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Anti-inflammatory Activity of 1-docosanoyl Cafferate Isolated from *Rhus verniciflua* in LPS-stimulated BV2 Microglial Cells

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Although various derivatives of caffeic acid have been reported to possess a wide variety of biological activities such as protection of neuronal cells against excitotoxicity, the biological activity of 1-docosanoyl cafferate (DC) has not been examined. The objective of the present study was to evaluate the anti-inflammatory effects of DC, isolated from the stem bark of *Rhus verniciflua*, on lipopolysaccharide (LPS)-stimulated BV2 microglial cells. Pretreatment of cells with DC significantly attenuated LPS-induced NO production, and mRNA and protein expression of iNOS in a concentration-dependent manner. DC also significantly suppressed LPS-induced release of cytokines such as TNF-α and IL-1β. Consistent with the decrease in cytokine release, DC dose-dependently and significantly attenuated LPS-induced mRNA expression of these cytokines. Furthermore, DC significantly suppressed LPS-induced degradation of IKB, which retains NF-kB in the cytoplasm. Therefore, nuclear translocation of NF-kB induced by LPS stimulation was significantly suppressed with DC pretreatment. Taken together, the present study suggests that DC exerts its anti-inflammatory activity through the suppression of NF-kB translocation to the nucleus.

Key Words: 1-Docosanoyl cafferate (DC), BV2 microglial cells, Lipopolysaccharide, Cytokines, iNOS, NF-kB

INTRODUCTION

Microglia, resident macrophages and immune surveillance cells in the central nervous system (CNS), have been reported to play a critical role in host defense and tissue repair in the CNS [1]. However, microglia have also been proposed to play a pathogenic role in neuroinflammatory conditions such as ischemia [2], multiple sclerosis [3], Parkinson's disease [4], Alzheimer's disease [5] and HIV-associated dementia [6]. It has been suggested that during these immunologically mediated CNS diseases, activated microglia contribute to neurodegenerative process by producing excessive amounts of proinflammatory molecules such as nitric oxide (NO) and several cytokines [7-10]. Therefore, novel pharmacological agents that can suppress microglia-induced overproduction of inflammatory mediators might provide a promising strategy for treating inflammation-related CNS diseases.

Rhus verniciflua, a deciduous tree in the anacardiaceae family, exhibits a variety of biological activities such as antioxidant [11,12], anti-microbial [13], anti-cancer [14,15], and anti-platelet properties [16]. However, the chemical components of *Rhus verniciflua* and their biological properties have not been fully elucidated. Previously, we reported that stigmasterols, which were isolated from *Rhus verniciflua*, were neuroprotective against kainic acid-induced excitotoxicity [17]. To further isolate and identify biologically active compounds from *Rhus verniciflua*, 1-docosanoyl cafferate (DC) was purified from the plant's stem bark.

DC is an ester derivative of caffeic acid, a simple phenylpropanoid that possesses a wide variety of biological activities: it has antioxidant and anti-inflammatory properties [18] and is neuroprotective against Aβ-induced neurotoxicity [19]. Ester derivatives of caffeic acid also exhibit various biological activities [20]. For example, caffeic acid phenylethyl ester (CAPE), an antioxidant component of propolis, protects against glutamate-induced neurotoxicity [21] and cerebral ischemia [22]. Although DC was previously identified in the root of *Sohora subprostrata* [23], its biological activity was never demonstrated.

In the present study, we investigated the anti-inflammatory activity of DC isolated from the stem bark of *Rhus verniciflua*. We studied a possible underlying mechanism by which DC exerts its anti-inflammatory action in LPS-stimulated BV2 microglial cells.

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ABBREVIATIONS: DC, 1-docosanoyl cafferate; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; NF-kB, nuclear factor kappa B; IKB, inhibitor of NF-kB; iNOS, inducible nitric oxide synthase; NO, nitric oxide; CAPE, caffeic acid phenylethyl ester.

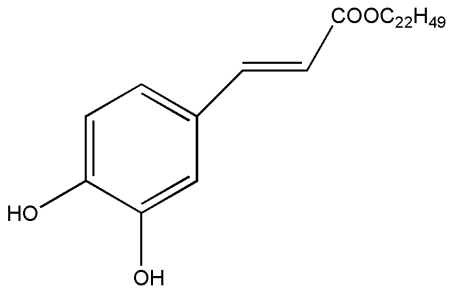

Fig. 1. Chemical structure of 1-docosanoyl cafferate (DC).

