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Suppression of inducible nitric oxide synthase expression in RAW 264.7 macrophages by two β-carboline alkaloids extracted from *Melia azedarach*

Byeong Gon Lee [a], Seung Hee Kim [b], Ok Pyo Zee [a], Kang Ro Lee [a], Hoi Young Lee [c], Jeung Whan Han [a], Hyang Woo Lee [a,*]

[a] *College of Pharmacy, Sungkyunkwan University, Suwon 440-746, South Korea*
[b] *National Institute of Toxicological Research, Korea Food and Drug Administration, Seoul 122-020, South Korea*
[c] *College of Medicine, Konyang University, Nonsan 320-030, South Korea*

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Abstract

We investigated the mechanism of suppression of inducible nitric oxide synthase (iNOS) by two β-carboline alkaloids isolated from *Melia azedarach*, 4,8-dimethoxy-1-vinyl-β-carboline (compound 1, C-1) and 4-methoxy-1-vinyl-β-carboline (compound 2, C-2). iNOS activity in a cell-free extract of lipopolysaccharide/interferon-γ-stimulated RAW 264.7 cells was found to be markedly increased, and this increase was prevented by C-1 and C-2, accompanied by the parallel reduction in nitrite accumulation in culture medium. However, C-1 and C-2 had no further effect on the iNOS activity prepared from fully lipopolysaccharide/interferon-γ-stimulated RAW 264.7 cells. Treatment with C-1 or C-2 decreased the levels of iNOS protein and mRNA in a concentration-dependent manner. In addition, prostaglandin E_2 production, cyclooxygenase-2 protein and DNA binding of nuclear factor-κB (NF-κB) in lipopolysaccharide-stimulated RAW 264.7 cells were reduced by these compounds. These results indicate that C-1 and C-2 primarily inhibit iNOS and cyclooxygenase-2 activities via the suppression of de novo synthesis of these two enzymes, and that the inhibition of iNOS expression may be associated with the inhibition of NF-κB activation. Taken together, the results suggest that suppression of iNOS and cyclooxygenase-2 induction by lipopolysaccharide is responsible for the anti-inflammatory activity of these alkaloids through selective inhibition of the expression of genes, which play important roles in inflammatory signaling pathways.

Keywords: Inflammation; Nitric oxide (NO); Inducible NO synthase; NF-κB (nuclear factor-κB); β-Carboline alkaloid

1. Introduction

Nitric oxide (NO) synthesized by nitric oxide synthase (NOS) is known to be involved in diverse physiological processes (Moncada et al., 1991; Radomski et al., 1987; Snyder and Bredt, 1992; Yap and Sher, 1999). Although NO has dual properties, as a pro-inflammatory or anti-inflammatory agent, excessive NO production has been implicated in a wide range of inflammatory diseases, such as sepsis, psoriasis, arthritis, multiple sclerosis, and systemic lupus erythematosus (Clancy et al., 1998; Kröncke et al., 1998). In mammalian cells, three isoforms of NOS, types I, II, and III, have been identified on the basis of physical and biochemical characteristics of the purified enzymes. Unlike type I (neuronal NOS, nNOS) and type III (endothelial NOS, eNOS), referred to as the constitutive NOS (cNOS), which are continuously present in the cells, type II, an inducible type NOS (iNOS), is expressed only after exposure to specific stimulants such as cytokines, bacterial lipopolysaccharide, and calcium ionophore in some cells (Vodovotz et al., 1993; Chesrown et al., 1994; Weisz et al., 1996; Denlinger et al., 1996). Moreover, once expressed, iNOS generates significantly large and sustained amounts of NO, and this excessive NO is known to be an important mediator of acute and chronic inflammation.

Nuclear factor-κB (NF-κB) is a transcription factor that acts as a central mediator of the human immune response and regulates the transcription of various inflammatory

* Corresponding author. Tel.: +82-31-290-7702; fax: +82-31-290-7722.
E-mail address: hylee@yurim.skku.ac.kr (H.W. Lee).

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cytokines such as interleukin-1, interleukin-2, interleukin-6, interleukin-8 and tumor necrosis factor-α (TNF-α) as well as genes encoding cell adhesion molecules, immunoreceptors, hematopoetic growth factors and growth factor receptors (Baeuerle and Henkel, 1994). In addition, NF-κB response elements have been demonstrated to be on the promoter regions of iNOS and cyclooxygenase-2 (Barnes and Karin, 1997; Crofford et al., 1997), which are involved in inflammatory processes by producing NO and prostaglandins, respectively.

