Bornyl Cinnamate Inhibits Inflammation-Associated Gene Expression in Macrophage Cells through Suppression of Nuclear Factor-κB Signaling Pathway

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Key words
- *Liquidamber formosana*
- Altingiaceae
- bornyl cinnamate
- anti-inflammatory activity
- NF-κB

Abstract

Formosan sweetgum (*Liquidamber formosana*) is an endemic tree species. Various parts of this tree are used as a traditional Chinese medicine for treating pain, inflammation, and rheumatic disorders. In this study, we investigated the anti-inflammatory potential of bornyl cinnamate, a cinnamic acid derivative from the essential oil of *L. formosana*. Pretreatment with bornyl cinnamate significantly inhibited lipopolysaccharide-induced proinflammatory molecules, including nitric oxide, prostaglandin-E2, tumor necrosis factor α, and interleukin-1β production, in murine macrophage RAW 264.7 cells. RT-PCR and immunoblotting analysis revealed that the inhibition of the proinflammatory molecules occurred through the downregulation of their corresponding mediator genes. Immunofluorescence and luciferase reporter assays revealed that the inhibition of proinflammatory genes by bornyl cinnamate was caused by the suppression of nuclear translocation and transcriptional activation of the redox-sensitive transcription factor nuclear factor κB. In addition, bornyl cinnamate increased the protein stability of the inhibitor of nuclear factor κB, an endogenous repressor of nuclear factor κB, through inhibition of its phosphorylation and proteasomal degradation. Furthermore, bornyl cinnamate significantly blocked the lipopolysaccharide-induced activation of I-κB kinase α, an upstream kinase of the inhibitor of nuclear factor κB α. Taken together, these results suggest that bornyl cinnamate could inhibit proinflammatory molecules through the suppression of the redox-sensitive nuclear factor κB signaling pathway.

Abbreviations

BC:	bornyl cinnamate
BSA:	bovine serum albumin
COX-2:	cyclooxygenase-2
CC:	curcumin
DAPI:	4′,6-diamidino-2-phenylindole dihydrochloride
FITC:	fluorescein isothiocyanate
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
IKK:	I-κB kinase
iNOS:	inducible nitric oxide synthase
Iκ-B:	inhibitor of nuclear factor κ-B
IL-1β:	interleukin-1β
LPS:	lipopolysaccharide
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide
NF-κB:	nuclear factor κB
NO:	nitric oxide
PGE_2:	prostaglandin-E_2
TNF-α:	tumor necrosis factor α

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Introduction

Inflammation is a necessary part of the body's response to many pathological conditions including tissue injury and microbial invasion. The inflammatory response is tightly regulated, with disruption leading to morbidity and a reduced life span [1,2]. Macrophages play a functional role in coordinating the immune response to invading pathogens through phagocytosis and cytokine secretion [3]. Activation of macrophages by endotoxins, such as lipopolysaccharides, the major constituents of the outer cell wall of gram-negative bacteria, has been widely used to investigate the mechanism of inflammation in both cell culture and animal models [4]. The activated macrophages subsequently trigger the activation of the redox-sensitive transcription factor NF-κB

through the inhibition of its endogenous repressor, I-κB. The cascade involves the phosphorylation of two serine residues (Ser32 and Ser36) on I-κBα, which results in its polyubiquitination and subsequent degradation by the 26S proteasome, permitting the unmasking of the nuclear localization signal and the translocation of the activated NF-κB into the nucleus. The translocated NF-κB bind to the DNA binding domain and transcribe a number of proinflammatory genes such as iNOS, COX-2, TNF-α, and IL-1β, which promote the production of proinflammatory molecules NO, PGE_2, TNF-α, and IL-1β [5–7]. Overproduction of these molecules elicits many inflammatory diseases, including rheumatoid arthritis, atherosclerosis, and hepatitis [8]. Therefore, inhibition of these proinflammatory molecules represents an ideal target for minimizing the burden of inflammatory diseases.

Clinically used anti-inflammatory drugs exhibit several side effects on humans and a high cost of treatment, as in the case of biologics. Plant extracts and derived compounds offer promising options for the development of drugs for treating inflammatory diseases [9]. *Liquidamber formosana* Hance (Altingiaceae) is an endemic deciduous tree native to Taiwan. *L. formosana* also produces a balsamic exudate named resina liquidambaris, which has numerous medical applications in Asian folk medicine, such as a promoter of blood circulation, alleviator of blood stasis, analgesic, anti-inflammatory, and wound-healing agent [10, 11]. A recent report shows that essential oil from the leaves of *L. formosana* inhibits LPS-induced inflammation in murine macrophage cells [12]. However, the active ingredients of the exudates involved in the protective effects of *L. formosana* were poorly understood.

