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Suppression by a sesquiterpene lactone from *Carpesium divaricatum* of inducible nitric oxide synthase by inhibiting nuclear factor-κB activation

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Abstract

Excessive nitric oxide (NO) produced by inducible NO synthase (iNOS) acts as a causative regulator in various inflammatory disease states. *Carpesium divaricatum* has been used in Korean traditional herbal medicine for its antipyretic, analgesic, vermifugic, and anti-inflammatory properties. We investigated the molecular mechanism for the suppression of lipopolysaccharide/interferon-γ (LPS/IFN-γ)-induced NO production in RAW 264.7 macrophages by the sesquiterpene lactone 2β,5-epoxy-5,10-dihydroxy-6α-angeloyloxy-9β-isobutyloxy-germacran-8α,12-olide (C-1), which has been identified recently as a new compound from *C. divaricatum*. C-1 decreased NO production in LPS/IFN-γ-stimulated RAW 264.7 cells in a concentration-dependent manner, with an IC_{50} of approximately 2.16 μM; however, it had no direct effect on the iNOS activity of fully LPS/IFN-γ-stimulated RAW 264.7 cells. Furthermore, treatment with C-1 led to a decrease in iNOS protein and mRNA. These effects appear to be due to inhibition of nuclear factor-κB (NF-κB) activation through a mechanism involving stabilization of the NF-κB/inhibitor of the κB (I-κB) complex, since inhibition of NF-κB DNA binding activity by C-1 was accompanied by a parallel reduction of nuclear translocation of subunit p65 of NF-κB and I-κBα degradation. Taken together, the results suggest that the ability of C-1 to inhibit iNOS gene expression may be responsible, in part, for its anti-inflammatory effects.

Keywords: Inducible nitric oxide synthase; Sesquiterpene lactone; NF-κB; I-κB

1. Introduction

NO synthesis, catalyzed by NOS (EC 1.14.13.39), is known to be involved in diverse pathological and physiological processes [1–3]. In mammalian cells, three isoforms of NOS have been identified on the basis of physical and biochemical characteristics of the purified enzymes. Unlike Ca^{2+}/calmodulin-dependent constitutive NOS (referred to as neuronal and endothelial NOS), which lies dormant in the cells until there is a rise in intracellular Ca^{2+}, Ca^{2+}/calmodulin-independent iNOS is largely expressed after exposure of some cells [4,5] to specific stimulants, such as cytokines and bacterial LPS. The excessive NO produced by iNOS mediates acute and chronic inflammation.

Many NOS inhibitors have been developed to regulate the adverse effects of excessive NO [6]. Most of the NOS inhibitors are substrate analogues acting as competitive inhibitors with little specificity toward NOS isoforms [7–9]. This lack of specificity restricts their use as therapeutics. Therefore, arduous efforts have been made to develop selective NOS-isoform inhibitors, with the primary focus being placed on iNOS because of its implication in the development of clinical disease conditions. A potential source for the development of selective iNOS inhibitors is medicinal plants used by indigenous cultures worldwide. Recently,

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase: iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; IFN-γ, interferon-γ; NF-κB, nuclear factor-κB; I-κB, inhibitor of κB; DTT, dithiothreitol: RT–PCR, reverse transcription-polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; IL, interleukin; TNF, tumor necrosis factor; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; and EMSA, electrophoretic mobility shift assay.

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parthenolide and isohelenin, sesquiterpene lactones derived from Mexican-Indian medicinal plants [10,11], and dehydrocostus lactone, isolated from *Saussurea lappa* [12,13], have been demonstrated to exert an inhibitory effect on NO produced by iNOS. This inhibitory effect appears to be due to the inhibition of NF-κB, which is a transcription factor acting as a central mediator of the human immune response, and which regulates the transcription of various inflammatory cytokines [14]. Based on the above considerations, we recently identified four new sesquiterpene lactones from *Carpesium divaricatum* [15], which has been used in Korean traditional herbal medicine for its antipyretic, analgesic, vermifugic, and anti-inflammatory properties [16].

This observation prompted us to study the inhibition of NO production by one of four new sesquiterpene lactones identified from *C. divaricatum*, 2β,5-epoxy-5,10-dihydroxy-6α-angeloyloxy-9β-isobutyloxy-germacran-8α,12-olide (C-1), by investigating the induction and catalytic activity of iNOS, protein and mRNA expression, and NF-κB activation in RAW 264.7 cells.

