI REAGENT™ according to the manufacturer's recommendations (Sigma Chemical Co., St. Louis, MO). From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM dNTP and 0.5 μg/μl oligo ($dT_{12-18}$). PCR analyses were performed on aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α, IL-6 and β-actin (as an internal standard). The reactions were carried out in a volume of 25 μl containing (final concentration) 1 U Taq DNA polymerase, 0.2 mM dNTP, 10× reaction buffer and 100 pmol of 5′ and 3′ primers. After an initial denaturation for 2 min at 95 °C, thirty amplification cycles were performed for iNOS (1 min 95 °C denaturation, 1 min 60 °C annealing and 1.5 min 72 °C extension), COX-1 (1 min denaturation at 94 °C, 1 min 60 °C annealing and 2 min 68 °C extension), COX-2 (1 min 94 °C denaturation, 1 min 60 °C annealing and 1 min 72 °C extension), 5-LOX (1 min 94 °C denaturation, 1 min 56 °C annealing and 1 min 72 °C extension), TNF-α (1 min 95 °C denaturation, 1 min 55 °C annealing and 1 min 72 °C extension) and IL-6 (1 min 94 °C denaturation, 1 min 56 °C annealing and 1 min 72 °C extension). PCR primers used in this study are listed below and were purchased from MWG Inc., (Bangalore, India): sense strand iNOS, 5′-AAT GGC AAC ATC AGG TCG GCC ATC ACT-3′; anti-sense strand iNOS, 5′-GCT GTG TGT CAC AGA AGT CTC GAA CTC-3′; sense strand COX-1, 5′-ACT GGC TCT GGG AAT TTG TG-3′; anti-sense strand COX-1, 5′-AGA GCC GCA GGT GAT ACT GT-3′; sense strand COX-2, 5′-GGA GAG ACT ATC AAG ATA GT-3′; anti-sense strand COX-2, 5′-ATG-GTC AGT AGA CTT TTA CA-3′; sense strand 5-LOX, 5′-GGC ACC GAC GAC TAC ATC TAC-3′; anti-sense strand 5-LOX, 5′-CAA TTT TGC ACG TCC ATC CC-3′; sense strand TNF-α, 5′-ATG AGC ACA GAA AGC-ATG ATC-3′; anti-sense strand TNF-α, 5′-TAC AGG CTT GTC ACT CGA ATT-3′; sense strand IL-6, 5′-GAG GAT ACC ACT CCC AAC AGA CC-3′; anti-sense strand IL-6, 5′-AAG TGC-ATC ATC GTT GTT CAT ACA-3′; sense strand β-actin, 5′-TCA TGA AGT GTG ACG-TTG ACA TCC GT-3′; anti-sense strand β-actin, 5′-CCT AGA AGC ATT TGC GGT GCA CGA TG-3′. After amplification, PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining and UV irradiation.

*Measurement of reactive oxygen species (ROS) production.* ROS production in control, LPS- and CA-treated RAW 264.7 cells was measured using 2, 7-dichloro dihydro fluorescein diacetate (DCFH-DA) dye. Cells seeded at a density of $2 \times 10^6$ per 60 mm culture dish were first pre-incubated with CA (10 and 20 μM) and the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) (10 μM) for 1 h, and then LPS (1 μg/ml) was added to all dishes except those containing control cells. Cells were harvested 30 min after LPS treatment and incubated with DCFH-DA (10 μM) for 10 min. Cells were then washed and harvested in PBS, and ROS measurement was carried out on a FACS Calibur flow cytometer. Data were collected using CELL Quest software (Becton Dickinson, San Jose, CA). DCF was measured with the following excitation and