BC (**Fig. 1**) is one of the major compounds in the balsamic exudate of *L. formosana* [10]. Biological effects of BC have been rarely studied, and the compound may be related to other organic molecules found in plants and microbes that also exhibit anti-inflammatory and antioxidant properties. For example, BC was identified in the rhizomes of the kava kava plant (*Piper methysticum* Forst), which has been adopted in traditional treatment for its anti-inflammatory and anxiolytic properties [13]. A previous study by Lobitz et al. [14] reported that BC derivatives from *Verbesina turbacensis* showed potent anti-inflammatory and antioxidant effects in human polymorphonuclear granulocytes. In the present study, we investigated whether BC could attenuate an inflammatory response in LPS-challenged RAW 264.7 macrophage cells, and if so, how it exerted this effect. Results of this study showed that pretreatment with BC significantly inhibited the LPS-induced production of proinflammatory molecules through the downregulation of their corresponding mediators via suppressing the transcriptional activation of NF-κB in murine macrophage cells.

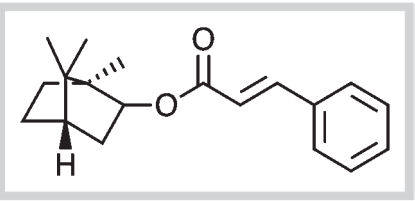

Fig. 1 Structure of bornyl cinnamate.

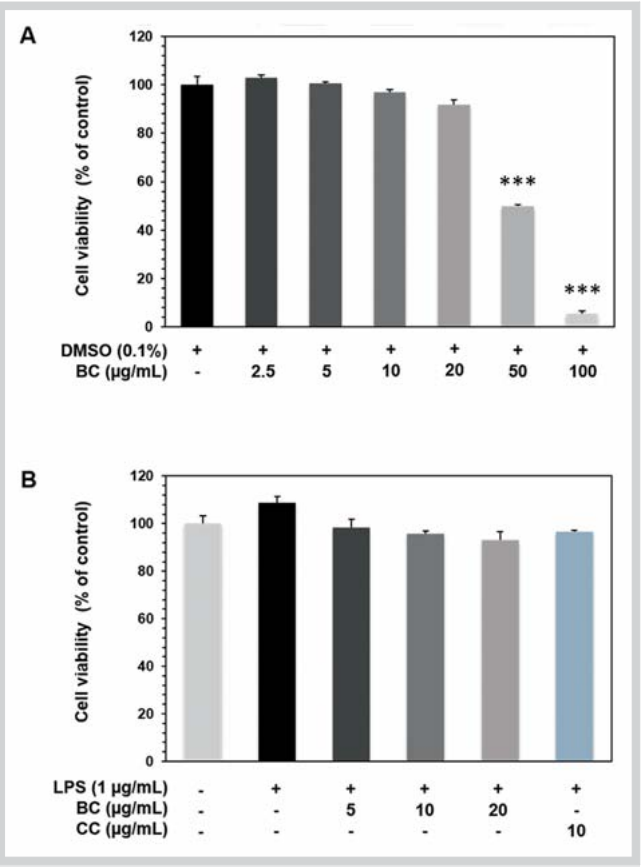

Fig. 2 Effect of bornyl cinnamate on macrophage cell viability. **A** RAW 264.7 cells were incubated with increasing concentrations (2.5–100 μg/mL) of BC for 24 h. **B** Cells were pretreated with BC (5–20 μg/mL) or curcumin (CC, 10 μg/mL) for 2 h, and then stimulated with LPS (1 μg/mL) for 24 h. The cell viability was determined by the MTT colorimetric assay as described in Materials and Methods. Data are reported as mean ± SD of three independent experiments; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ show significant differences between the control and sample-treated groups. (Color figure available online only.)

Results and Discussion

Complementary and alternative therapies have been attractive, as accumulating evidence elucidated the efficacy, safety, and functional mechanisms of herbal medicine. *L. formosana* is used in traditional Chinese medicine for treating rheumatoid arthritis, dermatosis, inflammation, and wounds. A previous study shows that essential oil isolated from *L. formosana* inhibits LPS-induced proinflammatory genes in murine macrophage cells [12]. BC isolated from the essential oil of *L. formosana* also showed potent antifungal activity against *Lenzites betulina* [10]. In this study, we found that BC treatment inhibits LPS-induced proinflammatory molecules through the suppression of the NF-κB signaling pathway. Therefore, we believe that BC could be responsible for the anti-inflammatory effect of the essential oil of *L. formosana*.