A large number of NOS inhibitors have been developed to regulate the adverse effects of excessive NO (for review, see Fukuto and Chaudhuri, 1995). Almost all of the developed inhibitors are substrate analogues for NOS, and they act as competitive inhibitors with little specificity for NOS isoforms. This lack of specificity restricts their use as therapeutics, and for this reason, efforts have been directed at the development of selective NOS-isoform inhibitors, and primary focus has been placed on iNOS because of its implication in the development of clinical diseases. Based on the above considerations, we screened iNOS inhibitory materials from many traditional herbal medicines, which have been used as traditional remedies for inflammatory diseases, and found that two β-carboline alkaloids (Fig. 1) isolated from the plant *Melia azedarach* L. var *japonica* inhibited iNOS activity in RAW 264.7 cells, a murine macrophage cell line (Kwon et al., 1999). Although these two compounds, 4,8-dimethoxy-1-vinyl-β-carboline (compound-1, C-1) and 4-methoxy-1-vinyl-β-carboline (compound-2, C-2), were first isolated from *Picrasma quassioides* Bennet (Simaroubaceae) by Ohmoto and Koike (1982), there is little information available on the biological activities of β-carboline alkaloid compounds.

In this study, in order to elucidate the mechanism of NOS inhibition by these β-carboline alkaloids, we investigated the effects of these compounds on the induction and catalytic activity of iNOS, protein and mRNA expressions, transcription factor, and NF-κB activation in RAW 264.7 cells. Furthermore, their effects on the induction, catalytic activity, and protein expression of cyclooxygenase-2, a pivotal enzyme in the biosynthesis of prostaglandins (for review, see Vane et al., 1998), were also examined to confirm the anti-inflammatory properties of these two compounds.

2. Materials and methods

2.1. Reagents

Two β-carboline derivatives, C-1 and C-2, were isolated from the cortex of *M. azedarach* L. var *japonica* Makino according to the method previously described, and their structures were confirmed by ^{1}H- and ^{13}C-nuclear magnetic resonance spectral data (Kwon et al., 1999). Dulbecco's modified Eagle's medium (DMEM) with high glucose and other reagents for cell culture were obtained from Gibco BRL Life Biotechnologies (Grand Island, NY, USA). [2,3,4,5-^{3}H]-L-arginine monohydrochloride (57 Ci/mmol) was obtained from Amersham International (Amersham, Bucks., UK) and [^{32}P]-labeled dATP (3000 Ci/mmol) was from NEN (NEN Life Science Products, Boston, MA, USA). Mouse anti-mouse iNOS monoclonal antibody and mouse anti-rat cyclooxygenase-2 antibody were obtained from Transduction Lab. (Lexington, KY, USA). Lipopolysaccharide (from *Escherichia coli*, 011:B4), interferon-γ (recombinant mouse interferon-γ, expressed in *E. coli*), *N*-(1-naphthyl) ethylenediamine hydrochloride, sulfanilamide, (6*R*)-5,6,7,8-tetrahydrobiopterin (6*R*-H_4B), aprotinin, leupeptin, pepstatin A, phenylmethylsulfonylfluoride, β-NADPH, dithiothreitol, calmodulin, L-citrulline, Dowex 50WX8-200 resin, FAD, FMN, Igepal CA-630, and N^{ω}-methyl-L-arginine (L-NMA) were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

The murine macrophage/monocyte cell line RAW 264.7 cells (from ATCC) were maintained at 37°C and 5% CO_2 in DMEM containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were plated at a density of 1.0×10^{6} cells/ml and allowed to attach for 2 h. For stimulation, the medium was replaced with fresh DMEM without phenol red, and the cells were then stimulated with

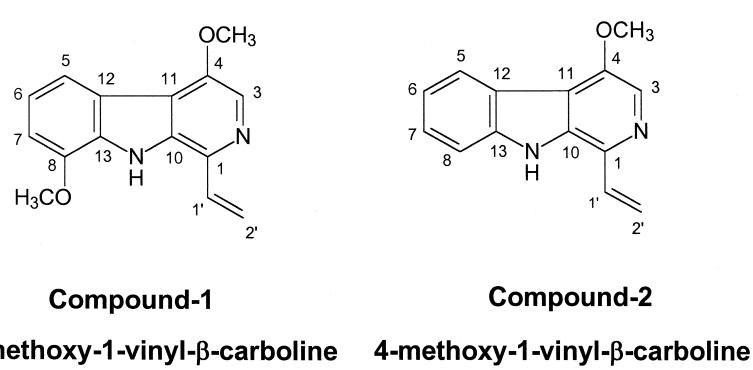

Fig. 1. Chemical structures of C-1 and C-2 isolated from *M. azedarach* L. var *japonica*.

1 μg/ml of lipopolysaccharide and 10 U/ml of interferon-γ in the presence or absence of test compounds for the indicated periods.

2.3. Measurement of nitrite production using Griess reagent

NO production in culture supernatant was spectrophotometrically evaluated by measuring nitrite, an oxidative product of NO. Nitrite was determined with the Griess reaction (Green et al, 1982) by mixing 100 μl of culture supernatant with 100 μl of Griess reagent containing equal volumes of 1% (w/v) sulfanilamide in 5% (w/v) phosphoric acid and 0.1% (w/v) of *N*-(1-naphthyl)ethylenediamine solution. Absorbance was measured in a microplate reader (Bio-Tek ELx 800, Bio-Tek Instrument, USA) at 540 nm against a calibration curve with sodium nitrite standards.