METHODS

Reagents and cell culture

Bacterial lipopolysaccharide (LPS, E. coli serotype 055: B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). DC was isolated and identified from the stem bark of *Rhus verniciflua* (Fig. 1). The compound was dissolved in ethanol and added to the cell culture at the desired concentrations. The BV2 microglia cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco BRL) and 50 μg/ml gentamicin (Sigma, St. Louis, MO, USA). Cells were maintained in a humidified incubator at 37°C with 5% CO_2. In all experiments, cells were incubated in the presence of the indicated concentration of DC before the addition of LPS (200 ng/ml).

Cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. BV2 microglial cells were plated into 96-well plates at a density of 5×10^4 cells per well for 24 hr. BV2 microglial cells were incubated with various concentrations of DC for 24 hr. MTT (0.5 mg/ml in PBS) was added to each well and the cells were incubated for 3 hr at 37°C. The resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The optical density was measured at 540 nm. The results were expressed as a percentage of surviving cells over control cells.

Nitrite quantification assay

The production of NO was estimated by measuring the amount of nitrite, a major stable metabolite of NO, using the Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). DC-pretreated BV2 microglial cells were stimulated with LPS in 12-well plates for 24 hr, and then 100 μl of each culture medium was mixed with an equal volume of Griess reagent. The absorbance at 540 nm was measured on a microplate reader. The results were expressed as a percentage of released NO from LPS-stimulated BV2 cells. To prepare a standard curve, sodium nitrite was diluted in culture medium to the desired concentrations.

TNF-α and IL-1β ELISA

BV2 microglia cells were treated with DC in the absence or presence of LPS. After 24 hr incubation, TNF-α and IL-1β levels in culture media were quantified using monoclonal anti-IL-1β or anti-TNF-α antibody according to the manufacturer's instruction (R & D Systems).

Western blot analysis

BV2 microglial cells were washed twice with PBS and lysed with lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate, 1 mM phenyl methylsulfonyl fluoride]. Equal amounts of protein were separated on 10% SDS-polyacrylamide gel. Proteins were transferred to Hypond PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked using 5% skim milk in TBST for 1 hr at room temperature and sequentially incubated with an appropriate antibody: anti-iNOS (BD Bioscience, Franklin Lakes, NJ), anti-IkB-α (Santa Cruz Biotechnology Inc., Eugene, OR, USA), or anti-β-actin (Sigma, St. Louis, MO, USA). After thoroughly washing with TBST, the membranes were washed three times with TBST and incubated with HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) or HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) for 2 hr at room temperature. The blots were developed using enhanced chemiluminescence detection kits (ECL Amersham Biosciences, Piscataway, NJ, USA).

Immunofluorescence assay

The effect of DC on the translocation to the nucleus of the p65 subunit of NF-kB was examined by immunofluorescence assay using confocal microscopy. BV2 microglial cells were cultured on? under? sterile coverslips, pretreated with DC for 1 hr, and stimulated with LPS. At 30 min after the LPS treatment, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were then permeabilized with 0.1% Triton X-100 in PBS and blocked with 3% BSA. Afterwards, the cells were sequentially incubated with rabbit p65 antibody (Santa Cruz Biotechnology Inc., Eugene, OR, USA) at room temperature and Alexa 546-labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) at room temperature for 1 hr. Cells were stained with 0.5 μg/ml of Hoechst staining solution (Hoechst 33258) for 10 min at room temperature. After washing with PBS, the samples were mounted and observed by confocal microscopy.

RNA isolation and quantitative real time RT-PCR

Total RNA was isolated using the Total RNA Extraction Kit (iNtRON Biotechnology, Inc, USA) according to the manufacturer's instruction. Total RNA (2 μg) obtained from cells was reverse-transcribed using oligo-(dT) 15 primers (Promega, Madison, WI, USA). The cDNA products were used immediately for SYBR (Takara, JAPAN) real time RT-PCR using primers specific for iNOS, TNF-α, IL-1β and β-actin (BIO NEER, KOREA). Quantitative changes in mRNA levels were estimated by real time PCR using the following cycling conditions: 35 cycles of denaturation at 94°C for 10 sec; annealing at 61°C for 15 sec; and ex-

tension at 72°C for 20 sec; followed by 2 min at 72°C, in the presence of SYBR Green (1 : 10,000 dilution of a stock solution from Molecular Probes) carried out in a 20 μL reaction volume.