2. Materials and methods

2.1. Reagents

L-[2,3,4,5-^{3}H]-Arginine monohydrochloride (57 Ci/mmol) was obtained from Amersham Life Science, and ^{32}P-labeled dATP (3000 Ci/mmol) was from NEN Life Science Products. LPS (from *Escherichia coli*, 011:B4) was purchased from the Sigma Chemical Co., and IFN-γ (recombinant mouse) was from Pharmingen International. RPMI 1640 medium with 25 mM HEPES and other reagents for cell culture were obtained from Gibco BRL Life Biotechnologies. Rabbit anti-mouse iNOS antibody was obtained from Transduction Laboratories. Rabbit polyclonal p65 (Rel A) antibody was from Calbiochem, and rabbit polyclonal I-κBα antibody was from New England BioLabs Inc. The sesquiterpene lactone containing an exomethylene group, compound-1 (C-1), was isolated from *C. divaricatum* by a method described previously, and its structure was confirmed by ^{1}H- and ^{13}C-NMR spectral data [17]. The rest of the chemicals were from various commercial sources and of the highest grade available.

2.2. Cell culture

RAW 264.7 cells (a murine macrophage cell line obtained from the American Type Culture Collection) were maintained at 37° and 5% CO_2 in RPMI 1640 medium (Gibco BRL) with 25 mM HEPES containing 10% heat-inactivated fetal bovine serum (HyClone), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were plated at a density of 1.0×10^5 cells/well in a 96-well-plate or at 5×10^6 cells in a 10-cm dish, and allowed to attach for 2 hr. For stimulation, the medium was replaced with fresh RPMI 1640, and the cells were then stimulated with 10 ng/mL of LPS and 10 U/mL of IFN-γ in the presence or absence of test compounds for the indicated periods.

2.3. Cell viability

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of MTT to formazan. Thus, the cells were seeded at 1×10^5 cells/well in a 96-well plate and treated with LPS/IFN-γ and C-1. After incubation for 20 hr, the medium was poured off, and macrophages were incubated with MTT (0.1 mg/mL) for 4 hr at 37°. The medium was removed, and 200 μL of DMSO was added to solubilize the formazan crystals; 25 μL of 0.1 M glycine buffer containing 0.1 M NaCl (pH 10.5) was then added. Absorbance was measured at 540 and 650 nm in a microplate reader.

2.4. Measurement of nitrite production using the Griess reagent

NO production in the culture supernatant was spectrophotometrically evaluated by measuring nitrite, a stable end-product of NO. Nitrite was determined by the Griess reaction [18]. One hundred microliters of the culture supernatant was mixed with an equal volume of the Griess reagent [1% (w/v) sulfanilamide in 5% (w/v) phosphoric acid:0.1% (w/v) *N*-(1-naphthyl)ethylenediamine solution = 1:1]. Absorbance was measured in a microplate reader (EL808, Bio-Tek Instrument Inc.) at 540 nm, using a calibration curve with sodium nitrite standards.

2.5. Measurement of NOS activity using the L-[^{3}H]citrulline formation assay

Cell-free extracts as enzyme sources were prepared from RAW 264.7 cells cultured for 20 hr in the presence of C-1 and stimulators (LPS/IFN-γ), and sonicated for 10 sec in 0.3 mL of lysis buffer [50 mM Tris buffer, 1 mM DTT, 0.1 mg/mL of PMSF, 10 μg/mL of trypsin inhibitor, and 10 μg/mL of leupeptin (pH 7.4)]. Specific activity of iNOS in cell-free extracts was measured by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline [19]. After preincubation of reaction mixtures that contained 50 mM Tris buffer (pH 7.4), 1 mM NADPH, 20 μM H4B, 5 μM FAD, 5 μM FMN, 1 mM DTT, and an appropriate amount of cell lysate protein for 3 min at 37°, 10 μL of 500 μM L-arginine containing L-[^{3}H]arginine (about 200,000 dpm) was added to the mixture (at a final concentration of 25 μM in 200 μL of total reaction mixture), and the mixture was further incubated for 10 min. Reaction was stopped by the addition of 1 mL of ice-cold 20 mM sodium acetate stop buffer (pH 5.5) containing 1 mM citrulline, 2 mM EDTA, and 2 mM EGTA. The reaction mixtures were then applied onto columns (1-cm diameter) containing 1 mL DOWEX 50W (Na^+ form) cation exchange resin, and the column was

eluted with 0.2 mL of the stop buffer. Radioactivity of L-[^{3}H]citrulline in the eluates was counted.