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

Cell-free extracts as enzyme sources were prepared from the RAW 264.7 cells cultured for 16–18 h in the presence of test compounds and stimulators, lipopolysaccharide (1 μg/ml) and interferon-γ (10 U/ml), using the method of Hayashi et al. (1997) with minor modifications. Specific activity of iNOS was measured in cell-free extracts by monitoring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline (Vodovotz et al., 1993). After preincubation of reaction mixtures which contained 50 mM Tris buffer (pH 7.4), 1 mM NADPH, 20 μM H_4B, 5 μM FAD, 5 μM FMN, 1 mM dithiothreitol and 20–40 μg of cell lysate protein for 3–5 min at 37°C, 10 μl of 500 μM arginine containing [^{3}H]L-arginine (about 200,000 cpm) was added to the mixture (at a final concentration of 25 μM in 200 μl of total reaction mixture), and was further incubated for 10–15 min. The reaction was stopped by the addition of 1 ml ice-cold 20 mM sodium acetate stop buffer (pH 5.5) containing 1 mM citrulline, 2 mM EDTA and 2 mM EGTA. Then the reaction mixtures were applied onto columns (1 cm diameter) containing 1 ml DOWEX 50WX8-200 (Na^+ form) cation exchange resin, and each column was eluted with 2 ml of water. The radioactivity of [^{3}H]L-citrulline in the eluates was measured on a liquid scintillation counter (LKB, RackBeta, Finland). For the nNOS assay, fresh rat brain extract as an enzyme source was prepared as previously published (Chan et al., 1997) and 10 μg/ml of calmodulin and 1.5 mM of $CaCl_2$ were used in addition to the above reaction mixture described for the iNOS assay.

2.5. Western blot analyses of iNOS and cyclooxygenase-2 protein

Whole-cell extracts containing equal quantities of proteins were loaded onto 4–20% gradient Tris–glycine gels (for iNOS) or 10% Bis-Tris gel (for cyclooxygenase-2), and electrophoresed with Tris–glycine sodium dodecylsulfate (SDS) running buffer or 2-(*N*-morpholino) ethane sulfonic acid (MES) SDS running buffer. Subsequently, the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using Hoefer™ Semi-Dry Transfer Units (SemiPhor™ TE 70, Hoefer Pharmacia Biotech, San Francisco, CA, USA). Immunoblot analysis for iNOS was carried out with Bio-Rad Western blot kit (Immuno-Blot™ Assay kit, Bio-Rad Lab., CA, USA) according to the manufacturer's instructions. Briefly, after blocking with 3% gelatin in Tris-buffered saline, the membranes were incubated with a 1:1000 dilution of murine anti-mouse iNOS monoclonal antibody for 2–3 h. The membranes were then incubated for 1–2 h with a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody, and the blots were finally developed with 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/nitroblue tetrazolium (NBT) color developing solution. In the case of cyclooxygenase-2, an enhanced chemiluminescence (ECL) method was used as Western blot detection system (ECL Plus™, Amersham). As a primary antibody, murine anti-rat cyclooxygenase-2 polyclonal antibody (Transduction Lab) was used at a 1:500 dilution. The secondary antibody, horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham), was diluted 1:2000 and the immunoblot was visualized using Amersham ECL-film.

2.6. Northern blot analysis

Total cellular RNA was extracted with TRIzol® reagent (Gibco) according to the manufacturer's instructions. Twenty micrograms of total RNA were separated on 1.0% (w/v) agarose gels containing 1.8% formaldehyde and were transferred to a positively charged-nylon membrane (Boehringer Mannheim, Mannheim, Germany) via downward capillary action. After UV cross-linking and prehybridization for 3 h at 45°C in 20 ml of hybridization solution (DIG Easy Hyb®, BM), the filters were hybridized to a digoxigenin-dUTP-labeled iNOS probe and digoxigenin-dUTP-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe overnight at 45°C in a hybridization incubator. Purified PCR products of iNOS (ca. 500 bps) and GAPDH (ca. 1000 bps) were used as DNA probes after labeling with digoxigenin-11-dUTP by random priming using a Dig-DNA labeling kit (BM). RNA blots hybridized with digoxigenin-labeled DNA probes were detected by EIA with luminescence using Dig-luminescent detection kit (BM) according to the manufacturer's instructions. Briefly, after hybridization and thorough washing, the membrane was blocked with non-fat milk for 30 min and then incubated for 30 min in 20 ml of antibody solution containing polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase

(BM). Alkaline phosphatase activity also was detected by the ECL system (BM) using Amersham ECL-film.