Natural products and phytocompounds have a fever-related cytotoxic effect on immune cells. Thus, prior to *in vitro* anti-inflammatory assessment, cytotoxicity of BC to the macrophages was examined. RAW 264.7 cells were incubated with increasing concentrations of BC (2.5–100 μg/mL) for 24 h. Then, the number of viable cells was quantified by the MTT colorimetric assay. As shown in **Fig. 2A**, cell viability significantly decreased with increasing concentrations of BC. The IC_{50} value was calculated as 48.3 μg/mL. Consequently, more than 20 μg/mL of BC had a substantial cytotoxic effect, therefore all subsequent experiments were executed with concentrations ranging from 5 to 20 μg/mL of BC. Moreover, incubation of the cells with BC (5–20 μg/mL) and LPS (1 μg/mL) showed a similar pattern of cell survival (**Fig. 2B**).

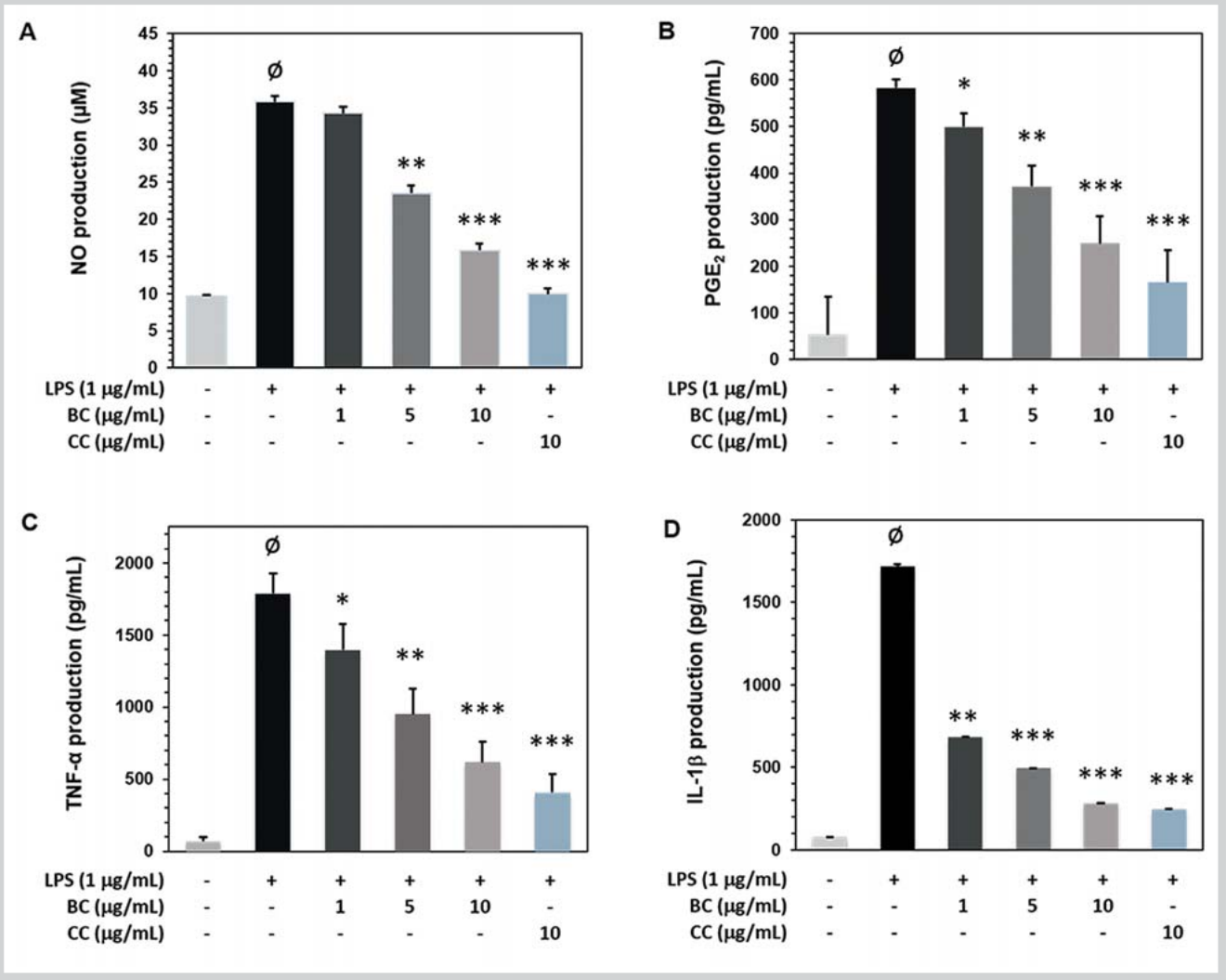

Fig. 3 Effect of bornyl cinnamate on lipopolysaccharide-induced nitric oxide, prostaglandin-E2, tumor necrosis factor α, and interleukin-1β secretion in macrophage cells. RAW 264.7 cells were preincubated with BC (5–20 μg/mL) or curcumin (CC, 10 μg/mL) for 2 h and then stimulated with LPS for 24 h. **A** The nitrite concentration in the culture media was determined by the Griess reagent assay. **B–D** PGE_2, TNF-α, and IL-1β levels in the culture media were measured by commercially available assay kits as described in Materials and Methods. Data are reported as mean ± SD of three independent experiments. $^{Ø}P < 0.001$ indicates a significant difference between the control and LPS-only treated groups. $^{*}P < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ show significant differences between the LPS-only and BC/curcumin treatment groups. (Color figure available online only.)