2.6. Western blot analysis

Whole cell extracts containing equal quantities of proteins (30 μg) were electrophoresed in a 10% polyacrylamide gel. Subsequently, the separated proteins were transferred to a PVDF membrane using a Semi-Dry Transfer Cell (TRANS-BLOT, Bio-Rad). Briefly, the membrane was blocked for 30 min with blocking buffer [5% skim milk in 50 mM Tris-HCl, 200 mM NaCl, and 0.05% Tween 20 (pH 7.5)], and was incubated overnight at 4° with appropriate dilutions of primary antibodies (against iNOS, p65 or I-κBα). After washing twice with the above buffer, the membrane was further incubated for 2 hr with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit antibody, and was developed with BCIP (5-bromo-4-chloro-3-indoyl phosphate)/NBT (nitroblue tetrazolium) color developing solution.

2.7. RT–PCR

After incubation of 5×10^6 cells with LPS/IFN-γ in the presence or absence of the sesquiterpene lactone for 20 hr, total cellular RNA was extracted with TRIzol® reagent (Gibco BRL). Reverse transcription was performed following the BRL protocol for Superscript TM II reverse transcriptase (Gibco BRL). Briefly, 5 μg of total RNA was incubated with 0.1 mM oligo-dT primers for 10 min at 70°. This was followed by incubation with 5 mM DTT, 0.5 mM deoxynucleotide triphosphate mixture (dNTPs, Promega), and 200 U of Superscript TM II reverse transcriptase for 50 min at 42°. The reverse transcription was terminated by heating for 15 min at 70°. To remove RNA complementary to the cDNA, RNase H (Gibco BRL) was added, and the sample was incubated for 20 min at 37°. The complementary DNA (cDNA) obtained was used for the PCR. The sense and antisense primers for iNOS were 5′-CCCTTC-CGAAGTTTCTGGCAGC-3′ and 5′-GGCTGTCAGAGC-CTCGTGGCTT-3′ (corresponding to nucleotides 3128–3149 and 3603–3624), respectively [20]. The sense and antisense primers for rat GAPDH mRNA expression (used as a control for total RNA content for each sample) were 5′-TGA-AGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CAT-GTAGGCCATGAGGTCCACCAC-3′ (corresponding to nucleotides 51–76 and 1010–1033), respectively [21]. PCR was performed using the AccuPower PCR PreMix (Bioneer, Korea) for 25 cycles: initial denaturation at 94° with an amplification profile of each cycle consisting of denaturation for 30 sec at 94°, annealing for 30 sec at 60°, and elongation for 1 min at 72°, followed by final extension for 7 min at 72°. Fifteen to forty cycles of PCR were performed to determine the linearity of the PCR amplification.

2.8. Northern blot analysis

Twenty micrograms of total RNA was separated on a 1% (w/v) formaldehyde-agarose gel, and transferred to a Nytran® immobilization membrane (Schleicher & Schuell) using 20× SSC (3 M NaCl in 0.3 M sodium citrate) via downward capillary action. After UV cross-linking, the membrane was prehybridized for 1 hr at 68° in 5 mL of QuikHyb solution (Stratagene Inc.), and hybridized to radioactive iNOS and GAPDH probes. Purified PCR products of iNOS and GAPDH were used as radioactive DNA probes after labeling with [α-^{32}P]CTP using the RadPrime DNA Labeling System (Life Technology Inc.) and following the manufacturer's instructions. After hybridization, the membrane was washed twice with 2× SSC in 0.1% SDS for 15 min at room temperature and once with 0.2× SSC in 0.1% SDS at 65°. RNA blots hybridized with radioactive DNA probes were exposed to x-ray film with an intensifying screen at −70°.

2.9. EMSA of NF-κB

RAW 264.7 cells were plated at a density of 1.0×10^7 cells/culture dish in RPMI 1640 medium supplemented with 3% fetal bovine serum and incubated at 37° in a CO_2 incubator. After pretreatment with the sesquiterpene lactone for 1 hr, the cells were stimulated with LPS/IFN-γ for 1 hr. Nuclear proteins were extracted by a modification of the method of Andrews and Faller [22]. All the procedures for nuclear protein extraction were performed at 4° with ice-cold reagents. Scrapped and pelleted cells were resuspended in 1 mL of ice-cold lysis buffer [10 mM Tris-HCl (pH 7.4), 3 mM $CaCl_2$, 2 mM $MgCl_2$, 1% NP-40, 0.5 mM PMSF, and 5 μg each of leupeptin, pepstatin A, and aprotinin/mL] and incubated for 15 min on ice with occasional vortexing. After centrifugation and rinsing of the nuclear pellet with wash buffer [10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and the above protease inhibitors], 30–50 μL of ice-cold hypertonic extraction buffer [20 mM HEPES-KOH (pH 7.9), 25% (w/v) glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, and the above protease inhibitors] was added, and incubated at 4° for 40 min with constant shaking. Nuclear extracts were then prepared by centrifugation at 20,000 *g* for 30 min and stored at −70° until used for EMSA. The oligonucleotide probe used for EMSA contained the NF-κB consensus sequence. The sequences of probes used were (binding site underlined) NF-κBU [5′-AGC-TTG-GGG-ACT-TTC-C-3′] and NF-κBL [3′-C-CCC-TGA-AAG-GTC-GGC-5′]. One nanomole of each oligonucleotide was annealed by heating at 95° for 5 min and cooling slowly to 30°, and diluted to 1.75 pmol/μL. The oligonucleotide probe was labeled with [α-^{32}P]dATP (Amersham Pharmacia) using Klenow fragment (Gibco BRL). The total volume of the labeling mixture was 25 μL, and the composition of the labeling mixture was as follows: 7 pmol oligomer (DNA