2.7. Measurement of cyclooxygenase-2 activity by enzyme immunoassay

Prostaglandin E_2 production was measured in culture medium in order to determine cyclooxygenase-2 activity. For the assay of cyclooxygenase-2 induction, RAW 264.7 cells were plated in 24-well plates at a density of 5×10^5 cells/well in 1 ml of DMEM and treated with 500 μM acetylsalicylic acid for cyclooxygenase inactivation. After a 2-h incubation, culture media were replaced with fresh DMEM containing 5% fetal bovine serum. The cells were stimulated with lipopolysaccharide (1 μg/ml) and incubated in the presence of test compounds for 16 h at 37°C. The culture supernatants were immediately used for prostaglandin E_2 determination or stored at −70°C until measurement. For the assay of intrinsic cyclooxygenase-2 activity, the cells incubated and pretreated with acetylsalicylic acid according to the same protocol as for the induction assay were stimulated with lipopolysaccharide (1 μg/ml) in the absence of test compounds. After a 16-h incubation and wash, the media were replaced with 0.9 ml of fresh media with or without test compounds. The cells were incubated for 20 min, and 100 μl of 100 μM arachidonic acid was then added to each well and the incubation was continued for another 12–13 min. The culture supernatants were used immediately or stored at −70°C until prostaglandin E_2 determination. Prostaglandin E_2 concentration was measured by a commercial competitive enzyme immunoassay kit (EIA, Caymann chem, Ann Arbor, MI, USA) according to the manufacturer's protocol. Briefly, after mouse monoclonal antibody pre-coated 96-well plates were washed, EIA buffer, rabbit prostaglandin E_2 antiserum, prostaglandin E_2 acetylcholine esterase tracer, and appropriately diluted culture supernatant were added to each well and the plate was incubated for 18 h at 4°C. After the plates were washed, Ellmann's reagent [0.8 mM acetylthiocholine iodide and 0.5 mM dithio-bis(2-nitrobenzoic acid) in 0.5 mM phosphate buffer] was added to each well. Color developed after incubation for about 2 h at room temperature was read at 412 nm using a microplate reader.

2.8. Electrophoretic mobility shift assay (EMSA) of NF-κB

Nuclear proteins were extracted by using a modification of the method of Andrews and Faller (1991). All the procedures for nuclear protein extraction were performed at 0–4°C 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% Igepal CA-630, 0.5 mM phenylmethylsulfonylfluoride, and 5 μg/ml of leupeptin, pepstatin, aprotinin, respectively) and incubated for 15 min on ice with occasional vortexing. After centrifugation and washing of the nuclei pellet, 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 dithiothreitol and protease inhibitors] was added and incubated at 4°C for 40 min with constant shaking. Nuclear extracts were isolated by centrifugation at 14,000 rpm for 30 min and the protein content in aliquots was determined by Bradford assay (Bradford, 1976). Nuclear extracts were stored at −70°C until use for EMSA. The oligonucleotide probe used for EMSA contained the NF-κB consensus sequence. Double-stranded NF-κB consensus sequence was obtained from Bioneer (Chungbuk, Korea) and used for radioactive labeling after annealing. The sequences of probes used in this work are shown as follows (binding site underlined).

NF-κBU 5′-AGC-TTG-GGG-ACT-TTC-C-3′

NF-κBL 3′ − C-CCC-TGA-AAG-GTC-GGC-5′.

One nanomole of each oligonucleotide was annealed by heating at 95°C for 5 min, cooled slowly to 30°C, and diluted to 1.75 pmol/μl. Oligonucleotide probe was labeled with α-[^{32}P]dATP using Klenow fragment (BM). The total volume of the labeling mixture was 25 μl and the composition of the labeling mixture was as follows: 7 pmol oligomer (DNA probe), 0.4 mM dNTPs (w/o dATP), labeling buffer (50 mM Tris–HCl, pH 8.0, 50 mM NaCl and 10 mM $MgCl_2$), 4 μl of α-[^{32}P]dATP (> 3000 Ci/mmol, 250 Ci/25 μl), and 1 μl of Klenow fragment (1 U/μl). The labeling reaction was performed for 40 min at 37°C 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 dithiothreitol, 10% glycerol, and 1 μg/ml of leupeptin, pepstatin and aprotinin) containing 1 μg of Poly[dI–dC · dI–dC] and 100,000 cpm [^{32}P]-labeled probe. The specificity of the binding reaction was confirmed by competition assay with a 100–200 fold molar excess of unlabeled oligonucleotide probe. DNA–protein complex was separated from unbound probe on native 6% polyacrylamide gels in 0.25X-TBE running buffer. After electrophoresis, the gel was vacuum-dried and autoradiographed by exposure to X-ray film at −70°C for 16–24 h.

3. Results

3.1. β-Carboline derivatives (C-1, C-2) decrease nitrite production and citrulline formation in RAW 264.7 cells

In order to evaluate the iNOS inhibitory capacities of C-1 and C-2, nitrite accumulation and citrulline formation were measured in lipopolysaccharide/interferon-γ-

stimulated RAW 264.7 cells. Thus, C-1 or C-2 was added to the culture media immediately prior to stimulation of RAW 264.7 cells with lipopolysaccharide/interferon-γ and the cells were incubated for 18 h. Thereafter, iNOS activity in the cell-free extract and nitrite accumulation in the culture medium were determined. As shown in Fig. 2, C-1 and C-2 inhibited nitrite accumulation and [^{3}H]L-citrulline formation in a concentration-dependent manner. Furthermore, C-1 had a more potent inhibitory effect on iNOS activity than C-2, and the inhibitory capacity determined by citrulline formation was a little higher than that determined by nitrite accumulation (Fig. 2B vs. A). The approximate IC_{50} value of C-1 was less than 2 μM and that of C-2 was 2.8 μM in citrulline formation assay.