Macrophages have long been considered to be important immune effector cells and the most sensitive targets of bacterial endotoxin LPS [15]. Upon stimulation of macrophages with LPS, bioactive products such as proinflammatory cytokines, chemokines, and adhesion molecules are released [16], NO, a prominent proinflammatory molecule, acts as an intracellular messenger, which regulates cellular functions such as vasodilation and elimination of pathogens and tumor cells [17]. However, the overproduction of NO is associated with several pathological processes including inflammation [17]. Induction of RAW 264.7 cells with LPS caused synthesis and secretion of NO into the culture media, which can be measured by a stable nonvolatile breakdown product, nitrite [18]. In this study, we found that untreated control cells released trace amount of NO (9.6 μM). Upon stimulation with LPS, NO production was markedly increased to 35.8 μM, whereas pretreatment with BC significantly decreased LPS-induced NO production to 34.2, 23.5, and 15.8 μM by 5, 10, and 20 μg/mL BC, respectively. A similar inhibitory effect was also observed in curcumin, a known anti-inflammatory agent in treated cells. Since PGE_2 is one of the stable prostanoids secreted by activated macrophages [19], we examined the PGE_2 level in culture media. Cell incubation with LPS for 24 h markedly increased PGE_2 production from 53.8 pg/mL to 582 pg/mL. On the other hand, PGE_2 production by LPS was significantly diminished by BC. Twenty μg/mL of BC inhibited nearly 50% of PGE_2 (249.5 pg/mL) production in RAW 264.7 cells.

It has been reported that activated macrophages secrete vast amounts of TNF-α and interleukins including IL-1β, IL-6, IL-12, and IL-18 [20]. Indeed, BC treatment significantly as well as dose-dependently inhibited LPS-induced TNF-α and IL-1β production in RAW 264.7 cells. As shown in **Fig. 3C**, cells incubated with LPS showed a remarkable increase of TNF-α from 66.8 pg/mL to 1788 pg/mL, whereas BC pretreatment significantly as well as dose-dependently decreased the elevated TNF-α. Furthermore, BC treatment dose-dependently inhibited the LPS-induced IL-1β production in RAW 264.7 cells. A similar inhibitory effect of curcumin was again noted in LPS-treated cells.