Statistical analysis

All values shown in the figures are expressed as mean± SD for at least three independent experiments. Statistical analysis was carried out by one-way analysis of variance (ANOVA) with Tukey's post-hoc test using SPSS software 12K (SPSS, Chicago, IL, USA). A value of $p<0.05$ was considered statistically significant.

RESULTS

1-Docosanoyl cafferate (DC) inhibits NO production and iNOS expression in LPS-stimulated BV2 cells

The effects of DC on NO production and iNOS expression were examined in LPS-stimulated BV2 microglial cells. No noticeable cell death was observed in the concentration range of DC used, although higher concentrations of DC appeared to be cytotoxic (Fig. 2A). Treatment of LPS resulted in excessive production of NO and up-regulation of iNOS as indicated by increases in both mRNA and protein levels (Fig. 2B~D). Pretreatment of cells with DC sig-

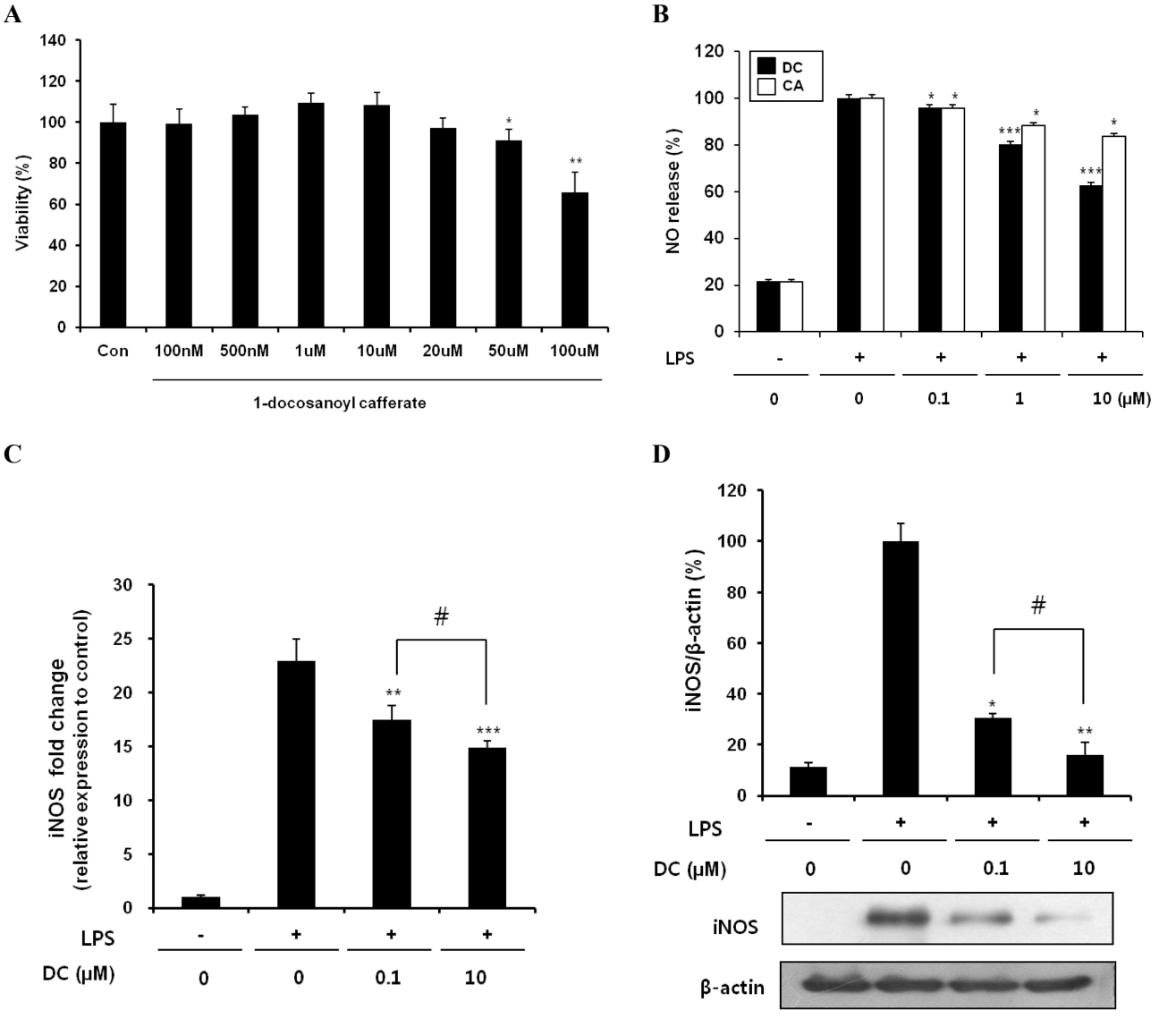

Fig. 2. Effects of DC on NO production and iNOS expression in LPS-stimulated BV2 microglial cells. (A) Effect of DC on the viability of BV2 microglial cells. No noticeable cell death was observed up to 20 μM. (B) Concentration-dependent suppression of LPS-induced NO production by DC and CA (caffeic acid). (C) Inhibitory effect of DC on LPS-induced upregulation of iNOS mRNA expression. (D) Suppression of LPS-induced iNOS protein expression by DC: top, quantitative analysis of immunoblots; bottom, representative immunoblot of iNOS. β-Actin was used as an internal control. Quantitative data represent three independent experiments and are expressed as mean±SD. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ indicate statistically significant differences compared to LPS alone. $^{\#}p<0.05$ indicate statistically significant differences between the indicated groups.

nificantly suppressed LPS-induced NO production in a concentration-dependent manner (Fig. 2B). Caffeic acid also caused a significant suppression of LPS-induced NO production. However, DC elicited a more robust attenuation compared to caffeic acid, presumably due to the increased lipophilicity of DC. Consistent with the decrease in NO production, DC also significantly attenuated LPS-induced up-regulation of iNOS expression - including both iNOS mRNA (Fig. 2C) and iNOS protein levels (Fig. 2D). DC inhibited LPS-induced NO production and iNOS expression without causing cell toxicity.

DC attenuates the expression of pro-inflammatory cytokines in LPS-stimulated BV2 cells

To determine the effect of DC on the expression of pro-inflammatory cytokines such as TNF-α and IL-1β, extracellular release and mRNA expression of these cytokines were examined using an ELISA assay and quantitative RT-PCR, respectively. DC significantly suppressed LPS-induced extracellular release of TNF-α and IL-1β (Fig. 3). Extracellular release of IL-1β was almost completely sup-