3.2. Two β-carboline derivatives (C-1, C-2) do not directly inhibit catalytic activity of iNOS

Since the result described in Fig. 2 reflects the cumulative consequence of iNOS expression and its activity, we investigated whether the inhibition by C-1 and C-2 of iNOS was due to a direct effect on the catalytic activity of the enzyme. We, therefore, directly assayed NOS activity by citrulline formation with cell-free extracts of RAW 264.7 cells stimulated with lipopolysaccharide (1 μg/ml) and interferon-γ (10 U/ml) for 18 h in the absence of the inhibitors. Unlike L-NMA, which is a well-known NOS inhibitor, neither C-1 nor C-2 had a significant inhibitory effect on iNOS activity (Fig. 3). They also did not significantly inhibit rat brain NOS activity (data not shown).

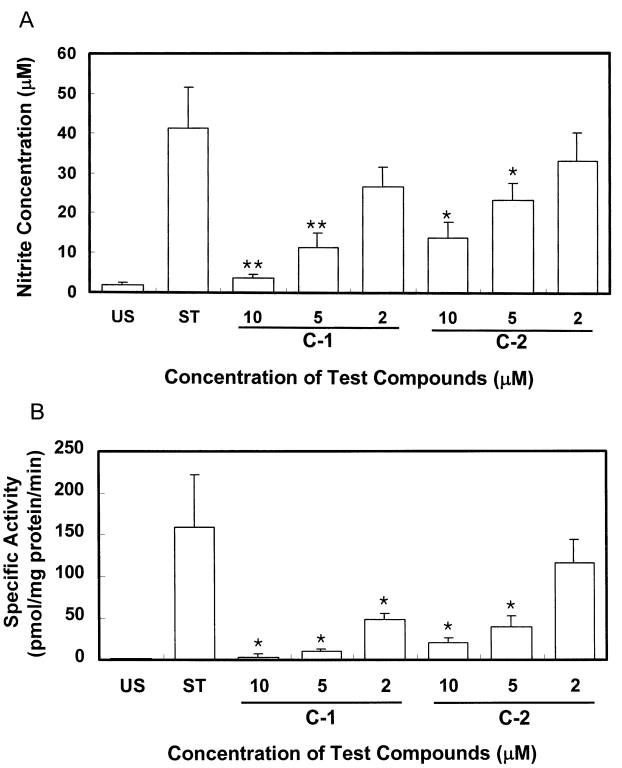

Fig. 2. Effects of C-1 and C-2 on nitrite accumulation and iNOS activity in stimulated RAW 264.7 cells. C-1 or C-2 was added to RAW 264.7 cells in 100-mm culture dishes immediately prior to the addition of lipopolysaccharide and interferon-γ, and the cells were incubated for 18 h. Nitrite accumulation (A) was determined by Griess reaction in culture medium and iNOS activity (B) was measured by [^{3}H]L-citrulline formation with cell-free extracts. US and ST represent unstimulated and lipopolysaccharide/interferon-γ-stimulated cells, respectively. Average ± S.D. values were obtained from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from induced control cells, analyzed by Student's *t*-test.

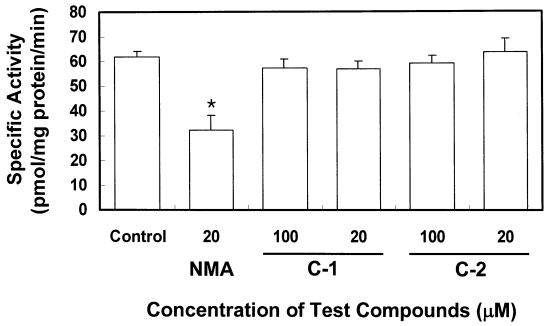

Fig. 3. Effects of C-1 and C-2 on iNOS activity in cell-free extracts of stimulated RAW 264.7 cells. Cell lysate was prepared from RAW 264.7 cells stimulated for 18 h with 1 μg/ml of lipopolysaccharide and 10 U/ml of interferon-γ. iNOS activity was measured by [^{3}H]L-citrulline formation in cell lysate with or without addition of L-NMA, C-1 or C-2. Average ± S.D. values were obtained from three separate experiments. $^{*}P < 0.05$, significantly different from buffer control, analyzed by Student's *t*-test.

3.3. Effects of C-1 and C-2 on iNOS protein and mRNA expression

Since both C-1 and C-2 inhibited iNOS when they were present together with stimulants, but had no inhibitory effect on iNOS or nNOS in vitro, we investigated iNOS protein and mRNA expression in order to elucidate the mechanism of inhibition of these two compounds (Fig. 4).