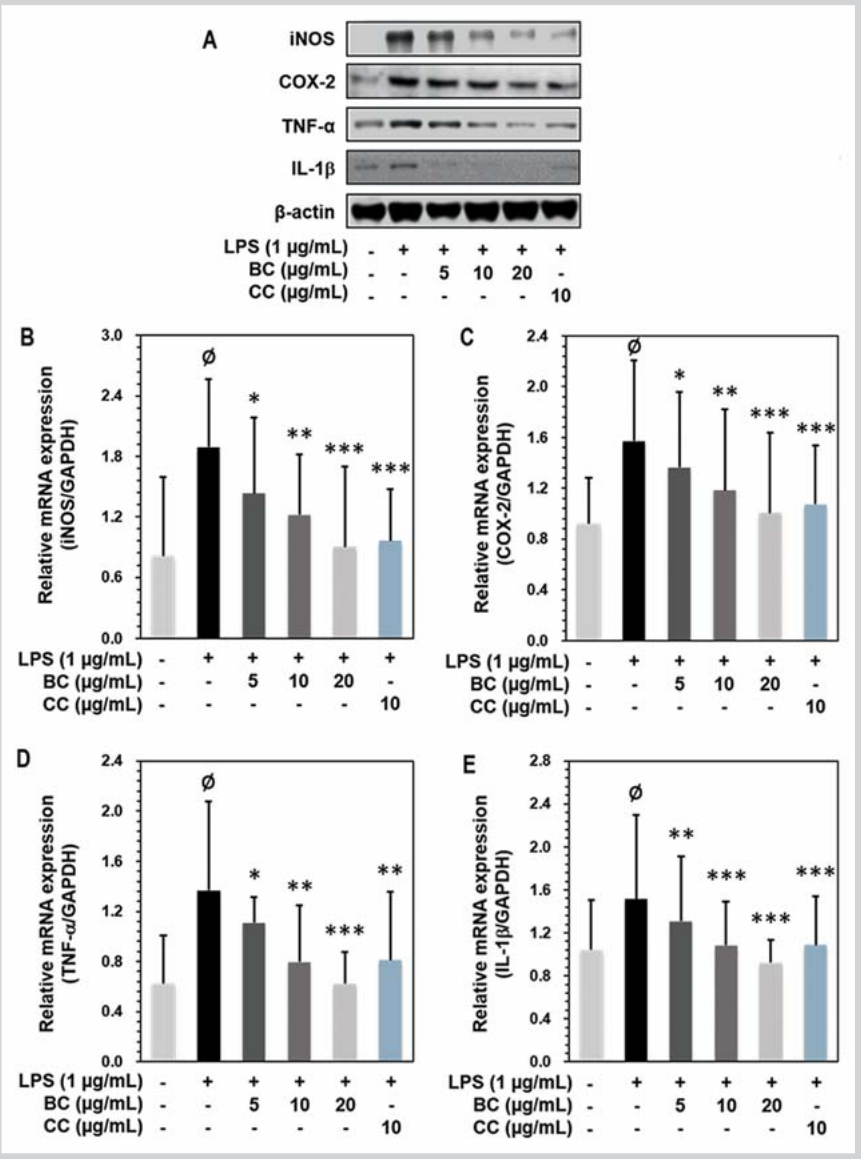

Fig. 4 Effect of bornyl cinnamate on lipopolysaccharide-induced proinflammatory genes and molecules in macrophage cells. RAW 264.7 cells were pretreated with various concentrations of BC (5–20 μg/mL) or curcumin (CC, 10 mg/mL) for 2 h and then stimulated with LPS (1 μg/mL) for 12 and 24 h. **A** Protein expression levels of iNOS, COX-2, TNF-α, and IL-1β were determined by Western blot analysis with specific antibodies. The housekeeping protein β-actin served as an internal loading control. **B–E** Total RNA was extracted, and the relative mRNA expression levels of iNOS, COX-2, TNF-α, and IL-1β were quantified by Q-PCR. Data are reported as mean ± SD of three independent experiments; $^{Ø}p < 0.001$ indicates a significant difference between the control and LPS-only treated groups; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ show significant differences between the LPS-only and BC/curcumin treatment groups. (Color figure available online only.)

Since BC was found to inhibit the LPS-induced elevation of NO, PGE_2, TNF-α, and IL-1β, we hypothesized that the inhibition may be associated with the downregulation of their corresponding mediator genes iNOS, COX-2, TNF-α, and IL-1β, respectively. As we expected, pretreatment with BC significantly inhibited the LPS-induced protein expression levels of iNOS, COX-2, TNF-α, and IL-1β in a dose-dependent manner (**Fig. 4A**). To further confirm this effect at the transcriptional level, RT-PCR analysis was performed. As shown in **Fig. 4B–E**, LPS-induced increases in mRNA levels of iNOS, COX-2, TNF-α, and IL-1β were significantly as well as dose-dependently inhibited by BC. A similar effect was also observed in curcumin-treated cells.

NF-κB, a redox-sensitive transcription factor, can be activated in macrophage cells stimulated with endotoxin LPS or other inflammatory agents that trigger the transcriptional activation of the responsive genes iNOS, COX-2, TNF-α, and IL-1β [21]. Thus, we examined whether BC treatment affects LPS-induced NF-κB transcriptional activity in RAW 264.7 cells by luciferase reporter gene analysis. LPS treatment caused a 6.2-fold increase in the NF-κB reporter activity, whereas pretreatment of cells with 5, 10, and 20 μg/mL of BC dose-dependently reduced the LPS-induced increase in NF-κB reporter activity 5-fold, 3.6-fold, and 2.7-fold, respectively (**Fig. 5A**).

The process of transcriptional activation of NF-κB is mediated through the nuclear translocation of the p65/p50 complex, which is regulated by phosphorylation and degradation of its repressor I-κB [21]. Therefore, the nuclear translocation of p65, an active subunit, was examined by immunofluorescence analysis. In control cells, NF-κB proteins were localized in the cytoplasm, while upon LPS stimulation, NF-κB proteins were largely accumulated in the nucleus. However, BC pretreatment significantly blocked LPS-induced nuclear translocation of NF-κB in a dose-dependent manner (**Fig. 5B**). Interestingly, BC treatment also significantly reduced the cytosolic NF-κB level in macrophage cells. In addition, Western blot analysis using specific antibodies that can detect I-κBα phosphorylation at Ser32/36 residues showed that a dramatic increase in phosphorylation of I-κBα was found after LPS treatment, whereas pretreatment with BC significantly blocked LPS-induced I-κBα phosphorylation, which is directly proportional to the significant increase of cytosolic I-κB by BC (**Fig. 5C**). Furthermore, BC treatment significantly inhibits LPS-induced phosphorylation of IKKα in a dose-dependent manner (**Fig. 5C**). However, the total IKKα level was not affected by either LPS or BC.