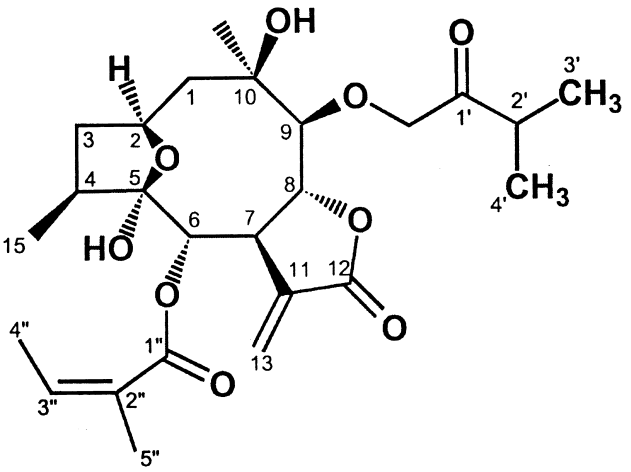

Fig. 1. Chemical structure of 2β,5-epoxy-5,10-dihydroxy-6α-angeloyloxy-9β-isobutyloxy-germacran-8α,12-olide (C-1) isolated from *C. divaricatum.*

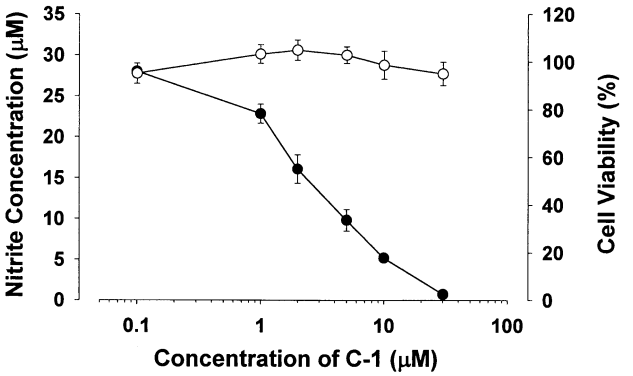

Fig. 2. Effect of C-1 on nitrite accumulation and cell viability in stimulated RAW 264.7 cells. Cells were plated at a density of 1.0×10^5 cells/well in a 96-well plate and allowed to attach for 2 hr. The cells were treated with the indicated concentrations of C-1 immediately prior to the addition of 10 ng/mL of LPS and 10 U/mL of IFN-γ, and the cells were incubated for 20 hr. Nitrite concentrations (●) in the culture medium were determined by the Griess reaction. Values (means ± SEM) were obtained from seven separate experiments. Nitrite concentrations in non-stimulated and LPS/IFN-γ-stimulated RAW 264.7 cells were 0.33 ± 0.11 and 32.75 ± 0.57 μM, respectively. Cell viability (○) was determined using the MTT assay and is expressed as a percentage of the control without the addition of C-1.

probe), 0.4 mM dNTPs (without dATP), labeling buffer [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 10 mM $MgCl_2$], 4 μL [α-^{32}P]dATP (>3,000 Ci/mmol), and 1 μL Klenow fragment (1 U/μL). The labeling reaction was performed for 40 min at 37°, and the labeled probes were purified by Sephadex G-25 spin-column chromatography. Binding reactions were performed at room temperature for 30 min with 5–10 μg of nuclear protein in 20 μL of binding buffer [10 mM HEPES-KOH (pH 7.7), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and 1 μg each of leupeptin, pepstatin A, and aprotinin/mL] containing 1 μg of poly[dI-dC · dI-dC] and 100,000 dpm of ^{32}P-labeled probe. The specificity of the binding reaction was confirmed by a competition assay with a 100-fold molar excess of unlabeled oligonucleotide probe. The DNA-protein complex was separated from the unbound probe on native 6% polyacrylamide gels in 0.5×-TBE running buffer [45 mM Tris base, 44 mM boric acid, and 1 mM EDTA (pH 8.0)]. After electrophoresis, the gel was vacuum-dried and autoradiographed by exposure to x-ray film at −70° for 24−48 hr.