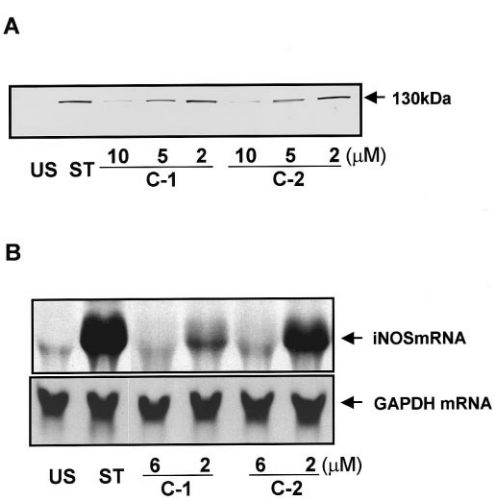

Fig. 4. Effects of C-1 and C-2 on the expression of iNOS. RAW 264.7 cells were stimulated with lipopolysaccharide (1 μg/ml)/interferon-γ (10 U/ml) and incubated for 18 h in the presence of test compounds. Western blot analysis (A) and Northern blot analysis (B) were carried out as described in Section 2. US — unstimulated, ST — stimulated.

Although hardly detected in unstimulated RAW 264.7 cells by Western blot analysis, iNOS protein was sufficiently expressed after stimulation with lipopolysaccharide (1 μg/ml)/interferon-γ (10 U/ml) for 18 h, and the presence of C-1 and C-2 in lipopolysaccharide/interferon-γ-stimulated cell cultures markedly decreased iNOS expression in a concentration-dependent manner (Fig. 4A). This result indicated that the decreased activity of iNOS by these two compounds could have resulted from the inhibition of iNOS protein expression. We then performed Northern blot analysis and found that both C-1 and C-2, decreased concentration dependently the steady state levels of iNOS mRNA (Fig. 4B).

3.4. C-1 and C-2 decrease lipopolysaccharide-induced prostaglandin E_2 production in RAW 264.7 cells, but do not inhibit cyclooxygenase-2 activity

Prostaglandin E_2 was measured in the supernatant of cultured RAW 264.7 cells stimulated with lipopolysaccharide (1 μg/ml) for 16 h in the presence of test compounds. As seen in Fig. 5A, while unstimulated cells synthesized 3–5 ng of prostaglandin E_2, lipopolysaccharide-stimulated cells had an about 15-fold increased prostaglandin E_2 synthesis. Since the cells had been pretreated with acetylsalicylic acid to irreversibly inactivate cyclooxygenase by acetylation, this increased prostaglandin E_2 production indicates increased de novo synthesis of cyclooxygenase-2 enzyme. However, C-1 and C-2 inhibited prostaglandin E_2 synthesis in lipopolysaccharide-stimulated RAW 264.7 cells concentration dependently, with approximate IC_{50} values of 0.39 and 1.39 μM, respectively. Indomethacin, a potent competitive inhibitor of cyclooxygenase at 0.03 μM, also blocked the prostaglandin E_2 production in response to lipopolysaccharide. In contrast to the significant concentration-dependent decrease of prostaglandin E_2 production by indomethacin (Table 1), there was no significant difference in prostaglandin E_2 production between the treated and non-treated cells when the cells were treated with these compounds after being fully stimulated with lipopolysaccharide. This result also demonstrates that these two compounds do not inhibit cyclooxygenase-2 activity.

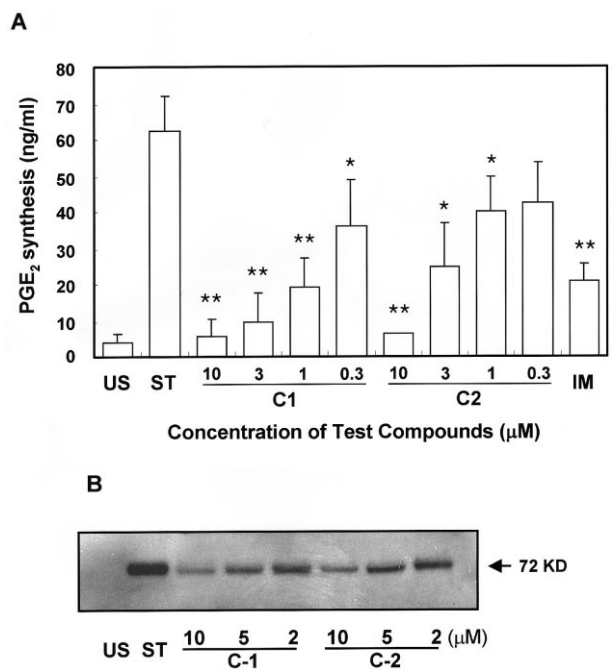

Fig. 5. Effects of C-1 and C-2 on the induction of cyclooxygenase-2 activity and cyclooxygenase-2 protein in lipopolysaccharide-stimulated RAW 264.7 cells. The cells were first treated with acetylsalicylic acid (500 μM) to inactivate cyclooxygenase followed by replacement of the medium with fresh medium together with lipopolysaccharide. The cells were further incubated in the absence or presence of test compounds. Detailed procedures for cell culture and cyclooxygenase-2 assay are described in Section 2. (B) To measure the level of cyclooxygenase-2 protein, the cells were stimulated with lipopolysaccharide (1 μg/ml) for 18 h in the presence of test compounds and analyzed by Western blot analysis as described in Section 2. US — unstimulated, ST — stimulated, IM — indomethacin 0.03 μM. Data are average ± S.D. values of three separate experiments and duplicate determinations were made for each experiment. $^{*}P < 0.05$, $^{*}P < 0.01$, significantly different from stimulated control.