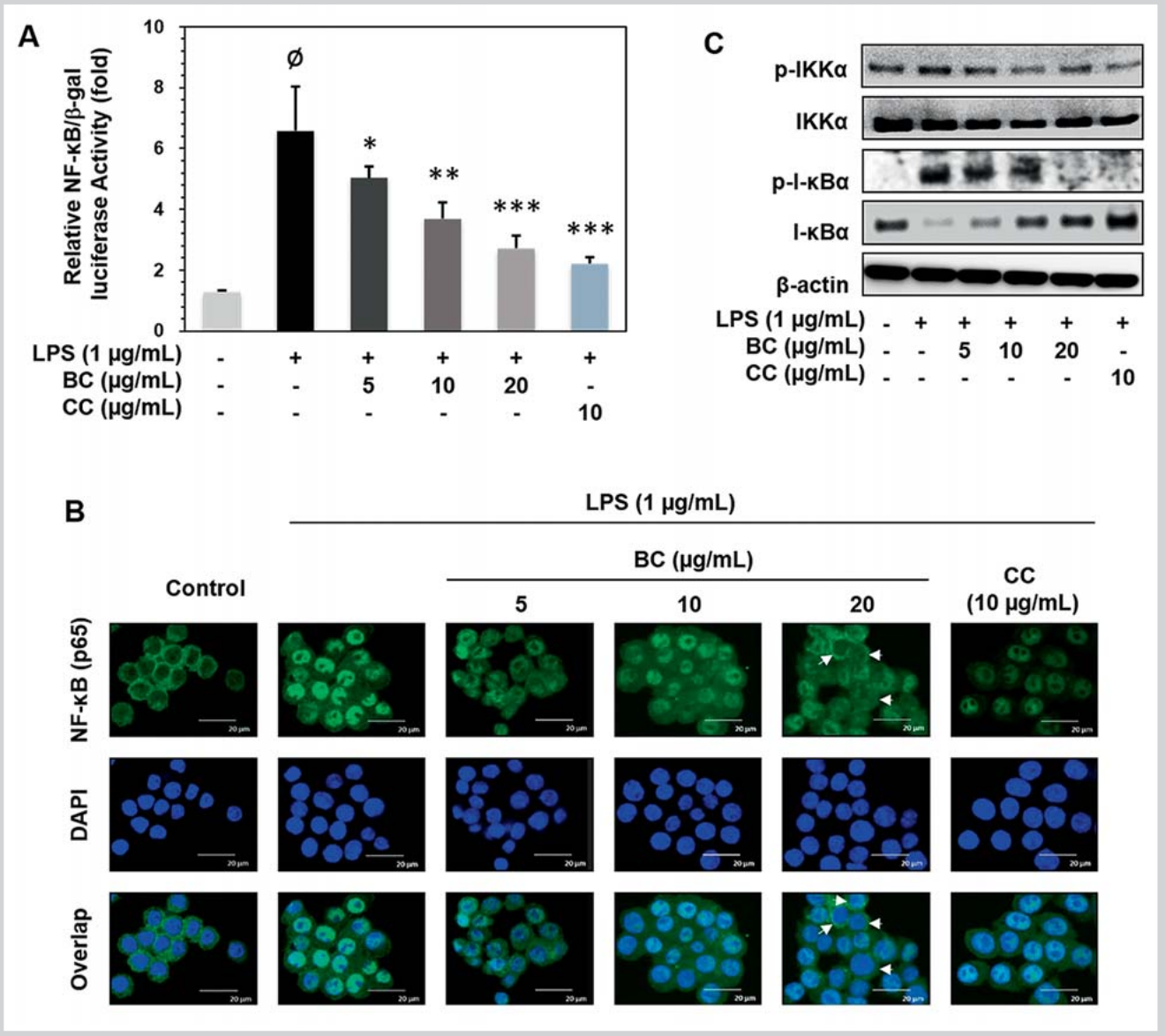

Fig. 5 Effect of bornyl cinnamate on lipopolysaccharide-induced transcriptional activation of nuclear factor κB in macrophage cells. **A** RAW 264.7 cells were cotransfected with NF-κB harboring luciferase reporter construct. After transfection, cells were preincubated with BC (5–20 μg/mL) or curcumin (CC, 10 μg/mL) for 2 h and then stimulated by LPS for 2 h. Luciferase activity was determined and normalized with β-gal activity. The histogram shows the relative luciferase activity (fold increase). **B** The nuclear localization of NF-κB in macrophage cells was determined by immunofluorescence staining. RAW 264.7 cells were seeded in an 8-well Tek chamber and allowed to adhere for 24 h. Then the cells were preincubated with BC (5–20 μg/mL) or curcumin (10 μg/mL) for 2 h and stimulated with LPS (1 μg/mL) for 2 h. After treatment, cells were fixed with paraformaldehyde and then incubated with NF-κB primary antibody overnight, followed by FITC secondary antibody for 1 h. The cellular DNA was stained with DAPI (1 μg/mL) and images were captured by a confocal microscope (magnification × 200). Arrows indicate NF-κB protein levels in the cytoplasm. **C** Cells were preincubated with BC (5–20 μg/mL) or curcumin (10 μg/mL) for 2 h and then treated with or without LPS for 1 h. The phosphorylated and total protein expression levels of I-κBα and IKKα were determined by Western blot analysis. The housekeeping protein β-actin served as an internal loading control. Data are reported as mean ± SD of three independent experiments, ^{Ø} $p < 0.001$ indicates a significant difference between the control and LPS-only treated groups; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ show significant differences between the LPS-only and BC/curcumin treatment groups. (Color figure available online only.)