2.10. Protein determination

Protein concentrations were determined by the Bradford method [23], using bovine serum albumin as a standard.

3. Results

3.1. Inhibition by C-1, the sesquiterpene lactone containing an exomethylene group, of nitrite production in RAW 264.7 cells, but not of iNOS activity

First, we evaluated the capacity of C-1 [2β,-5-epoxy-5,10-dihydroxy-6α-angeloyloxy-9β-isobutyloxy-germacran-8α12-olide] (Fig. 1) to inhibit NO synthesis. RAW 264.7 cells were treated with C-1 in the culture medium, immediately followed by stimulation with 10 ng/mL of LPS and 10 U/mL of IFN-γ for 20 hr. C-1 significantly inhibited nitrite production in a concentration-dependent manner (0.1 to 30 μM), with an IC_{50} of approximately 2.16 μM (Fig. 2). Cell viability was also measured by MTT assay to determine whether the inhibitory effects of C-1 on NO production were attributable to nonspecific cytotoxicity. In the presence of up to 30 μM C-1, a concentration at which NO synthesis was inhibited completely, cell viability was not different from that of LPS/IFN-γ-stimulated cells (Fig. 2).

Next, we investigated whether the inhibition of NO production by C-1 was due to direct effects on the catalytic activity of the enzyme or indirectly through suppression of iNOS protein expression. iNOS activity was assayed *in vitro* in the presence of inhibitors by measuring L-[^{3}H]citrulline formation in cell-free extracts prepared from RAW 264.7 cells that were stimulated with LPS/IFN-γ for 20 hr. Unlike N^G-monomethyl-L-arginine, a well-known NOS inhibitor, C-1 did not show any significant inhibitory effect on the specific activity of iNOS (Fig. 3).

3.2. Effect of C-1 on iNOS protein and mRNA expressions

Since C-1 inhibited NO production in the presence of stimulants, but had no effect on iNOS assayed *in vitro*, we investigated iNOS protein and mRNA expressions in order to elucidate the inhibitory mechanism of the compound (Fig. 4). Although hardly detected by Western blot analysis in unstimulated RAW 264.7 cells, iNOS protein was sufficiently expressed by stimulation with LPS/IFN-γ for 20 hr; however, the presence of C-1 in the LPS/IFN-γ-stimulated cell cultures markedly decreased iNOS expression (Fig.

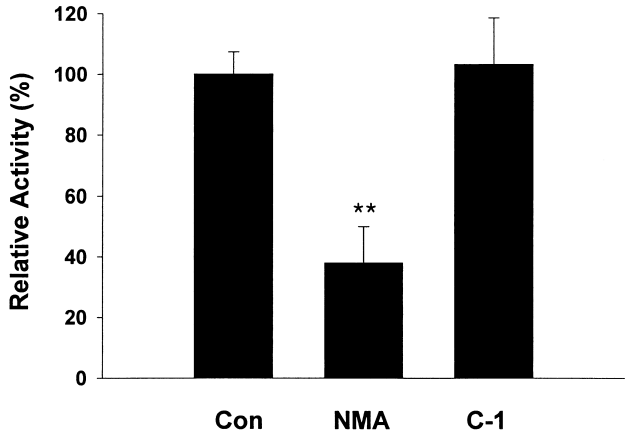

Fig. 3. Effect of C-1 on iNOS activity in cell-free extracts of stimulated RAW 264.7 cells. Cell lysate was prepared from RAW 264.7 cells stimulated for 20 hr with 10 ng/mL of LPS and 10 U/mL of IFN-γ, and iNOS activity in the lysate was measured by L-[^{3}H]citrulline formation with or without the addition of NMA (10 μM) or C-1 (10 μM). Results are expressed as percent inhibition of the LPS/IFN-γ-stimulated control (specific activity of 142.8 pmol [^{3}H]citrulline formed/mg protein/min) with the vehicle DMSO. Values (means ± SEM) were obtained from three separate experiments. Key: $^{**}P < 0.01$, significantly different from LPS/IFN-γ-stimulated cells, analyzed by Student's *t*-test.

4A). This result indicated that the decrease in NO production caused by C-1 could have resulted from inhibition of iNOS protein expression.

To determine whether the reduction of the iNOS protein level by C-1 was due to reduced expression of iNOS mRNA, we performed RT-PCR and Northern blot analysis. As shown in panels B and C of Fig. 4, iNOS mRNA was expressed at an undetectable level in untreated cells; however, there was a dramatic increase in LPS/IFN-γ-stimulated cells. The steady-state iNOS mRNA level in the presence of C-1 was reduced compared with that of LPS/IFN-γ-stimulated cells. Taken together, these results suggest that the decrease in iNOS protein expression by C-1 resulted from the instability of iNOS mRNA or the suppression of iNOS mRNA expression by impairing some upstream components of the pathways involved in NO production.