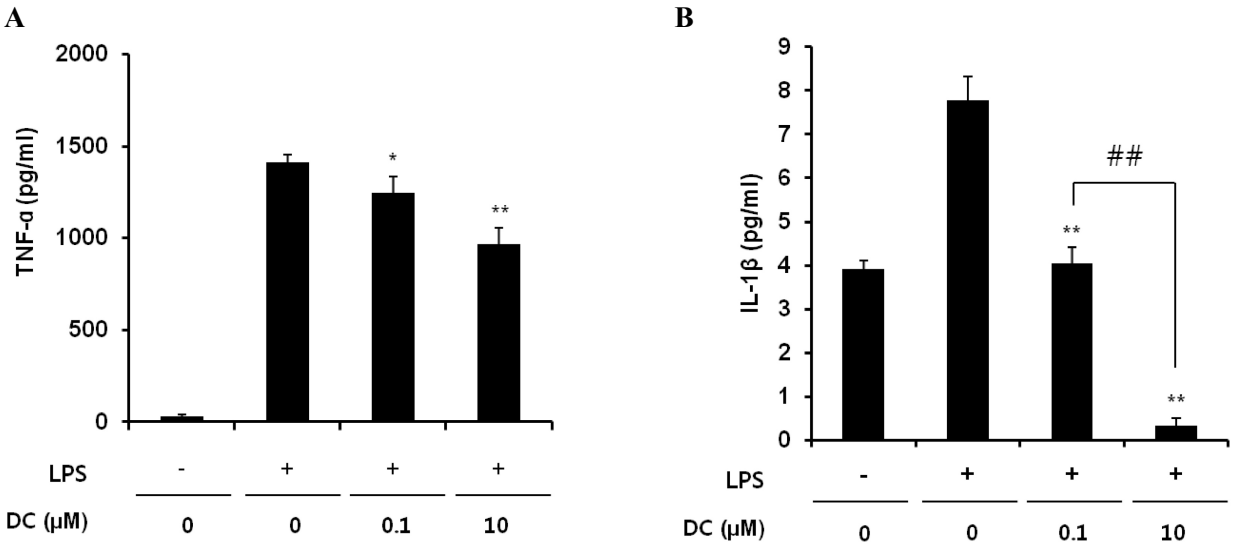

Fig. 3. Inhibitory effects of DC on LPS-induced release of TNF-α (A) and IL-1β (B) in BV2 microglial cells. BV2 microglia cells were incubated with 200 ng/ml of LPS in the presence or absence of the indicated concentrations of DC for 24 hr. Cell culture media were collected and subjected to TNF-α and IL-1β sandwich ELISAs. Data represent three independent experiments, each run in triplicate, and are expressed as mean±SD. *$p<0.05$ and **$p<0.01$ indicate statistically significant differences compared to LPS alone. $^{\#\#}p<0.01$ indicates a statistically significant difference between the indicated groups.

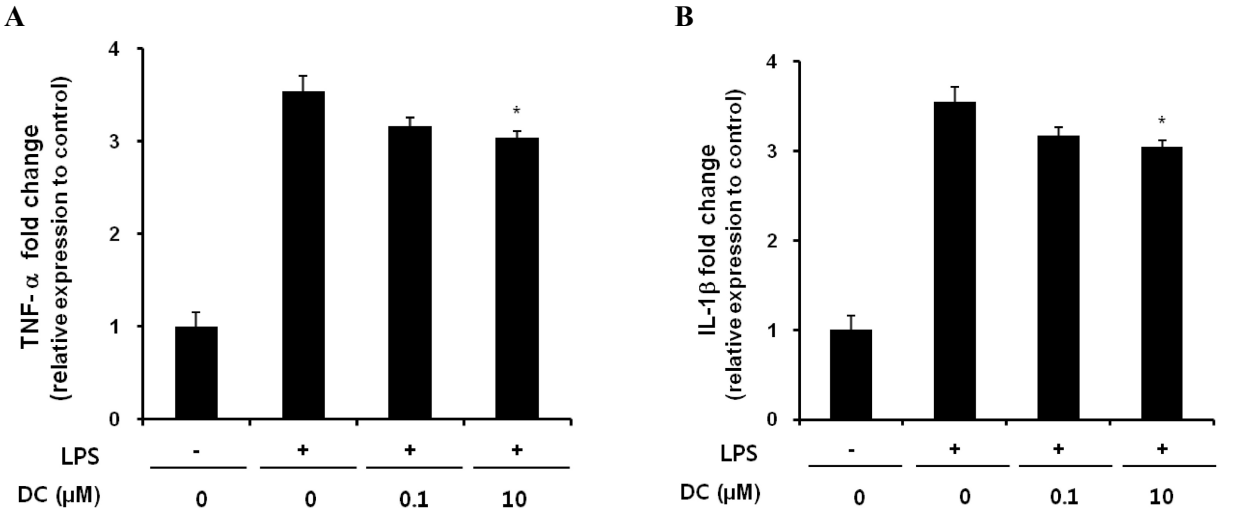

Fig. 4. Effects of DC on the gene expression of TNF-α (A) and IL-1β (B) in LPS-stimulated BV2 microglial cells. Cells were incubated with DC for 1 hr prior to exposure to 200 ng/ml LPS. Total RNA was isolated 6 hr after LPS treatment. TNF-α and IL-1β mRNA levels were determined by real time PCR. Data represent three independent experiments, each done in triplicate, and are expressed as mean±SD. *$p<0.05$ indicates a statistically significant difference compared to LPS alone.

pressed at a DC concentration of 10 μM. The inhibition by DC was attributable to the attenuated expression of these genes as DC decreased mRNA levels of these cytokines (Fig. 4).

DC suppresses LPS-induced degradation of IKB and nuclear translocation of NF-kB

Given the previous report that the transcription factor NF-kB is the predominant regulator of numerous inflammatory cytokine genes and that IKB inhibits the nuclear translocation of NF-kB by retaining it in the cytoplasm [24,25], we examined the effects of DC on LPS-induced nuclear translocation of NF-kB and degradation of cytosolic IKB. LPS significantly decreased intracellular levels of IKB; pretreatment with DC significantly attenuated this effect of LPS (Fig. 5). To further determine whether the change in the level of IKB affects the nuclear translocation of NF-kB, we used confocal microscopy. Immunostaining of the RelA (p65) subunit of NF-kB was present predominantly in the cytoplasm under basal conditions (Fig. 6). LPS increased nuclear translocation of the p65 subunit of NF-kB. Pretreatment with DC attenuated the effect of LPS (Fig. 6). Line scanning analysis of confocal images clearly demonstrated the intensity of the intracellular localization of the p65 subunit (Fig. 6B).