In conclusion, this study demonstrates that bornyl cinnamate inhibited the production of proinflammatory molecules NO, PGE_2, TNF-α, and IL-1β via the downregulation of their corresponding genes in LPS-induced RAW 264.7 macrophage cells. This inhibitory action resulted from the suppression of NF-κB transcriptional activation, since NF-κB is one of the critical transcription factors regulating the transcription of many inflammation-associated genes. Thus, inhibition of NF-κB by bornyl cinnamate would be a possible therapeutic approach to the treatment of inflammation-associated diseases.

Materials and Methods

Chemicals and reagents

Bornyl cinnamate was prepared according to the protocol described previously [10]. The purity was above 99%, as confirmed by HPLC and ^{1}H-NMR analysis. DMEM, FBS, glutamine, and penicillin/streptomycin were obtained from Gibco® Life Technologies, Inc. LPS, MTT, BSA, curcumin (purity: 96%), and Griess reagent were purchased from Sigma-Aldrich. DAPI was obtained from Calbiochem. Antibodies against iNOS and COX-2 were purchased

from Cayman Chemical. Antibodies against IKKα, phos-IKKα, I-κBα, phos-I-κBα, and NF-κB were obtained from Cell Signaling Technology, Inc. All other chemicals and solvents used in this study were of reagent grade or HPLC grade and supplied by either Merck or Sigma-Aldrich.

Cell culture and cell viability assay

RAW 264.7 murine macrophage cells were obtained from ATCC and cultured at 37 °C in DMEM supplemented with 10% FBS, 4.5 g/L glucose, 4 mM glutamine, penicillin (100 units/mL), and streptomycin (100 µg/mL) in an incubator with a humidified atmosphere of 5% CO_2 as recommended by ATCC. Cell viability was determined by the MTT colorimetric assay as described previously [22]. RAW 264.7 cells (2×10^5 cells/well) were seeded in a 96-well culture plate. Cells were incubated with various concentrations of BC (2.5–100 µg/mL) for 24 h. For other samples, cells were pretreated with BC (5–20 µg/mL) or curcumin (10 µg/mL) for 2 h and then incubated with LPS (1 µg/mL) for 24 h. Cells were pretreated with various concentrations of BC (5–20 µg/mL) or curcumin (10 µg/mL) for 2 h and then stimulated by LPS (1 µg/mL) for 1 to 24 h. After incubation, the culture medium was removed and stored at − 80 °C, which can be used for determining intercellular NO. The medium-deprived cells were incubated with MTT (10 µg/mL) in 100 µL of fresh DMEM for 1 h at 37 °C. The MTT-generated violet formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm (A_{570}) using an ELISA microplate reader (µQuant, Bio-Tek Instruments, Inc.). Cell viability (%) was calculated as: (A_{570} of treated cells/A_{570} of untreated cells) × 100.

Determination of nitric oxide, prostaglandin-E2, tumor necrosis factor α, and interleukin-1β levels in culture media

Accumulation of intercellular NO was determined by the Greiss reaction assay as described previously [22]. Culture supernatant derived from the cell viability assay was mixed with an equal volume of Griess reagent and incubated for 30 min at room temperature. The intercellular level of NO was determined by nitrate, a major stable product of NO and measured at 540 nm using an ELISA microplate reader. A standard curve was constructed using known concentrations of sodium nitrate. On the other hand, RAW 264.7 cells at a density of 4×10^5 cells/well in a 12-well plate were pretreated with or without BC (5–20 µg/mL) or curcumin (10 g/mL) for 2 h and then incubated with or without LPS for 24 h. The PGE_2, TNF-α, and IL-1β concentrations in the culture media were determined using an EIA kit (R&D Systems) according to the manufacturer's protocols.

Preparation of protein fraction and Western blot analysis

RAW 264.7 cells (1×10^6 cells/dish) were seeded in a 6-cm cell culture dish and preincubated with or without BC (5–20 µg/mL) or curcumin (10 µg/mL) for 2 h and then stimulated with LPS (1 µg/mL) for 1 to 24 h. The total cell lysates were obtained by mammalian protein extraction reagent (Cayman Chemicals). The protein content in each fraction was determined by Bio-Rad protein assay reagent, with BSA as the standard. Equal amounts (50 µg) of denatured protein samples were electrophoresed by 8–15% SDS-PAGE, followed by a transfer onto PVDF membranes overnight. The membranes were blocked with 5% nonfat dry milk for 30 min at room temperature and reacted with primary antibodies for 2 h. They were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody for 2 h and developed using the enhanced chemiluminescence substrate (Millipore). Images were captured with a VL Chemi-Smart 3000 gel documentation system (Vilber Lourmat).