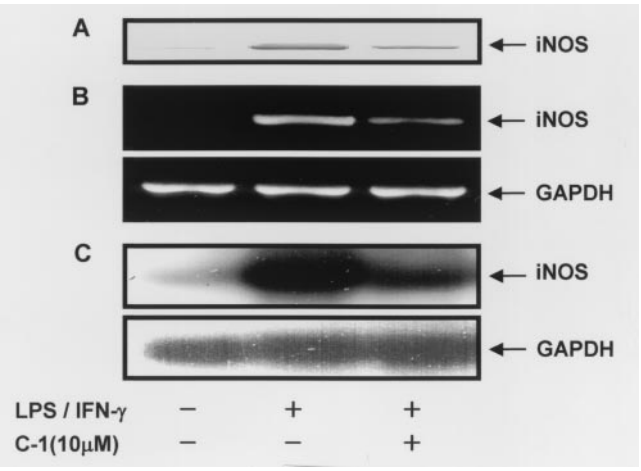

Fig. 4. Effect of C-1 on iNOS protein and mRNA expressions. Cells were stimulated with 10 ng/mL of LPS and 10 U/mL of IFN-γ, and incubated for 20 hr in the presence of 10 μM C-1. Western blot analysis (A), RT–PCR (B), and Northern blot analysis (C) were carried out as described under section 2. Data shown are typical of four separate experiments.

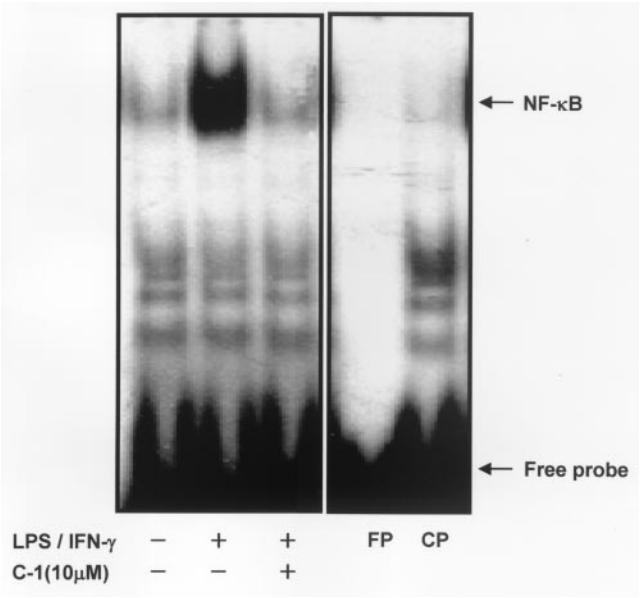

Fig. 5. Effect of C-1 on DNA binding activity of NF-κB in RAW 264.7 cells treated with LPS/IFN-γ. Cells were stimulated with 10 ng/mL of LPS and 10 U/mL of IFN-γ for 1 hr after a 1-hr pretreatment with 10 μM C-1. Nuclear extracts were subjected to EMSA as described under "Materials and methods". Band specificity was confirmed by cold-competition (CP) assay with a 100-fold molar excess of unlabeled oligonucleotide probe or by running free probe (FP) without nuclear extracts. Data shown are typical of four separate experiments.

3.3. Effect of C-1 on the activation of the transcription factor NF-κB

Since NF-κB activation is critical for the induction of iNOS by LPS or other inflammatory cytokines and since sesquiterpene lactones have been demonstrated previously to be potent inhibitors of NF-κB activation in immune-related cells [10,24,25], EMSA was performed to examine whether C-1 suppressed NF-κB activation. The nuclear binding assay of NF-κB was carried out with nuclear extracts prepared from RAW 264.7 cells that were stimulated with LPS/IFN-γ for 1 hr in the presence or absence of C-1. In LPS/IFN-γ-stimulated RAW 264.7 cells, NF-κB was found to be activated and translocated into the nucleus, where it was bound to the iNOS promoter region (Fig. 5). As shown in the lane of competition, DNA binding of NF-κB was blocked completely by the addition of a 100-fold molar excess of unlabeled oligonucleotide NF-κB DNA probes, which indicated the specificity of the binding reaction. Pretreatment with 10 μM C-1 significantly interfered with the LPS/IFN-γ-induced band shift. These results