DISCUSSION

In the present study, we demonstrated that DC isolated from the stem bark of *Rhus verniciflua* possesses anti-in-

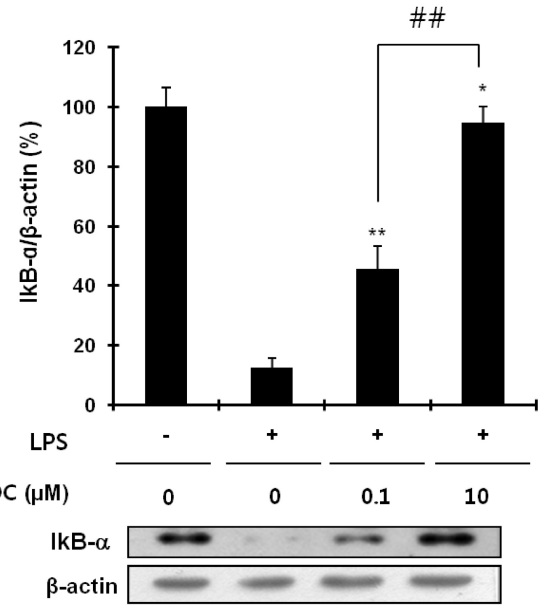

Fig. 5. Inhibitory effects of DC on LPS-induced IkB-α degradation in BV2 microglial cells. The intracellular level of IKB-α was determined using immunoblotting analysis: top, quantitative analysis of immunoblots; bottom, representative immunoblot of IKB-α. β-Actin was used as an internal control. Quantitative data represent three independent experiments and are expressed as mean±SD. *$p<0.05$ and **$p<0.01$ indicate statistically significant differences compared to LPS alone. ##$p<0.01$ indicates a statistically significant difference between the indicated groups.