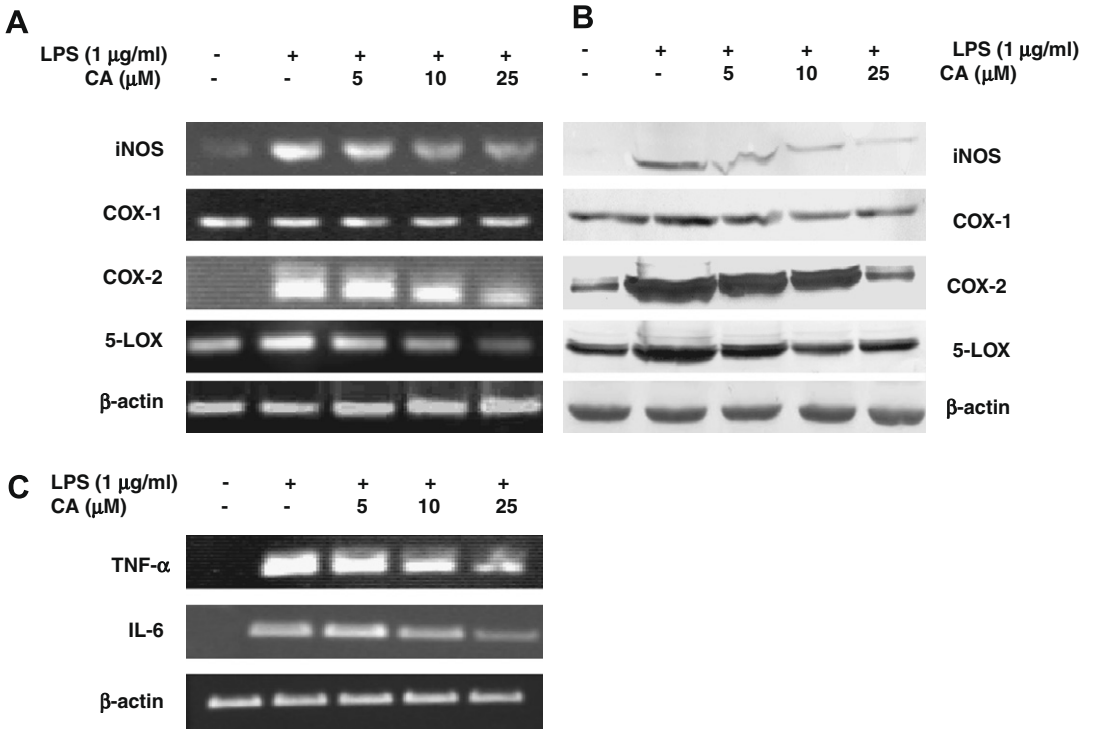


**Fig. 2.** Effect of CA on mRNA and protein expression in LPS-induced RAW 264.7 cells. (A,C) RT-PCR analysis of CA-mediated effects on iNOS, COX, 5-LOX, IL-6 and TNF-α mRNAs in LPS-induced RAW 264.7 cells. (B) Western blot analysis showing the effects of CA treatment on expression of iNOS, COX, 5-LOX proteins in LPS-induced RAW 264.7 cells. β-Actin was used as an internal control. The experiment was repeated two times and similar results were obtained.

emission wavelengths: $\lambda_{exc}$ = 488 nm and $\lambda_{emi}$ = 525 nm. 10,000 cells were analyzed per sample.

*Statistical analysis.* Data were presented as mean ± SEM and *P* values were determined using the unpaired Student's *t*-test. *P* values of less than 0.05 were considered significant.

## Results and discussion

### *CA treatment inhibited LPS-induced NO and $PGE_2$ secretion in RAW 264.7 macrophages*

In the present study, we found that CA potently inhibited the production of NO and prostaglandin $E_2$ ($PGE_2$) in a concentration-dependent manner. To examine the potential anti-inflammatory properties of CA on LPS-induced NO and $PGE_2$ production in RAW 264.7 cells, cells were treated with or without CA (5, 10, 15, 20 and 25 μM) for 1 h and then treated with LPS (1 μg/ml) for 16 h. NO and $PGE_2$ concentrations were measured in the culture supernatants by the Griess reaction and ELISA assays, respectively. LPS treatment significantly increased the concentrations of NO and $PGE_2$. As shown in Fig. 1, CA significantly inhibited LPS-induced NO (Fig. 1B) and $PGE_2$ (Fig. 1C) production in a concentration-dependent manner.