RNA extraction and Q-PCR analysis

RAW 264.7 cells (1×10^6 cells/dish) were seeded in a 6-cm cell culture dish and preincubated with or without BC (5–20 µg/mL) or curcumin (10 µg/mL) for 2 h and then stimulated with LPS (1 µg/mL) for 12 h. RNA extraction and quantitative polymerase chain reaction (Q-PCR) were performed as described previously [23]. In brief, total RNA was extracted from cultured macrophage cells using Trizol reagent according to the supplier's instructions (Invitrogen Corporation). For cDNA preparation, total RNA (5 µg) was incubated at 37 °C for 90 min using a first-strand cDNA synthesis kit (Invitrogen Corporation). Q-PCR analysis for iNOS, COX-2, and GAPDH mRNA were performed using the Applied Biosystems detection instrument and software. This system incorporates a gradient thermocycler and a 48-channel optical unit. For the quantitative analysis of mRNA expression, the same system was employed using a DNA binding dye, SYBR Green, for the detection of PCR products. The melting point, optimal conditions, and specificity of the reaction were first determined using a standard procedure. The working stock solution of SYBR Green was 1 : 100 (Invitrogen Corporation). Quantitative PCR was carried out in a 48-well plate with 10 pmol forward and reverse primers and the working solution SYBR green, using a PCR master mix, under the following conditions: 95 °C for 5 min, followed by 40 cycles at 95 °C for 1 min, 55 °C for 45 s, 72 °C for 30 s. GAPDH, a housekeeping gene, was chosen as an internal control. The sequences of the primers are summarized in **Table 1**. The copy number of each transcript was calculated as the relative number normalized by the GAPDH copy number.

Luciferase reporter assay

The NF-κB transcriptional activity was measured using a dual-luciferase reporter assay system (Promega). RAW 264.7 cells were cultured in 24-well plates that had reached 70–80% confluence and incubated with serum-free DMEM that did not contain antibiotics for 5 h. The cells were then transfected with either a pcDNA vector or an NF-κB with β-galactosidase using Lipofectamine 2000 (Invitrogen). Cells were treated with BC (5–20 µg/mL) or curcumin (10 µg/mL) for 2 h, after which they were incubated with or without LPS for 2 h. The relative fluorescence intensity was quantified using a luminance ELISA reader at 405 nm (A_{405}). The fold increase of luciferase activity was calculated as (A_{405} of treated cells/A_{405} of untreated cells) × 100. The luciferase activity was normalized to β-galactosidase activity in the cell lysates.

Immunofluorescence staining

RAW 264.7 cells at a density of 1×10^4 cells/well were cultured in an 8-well glass Tek chamber and pretreated with BC (5–20 µg/mL) or curcumin (10 µg/mL) for 2 h and then incubated with the presence or absence of LPS for 2 h. The cells were then fixed in 2% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, washed, blocked with 10% FBS in PBS, and then incubated with anti-NF-κB primary antibody in 1.5% FBS for 2 h, followed by incubation with FITC (488 nm)-conjugated secondary antibody for 1 h in 6% BSA. Cells were stained with 1 µg/mL of DAPI for 5 min. The stained cells were washed with PBS and visualized using a confocal microscope at 630× magnification.

Gene	Primer	Sequence	Reference
iNOS	Forward	5′-TCCTACACCACACCAAAC-3′	[24]
	Reverse	5′-CTCCAATCTCTGCCTATCC-3′	
COX-2	Forward	5′-CCTCTGCGATGCTCTTCC-3′	[24]
	Reverse	5′-TCACACTTATACTGGTCAAATCC-3′	
TNF-α	Forward	5′-GACGTGGAAGTGGCAGAAGAG-3′	[25]
	Reverse	5′-TGCCACAAGCAGGAATGAGA-3′	
IL-1β	Forward	5′-TCGTGCTGTCGGACCCATAT-3′	[26]
	Reverse	5′-GTCGTTGCTTGGTTCTCCTTGT-3′	
GA3PDH	Forward	5′-TCAACGGCACAGTCAAGG-3′	[24]
	Reverse	5′-ACTCCACGACATACTCAGC-3′	

Table 1 Primers used for Q-PCR analysis.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Duncan's test for multiple comparisons and a Student's t-test for single comparison. The data are reported as mean ± SD. The numbers of independent experiments assessed are given in the figure legends.

Acknowledgements

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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