Table 1
Effects of C-1 and C-2 from *M. azedarach* on prostaglandin E_2 generation in LPS-stimulated RAW 264.7 cells

LPS and arachidonic acid (AA) treatment of cells[a]		Test materials (μM)	PGE_2 synthesis (ng/ml)[b]
None	AA	DMSO	2.2 ± 0.3
LPS	AA	DMSO	26.3 ± 6.5
		Indomethacin	
		2	4.5 ± 1.9[c]
		0.4	6.8 ± 5.5[c]
		0.08	10.2 ± 3.2[c]
		C-1	
		30	34.1 ± 6.8
		10	33.6 ± 5.0
		5	25.3 ± 0.7
		C-2	
		30	36.1 ± 7.2
		10	28.9 ± 4.1
		5	27.2 ± 3.6

[a] RAW 264.7 cells were stimulated with LPS (1 μg/ml) for 16 h After removal of LPS by replacement with fresh medium, C-1, C-2 or indomethacin was added and equilibrated for 20 min. The cells were incubated further with arachidonic acid (10 μM) for 13 min, and then the prostaglandin E_2 (PGE_2) content was determined in the culture medium.

[b] Data are average ± S.D. values of four independent experiments and duplicate determinations were made for each experiment.

[c] $P < 0.01$, significantly different from buffer control, analyzed by Student's *t*-test.

3.5. Effects of C-1 and C-2 on cyclooxygenase-2 protein expression

To assess whether de novo protein synthesis is involved in the inhibition of cyclooxygenase-2 by C-1 and C-2,

Western blot analysis was performed with RAW 264.7 cells treated with these two compounds together with lipopolysaccharide stimulation for 18 h. Fig. 5B shows that these compounds inhibited lipopolysaccharide-induced cyclooxygenase-2 protein expression in a concentration-dependent manner. This result is in good agreement with the above results that C-1 and C-2 decreased cyclooxygenase-2 induction by lipopolysaccharide, but did not inhibit cyclooxygenase-2 activity.

3.6. Effect of C-1 and C-2 on activation of transcription factor NF-κB

Since the activation of NF-κB is critical for the induction of iNOS or cyclooxygenase-2 by lipopolysaccharide or other inflammatory cytokines, EMSA was performed in order to examine whether the compounds suppress NF-κB activation. Thus, the nuclear binding assay of NF-κB was carried out with nuclear extracts obtained from RAW 264.7 cells stimulated with lipopolysaccharide (1 μg/ml)/interferon-γ (10 U/ml) for 1 h in the presence or absence of the test compounds. Stimulation with lipopolysaccharide/interferon-γ markedly activated NF-κB (Fig. 6). As seen in the lane of competition, DNA binding of NF-κB was completely blocked by the addition of a 100–200 fold molar excess of unlabeled oligonucleotide NF-κB DNA probes, which indicated the specificity of the binding reaction. In contrast, C-2 showed only a slight inhibition at 6 μM, whereas C-1 markedly decreased the lipopolysaccharide/interferon-γ stimulation of DNA binding of NF-κB.

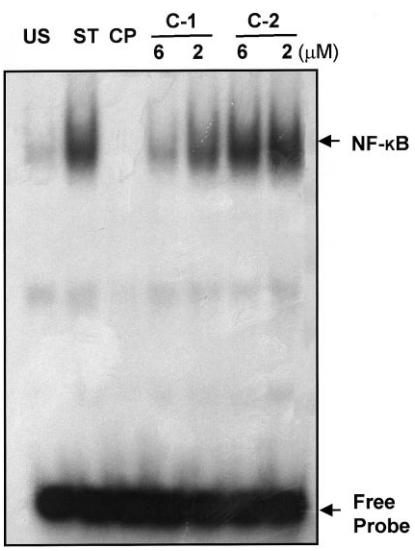

Fig. 6. Effect of C-1 and C-2 on the activation of NF-κB in RAW 264.7 cells. The cells were stimulated with 1 μg/ml lipopolysaccharide plus 10 U/ml interferon-γ for 1 h after a 1-h pretreatment with test compounds. Nuclear extracts were subjected to EMSA as described in Section 2. US — unstimulated, ST — stimulated, CP — competition.

4. Discussion

Since increased NO production is associated with acute and chronic inflammation and the intracellular level of iNOS plays a central role in determining NO production rates in activated macrophages and several other cell types, iNOS regulation became an important target in the treatment of inflammation (Szabo and Thiemermann, 1995; MacMicking et al., 1997). Therefore, in the present study, we investigated two β-carboline alkaloids, C-1 and C-2, isolated from *M. azedarach* for their potential therapeutic value by determining their effect on iNOS.