### *CA treatment attenuated LPS-induced expression of iNOS, COX and 5-LOX mRNAs and proteins*

iNOS, COX-2 and 5-LOX enzymatic products (NO, $PGE_2$ and $LTB_4$, respectively) serve as key mediators of inflammation; thus agents that suppress their expression have therapeutic potential for inflammatory diseases. iNOS, COX-2 and 5-LOX protein levels were markedly up-regulated in response to LPS treatment (Fig. 2B), and CA inhibited the expression of these proteins in a concentration-dependent manner. Moreover, RT-PCR analysis showed that the expression of iNOS, COX-2 and 5-LOX mRNAs was correlated with their protein levels (Fig. 2A). Furthermore, CA treatment did not change the expression level of COX-1, which is constitutively expressed in most tissues and appears to be responsible for housekeeping and normal physiological functions including maintenance of the integrity of the gastric mucosa and regulation of renal blood flow.

### *CA treatment inhibited LPS-induced TNF-α and IL-6 mRNA expressions*

TNF-α and IL-6 are mainly produced by macrophages and are known to have a key role in inflammatory processes [22]. In the present study, pre-treatment with CA (5, 10 and 25 μM) significantly reduced TNF-α and IL-6 mRNA expression in a concentra-

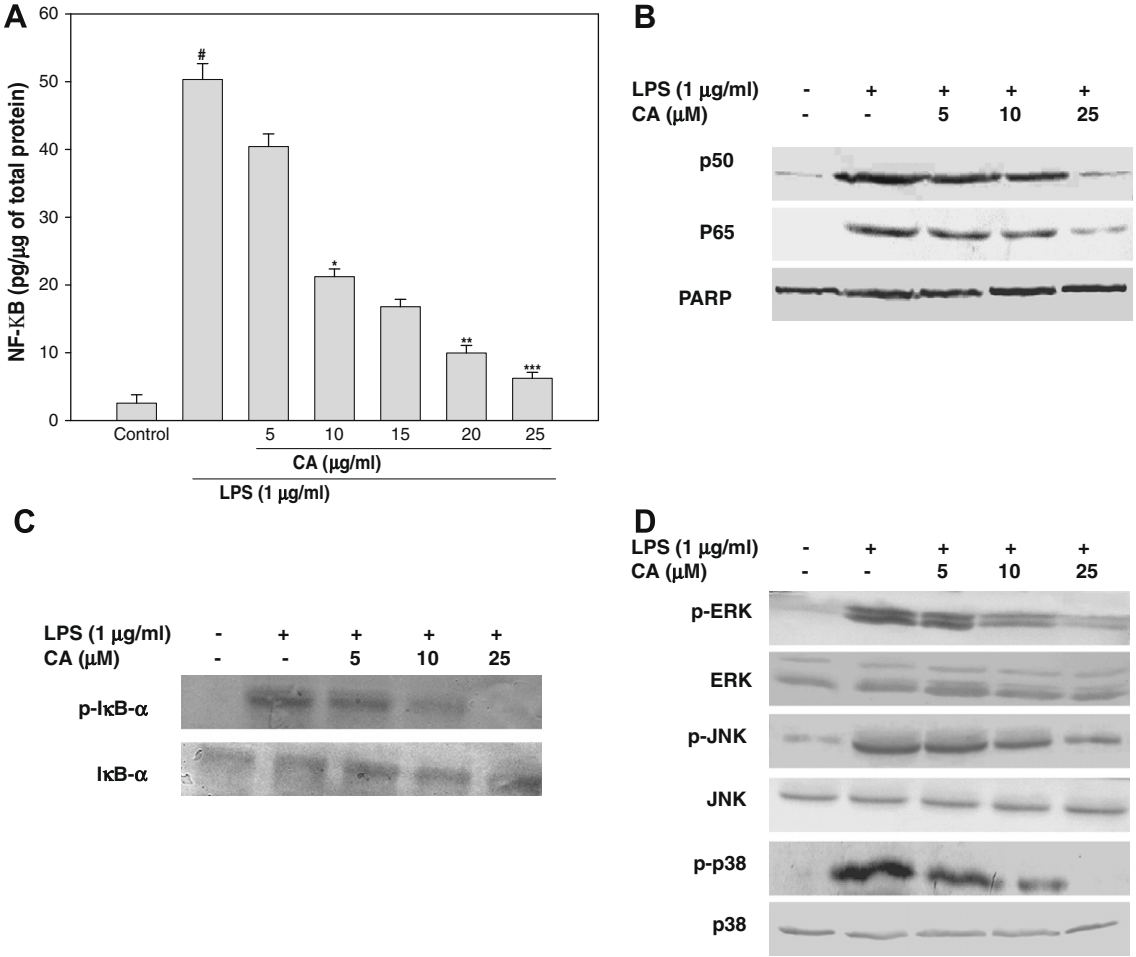


**Fig. 3.** Effect of CA on nuclear translocation, activation of NF-κB and phosphorylation of IκB-α and MAP kinases in LPS-stimulated RAW 264.7 macrophages. (A) Nuclear NF-κB (p65) levels were estimated by EIA as described in Materials and methods. Data represent the mean ± SEM of three independent experiments ($N = 3$). $^{\#}P < 0.05$ compared with control group (Con), $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared with the groups treated with LPS (1 μg/ml) alone. (B) Western blot analysis showing the effect of CA treatment on expression of NF-κB p65 and p50 in nuclear extracts of LPS-induced RAW 264.7 cells. (C,D) Western blot analysis showing the effect of CA on the phosphorylation of IκB-α and MAPKs.

tion-dependent manner in RAW 264.7 cells stimulated with LPS (Fig. 2C).