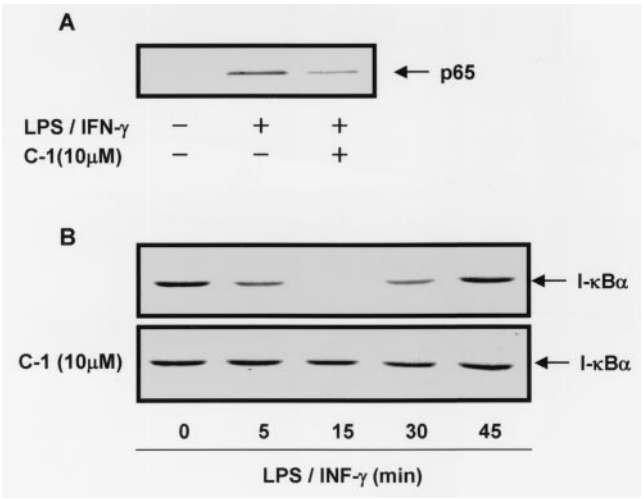

Fig. 6. (A) Effect of C-1 on the nuclear translocation of NF–κB in RAW 264.7 cells treated with LPS/IFN-γ. Cells were stimulated with 10 ng/mL of LPS and 10 U/mL of IFN-γ for 1 hr after a 1-hr pretreatment with 10 μM C-1. The nuclear extracts prepared from the experiments in Fig. 5 were analyzed by western blotting with an anti-p65 polyclonal antibody. (B) Effect of C-1 on the degradation of I-κBα in RAW 264.7 cells treated with LPS/IFN-γ. The cells were stimulated with LPS/IFN-γ for the indicated times after a 1-hr pretreatment with 10 μM C-1. Cellular extracts were analyzed by western blotting with an anti-I-κBα polyclonal antibody. Data shown are typical of four separate experiments.

are similar to those found with other sesquiterpene lactones such as helenalin, parthenolide, isohelenin, and dehydrocostus lactone [10,11,13,14,24,26].

3.4. Effect of C-1 on the translocation of subunit p65 into the nucleus and LPS/IFN-γ-mediated degradation of I-κBα

The active NF-κB heterodimer, frequently composed of a p50 and a Rel A (p65) subunit, rapidly translocates to the nucleus where it activates transcription of its target genes. In most cell types, NF-κB is sequestered in an inactive, cytoplasmic complex by binding to a group of inhibitory proteins belonging to the I-κB family [27]. Active NF-κB is released from the cytoplasmic complex by phosphorylation and proteolytic degradation of I-κBα in response to proinflammatory signals, thus unmasking NF-κB nuclear translocation sequences and freeing it to enter the nucleus. The inhibitory effect of C-1 on NF-κB-DNA binding may be due to either direct modification of NF-κB or inhibition of upstream events leading to NF-κB activation. Western blot analysis was used to determine whether the translocation of subunit p65 of NF-κB was associated with inhibition of DNA binding of NF-κB by C-1. As shown in Fig. 6A, LPS/IFN-γ treatment caused the translocation of subunit p65 into the nucleus. However, the amount of p65 translocated into the nucleus in response to LPS/IFN-γ was reduced significantly in the presence of C-1, indicating that the sesquiterpene lactone (C-1) suppresses the induction of the iNOS gene by inhibiting the translocation of subunit p65 in LPS/IFN-γ-induced NF-κB activation. We next determined the effects of C-1 on LPS/IFN-γ-mediated degradation of I-κBα by Western blot analysis using polyclonal antibody. Treatment with LPS/IFN-γ led to the rapid disappearance of the immunoreactive I-κBα band within 15 min; this band returned to basal levels within 45 min (Fig. 6B). However, LPS/IFN-γ-induced degradation of I-κBα was attenuated in the presence of C-1. Taken together, these results suggest that suppression of iNOS gene expression by the sesquiterpene lactone C-1 may be due to the attenuation of DNA binding of NF-κB by inhibiting the degradation of I-κBα.

4. Discussion

Excessive production of NO is associated with various diseases such as septic shock, stroke, arthritis, chronic inflammatory diseases, and autoimmune disease [2,3]. Since *de novo* synthesis of iNOS in activated macrophages and several other cell types by various stimuli including LPS, IFN-γ, or TNF-α plays a key role in determining NO production rates, iNOS became an important target in the remedy of inflammation [28,29]. While many plant materials have been found to inhibit NO production by suppressing iNOS protein expression, but not by directly inhibiting enzyme activity [30–33], some phytochemicals having antioxidative properties have shown a dual function of suppressing iNOS protein expression and directly inhibiting catalytic activity [34–37]. These considerations led us to investigate the sesquiterpene lactone 2β,5-epoxy-5,10-dihydroxy-6α-angeloyloxy-9β-isobutyloxy-germacran-8α12-olide (C-1), a new compound isolated from *C. divaricatum*, for its potential therapeutic value.