While a number of plant materials have been reported to inhibit iNOS activity by suppressing iNOS expression, but not by directly inhibiting enzyme activity (Yu, 1994; Dirsch et al., 1997; Jun et al., 1998), some phytochemicals having anti-oxidative properties, such as procyanidines (Virgili et al., 1998), catechins (Chan et al., 1997) and *Ginko biloba* extract (Kobuchi et al., 1997), cause inhibition by a dual mechanism, namely suppression of iNOS expression and direct inhibition of iNOS catalytic activity. However, they inhibit iNOS activity only at relatively high concentrations of more than 100 μM. Antioxidant molecules are also known to modulate the gene expression of iNOS directly by affecting transcription factors (Suzuki et al, 1997) or indirectly by modulating the cellular redox status (Suzuki and Packer, 1993).

The results presented in this study showed that treatment of RAW 264.7 cells, which were activated with lipopolysaccharide/interferon-γ, with C-1 or C-2 potently decreased [^{3}H]L-citrulline formation as well as nitrite accumulation (Fig. 2). However, these compounds did not directly inhibit the iNOS activity of fully activated RAW 264.7 cells (Fig. 3) and rat brain NOS, a kind of cNOS (data not shown). Therefore, the results strongly suggested that the inhibitory effects of these compounds in lipopolysaccharide/interferon-γ-activated RAW 264.7 cells were mediated via a reduction of iNOS mRNA expression rather than a direct effect on the catalytic activity of iNOS. This suggestion was also strengthened by evidence that iNOS protein and mRNA levels were reduced by these compounds, as analyzed by Western and Northern blot, respectively (Fig. 4).

It is known that the inactive NF-κB normally binds to IκB 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, which induce increased protein phosphorylation and proteolysis of IκB protein (for reviews, see Ghosh et al., 1998). The free and activated NF-κB is in turn translocated into the nucleus to bind to relevant DNA sites on the promoter region of genes. Accordingly, there may be a number of points for suppression of NF-κB activation, such as signal cascade, protein kinases and redox state in the cytoplasm. NF-κB is also a crucial transcription factor for mRNA expression of iNOS (Xie et

al., 1993, 1994) and its response elements are on the promoter regions of iNOS (Barnes and Karin, 1997; Crofford et al., 1997). To identify the effect of C-1 and C-2 on NF-κB, EMSA was carried out. The DNA-binding activity of NF-κB was significantly decreased by treatment with C-1 and C-2 for 2 h subsequent to stimulation with lipopolysaccharide (Fig. 6). However, further investigation is needed to discern how C-1 and C-2 suppress NF-κB activation and which components of NF-κB are suppressed.

Cyclooxygenase is the key enzyme in the prostaglandin synthetic pathway, and the prostaglandins belong to a group of bioactive mediators that modulate a number of cellular functions. Like NOS, cyclooxygenase also exists in both constitutive (cyclooxygenase-1) and inducible (cyclooxygenase-2) forms. Both NO and prostaglandins are involved in inflammatory processes and regulate each other either positively or negatively (Tetsuka et al., 1994; Kanematsu et al., 1997). NO has been shown to stimulate cyclooxygenase-2 activity via the heme component, which binds to the active site of the cyclooxygenase-2 enzyme (Salvemini et al., 1993, 1994; Corbett et al., 1993; Franchi et al., 1994). In contrast to their positive effect on cyclooxygenase-2 activity, prostaglandins, cyclooxygenase-2 products, have been demonstrated to downregulate iNOS induction (Tetsuka et al., 1994), thus demonstrating cross-talk between the cyclooxygenase-2 and NOS pathways. Here, we tested C-1 and C-2 for their ability to suppress the enzyme activity and de novo synthesis of cyclooxygenase-2 in lipopolysaccharide-stimulated RAW 264.7 cells. Our results showed that C-1 and C-2 inhibited prostaglandin E_2 biosynthesis in the presence of stimulators and that this inhibition was due to the inhibition of enzyme induction, and not to the inhibition of cyclooxygenase-2 activity (Fig. 5 and Table 1). The effects of C-1 and C-2 on the expression of cyclooxygenase-2 protein could be partially due to the inhibition of NF-κB activity because NF-κB response elements are reported to be on the promoter region of cyclooxygenase-2 (Barnes and Karin, 1997; Crofford et al., 1997) and NF-κB is known to be crucial for the transcription of the cyclooxygenase-2 gene in murine macrophages (Xie et al., 1993, 1994).

In conclusion, the study described herein suggests that two β-carboline alkaloids, C-1 and C-2, from *M. azedarach* suppress NO synthesis in lipopolysaccharide/interferon-γ-activated RAW 264.7 cells through the inhibition of iNOS protein expression due to decreased mRNA transcription. Furthermore, the inhibition of mRNA transcription of iNOS is, at least in part, associated with the inhibition of NF-κB activation.

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