*CA treatment inhibited LPS-induced NF-κB nuclear translocation and phosphorylation of IκB-α*

NF-κB, a mammalian transcription factor activated by LPS, is known to control the expression of cell survival genes as well as pro-inflammatory enzymes and cytokines such as iNOS, COX-2, TNF-α and IL-6. In the present study, CA treatment inhibited the LPS-induced translocation of NF-κB/p65 and the nuclear translocation of p65 and p50 proteins in a concentration-dependent manner as evidenced by ELISA (Fig. 3A) and Western blotting (Fig. 3B). These results suggest that the inhibition of LPS-induced iNOS and COX-2 expression was mediated by blockade of NF-κB activation.

Translocation of NF-κB to the nucleus requires IκB-α protein phosphorylation, ubiquitination and degradation, since nuclear translocation of NF-κB is directly linked to IκB-α degradation and phosphorylation [23]. The signal-induced phosphorylation of IκB-α is considered a key event that ultimately leads to the activation of NF-κB. Reactive oxygen species are one of the important activators, that activates the IκB kinase complex by phosphorylating IκB-α on serines 32 and 36, followed by ubiquitination on lysine residues 21 and 22 [24]. The ubiquitinated IκB-α is degraded by the proteasome. Active NF-κB (p50/p65 dimer) translocates to the nucleus and activates the expression of pro-inflammatory genes. Anti-oxidants like curcumin and ascorbic acid were reported to down regulate the activation of NF-κB by inhibiting phosporylation of IκB-α [25] and [26]. We therefore investigated whether CA could prevent the phosphorylation of IκB-α. It was found that pre-treatment with CA prior to LPS induction attenuated IκB-α phosphorylation in a concentration-dependent manner as revealed by Western blotting (Fig. 3C). Taken together, these results suggest that CA treatment inhibited LPS-induced NF-κB translocation by attenuating the phosphorylation of IκB-α.