The results presented in this study showed that treatment of LPS/IFN-γ-stimulated RAW 264.7 cells with C-1 potently decreased nitrite accumulation in a concentration-dependent manner, with an IC_{50} of approximately 2.16 μM (Fig. 2). The IC_{50} of C-1 was comparable to that of dehydrocostus lactone (3 μM) in LPS-activated RAW 264.7 cells, and lower than that of parthenolide and isohelenin observed in cultured rat aortic smooth muscle cells [11]. These inhibitory effects were not the result of cytotoxicity (Fig. 2), because in the presence of C-1 (up to 30 μM) cell viability was not different from that of LPS/IFN-γ-stimulated cells (Fig. 2). Cell viability was comparable to that of parthenolide, which was reduced modestly only at a concentration of 30 μM. However, C-1 did not directly inhibit iNOS activity of fully activated RAW 264.7 cells (Fig. 3), and the results strongly suggest that the inhibitory effects of this compound are mediated via a reduction in iNOS mRNA expression: iNOS protein and mRNA levels were reduced by this compound, as determined, respectively, by Western and Northern blot analyses (Fig. 4). Taken together, it is quite obvious to presume that the decrease in iNOS protein

expression by C-1 resulted from the suppression of iNOS mRNA expression or instability of iNOS mRNA.

It is known that inactive NF-κB normally binds to I-κB in the cytosol, and that NF-κB can be activated by various factors such as cell-damaging signals, bacterial infection, pro-inflammatory cytokines, and reactive oxygen intermediates, all of which increase protein phosphorylation and the proteolysis of I-κB protein [38]. The free and activated NF-κB is translocated into the nucleus to bind to relevant DNA sites on the promoter region of its target genes including various inflammatory cytokine genes such as IL-1, IL-2, IL-6, IL-8, and TNF-α, as well as genes encoding cell adhesion molecules, immunoreceptors, hematopoetic growth factors, and growth factor receptors [14]. Furthermore, NF-κB is also a crucial transcription factor for mRNA expression of iNOS, and its response elements are on the promoter regions of iNOS [39–41]. The central role of NF-κB in mediating inflammatory processes is evident from both the importance of its target genes and from the phenotypes of mice lacking the NF-κB subunit [42]. Therefore, compounds inhibiting NF-κB are potentially of great interest for developing lead structures for the treatment of acute and chronic inflammation. There are at least four groups of NF-κB inhibitors. The first group of NF-κB inhibitors exerts their inhibitory effects by scavenging reactive oxygen intermediates, which share the property of being anti-oxidative. The second group of inhibitors interferes with the induced degradation of I-κB-family members by affecting the functioning of the 26-S proteasome [42–44]. The third group of inhibitors exerts their effects only in the cell nucleus by impairing the transcriptional activity of NF-κB already bound to DNA [45,46]. The fourth group of inhibitors, such as helenalin, have been shown to exert their effects by directly modifying the transcription factor NF-κB [47]. Indeed, it has been reported recently that the sesquiterpene lactones parthenolide and isohelenin, isolated from extracts of Mexican-Indian medicinal plants, inhibit iNOS gene expression by stabilizing the association between I-κBα and NF-κB [11]. Therefore, to investigate the molecular mechanism for the suppression of LPS/IFN-γ-induced NO production in RAW 264.7 macrophages by the sesquiterpene lactone C-1, isolated from *C. divaricatum*, an EMSA was carried out. When treated with C-1 for 1 hr subsequent to stimulation with LPS/IFN-γ, LPS/IFN-γ-induced NF-κB DNA binding activity was nearly completely abolished at a C-1 concentration of 10 μM, which was lower than that of parthenolide and isohelenin [11] and comparable to that of helenalin [25]. Consistent with this result, this effect was accompanied by the parallel reduction of nuclear translocation of subunit p65 of NF-κB as well as I-κBα degradation. Therefore, our results favor a mechanism by which the sesquiterpene lactone C-1 exerts its inhibitory effect on NO production by stabilizing the NF-κB/I-κB complex rather than by directly targeting the active NF-κB. Further investigation is needed to discern which components or which upstream events leading to NF-κB activation were suppressed by C-1 and to determine whether C-1 can inhibit LPS/IFN-γ-stimulated iNOS expression through other transcription factors such as AP-1 and Oct-1 [48–51].

In conclusion, the study described herein suggests that the new sesquiterpene lactone C-1, isolated from *C. divaricatum*, suppresses NO synthesis in LPS/IFN-γ-activated RAW 264.7 cells through the inhibition of NF-κB activation, iNOS mRNA, and protein expression.

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