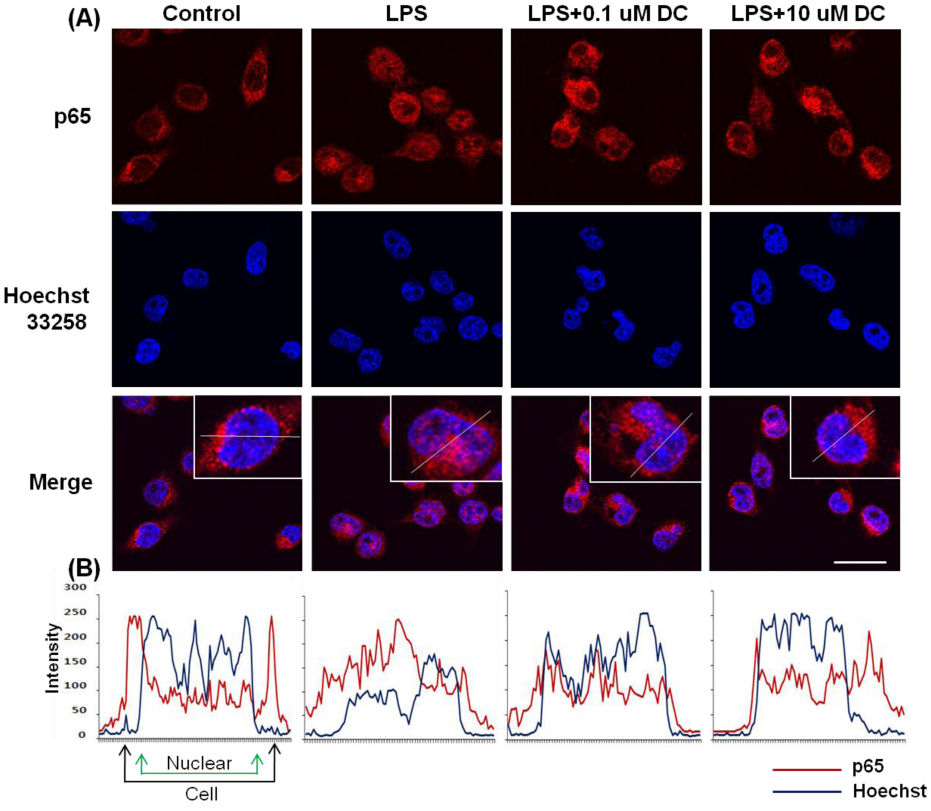

Fig. 6. Blockade by DC of nuclear translocation of the p65 subunit of NF-kB in LPS-stimulated BV2 microglial cells. (A) Localization of the NF-kB p65 subunit was determined using a p65 antibody and an Alexa 546-labeled goat anti-rabbit IgG antibody. Nuclei were visualized by Hoechst staining (Hoechst 33258). In basal conditions, immunostaining of p65 subunit was diffuse throughout the cytoplasm. LPS stimulation resulted in the translocation of p65 subunits into the nucleus. Pretreatment with DC attenuates LPS-induced nuclear translocation of the p65 subunit. (B) Line scanning analysis of confocal images further visualizes the intracellular localization of the p65. Scale bar, 20 μm.

flammatory activity in LPS-stimulated BV2 microglial cells. DC significantly suppressed LPS-induced increases in the production of NO, upregulation of iNOS expression, and release of cytokines such as TNF-α and IL-1β. Furthermore, DC significantly attenuated LPS-induced IKB degradation and subsequent nuclear translocation of NF-kB.

Numerous ester derivatives of caffeic acid such as CAPE [21], chlorogenic acid [26], pedicularioside A [27], forsythoside B [28], and echinachoside [29] exhibit a wide range of biological activities including antioxidant and anti-inflammatory properties, inhibition of caspase-3, and maintenance of mitochondrial function. However, ester derivatives of caffeic acid with high molecular weight aliphatic alcohols such as DC and 1-eicosanoyl cafferate have rarely been isolated. DC was isolated from *sophora subprostrata* [23] and 1-eicosanoyl cafferate from *Echinosophora koreensis* [30]. Furthermore, their biological activities have not been demonstrated. High molecular fatty alcohols in their chemical structures are thought to increase the lipid solubility of the compounds, leading to enhanced penetration of the compounds through the plasma membrane and into cells. In the present study, DC exhibited a significant anti-inflammatory activity in the micromolar range, suggesting that DC might efficiently enter cells due to its high lipid solubility. Although DC was not first isolated from *Rhus verniciflua*, the present study demonstrated its anti-inflammatory activity for the first time.

Aberrant activation of microglia contributes to neuronal damage in several pathologic conditions by releasing proinflammatory cytokines and oxidants such as TNF-α, interleukin (IL)-6, IL-1β, IL-6, IL-10, IL-12, interferon-γ, and NO [8,31]. Not surprisingly, then, suppression of microglial activation and subsequent release of cytokines protected neuronal damage in several inflammation-related CNS diseases [2,32-34]. In the present study, DC significantly attenuated LPS-induced NO production through the suppression of iNOS expression. In addition, DC suppressed the LPS-induced increases in the release of TNF-α and IL-1β and in their gene expression. To our knowledge, effects of DC on other proinflammatory cytokines have not been reported. Therefore, further studies will be necessary to examine the effect of DC on the release and gene expression of other cytokines.

The inappropriate regulation of NF-kB and its downstream genes has been associated with various pathological conditions including cancer and autoimmune diseases [35,36]. For example, NF-kB-dependent microglial activation was a crucial contributor to ischemia [37]. Consistent with these reports, DC significantly attenuated LPS-induced nuclear translocation of NF-kB and subsequent release of proinflammatory cytokines in the present study. However, it has also been suggested that NF-kB is anti-apoptotic [38] and that activation of NF-kB can provide neuroprotection against amyloid β-induced toxicity [39] and against excitotoxic or oxidative stress [40,41]. Overall, these studies suggest diverse functions for NF-kB in the nervous system depending on the cellular context.

In conclusion, our results demonstrate that DC exerts anti-inflammatory activity such as suppression of NO production and cytokine release by inhibiting nuclear translocation of NF-kB in LPS-stimulated BV2 microglial cells. This suggests that DC might be a valuable therapeutic agent in the treatment of inflammation-related brain pathologies such as ischemia and neurodegenerative diseases.

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