*CA treatment attenuated LPS-induced MAP kinase phosphorylation*

MAP kinases play a critical role in the regulation of cell growth and differentiation, and they control cellular responses to cytokines and stress [27]. In addition, they play a critical role in the modulation of NF-κB activity [28]. To investigate the molecular mechanism of NF-κB inhibition by CA in LPS-stimulated RAW 264.7 cells, we studied the inhibition of the phosphorylation of p38, ERK1/2 and JNK. Maximal MAPK (ERK1/2, p38 and JNK) expression is known to occur 10–30 min after LPS treatment in human and murine monocytes and macrophages [29]. RAW 264.7 cells were pretreated with CA at the indicated concentrations for 30 min and then stimulated with 1 μg/ml LPS for 30 min. The total cell lysates were then probed with phosphospecific antibodies for p38, ERK1/2 and JNK. Phosphorylation of p38, ERK1/2 and JNK was increased in cells treated with LPS alone. However, CA treatment reduced phosphorylated p38, ERK1/2 and JNK levels in LPS-stimulated RAW 264.7 cells in a concentration-dependent manner (Fig. 3D). No changes in the expression of non-phosphorylated ERK, JNK and p38 kinase were observed in cells treated with LPS or LPS and CA. These results suggest that suppression of phosphorylation of MAP kinases might be involved in the inhibitory effect of CA on LPS-stimulated NF-κB activation in RAW 264.7 cells.

*CA reduced ROS (reactive oxygen species) production via inhibition of NADPH oxidase*

ROS have an important role in NF-κB activation and pro-inflammatory cytokine production of LPS-treated macrophages. Macrophages produce reactive oxygen species (ROS) during stimulation with a wide variety of agents through activation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase. These ROS are assembled at the plasma membrane from resident plasma membrane and cytosolic protein components [30]. In view of the anti-oxidant characteristics of CA, we investigated whether CA could inhibit LPS-induced ROS generation in RAW 264.7 cells using FACS analysis. As shown in Fig. 4, ROS production was markedly increased in LPS-induced cells and treatment with CA attenuated ROS production in a concentration-dependent manner. DPI, a NADPH oxidase inhibitor, reversed ROS production in LPS-induced RAW 264.7 cells.

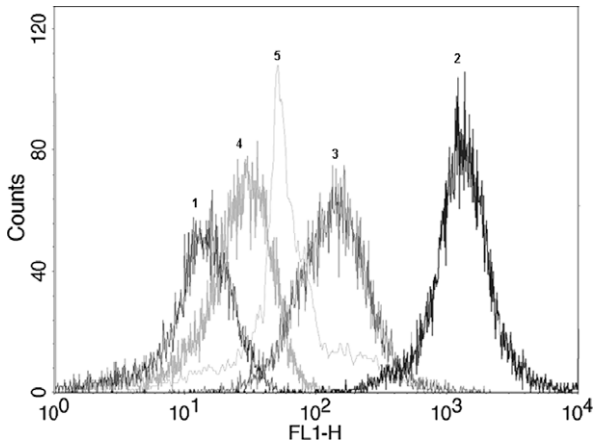


**Fig. 4.** FACS analysis of ROS generation using DCFH-DA in cells exposed to LPS (1 μg/ml) with or without CA and DPI pre-treatment. (1) Control RAW 264.7 cells, (2) RAW 264.7 cells treated with LPS (1 μg/ml) only, (3) LPS + CA (10 μM), (4) LPS + CA (25 μM) and (5) LPS + DPI (10 μM). The figure was obtained from three independent experiments with similar patterns. DPI, an NADPH oxidase inhibitor, was used as a positive control at 10 μM.

In conclusion, our observations suggest that CA, a natural antioxidant, exerts potent anti-inflammatory effects by inhibiting LPS-induced gene expression (TNF-α, IL-6, iNOS, 5-LOX and COX-2) in RAW 264.7 macrophages. These effects are exerted by suppression of NF-κB activation and phosphorylation of MAP kinases. CA could thus represent a potential anti-inflammatory drug candidate. Further studies are required to elucidate the mechanism and therapeutic potential of CA.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.02.022.

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