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β-CARBOLINE ALKALOID SUPPRESSES NF-κB TRANSCRIPTIONAL ACTIVITY THROUGH INHIBITION OF IKK SIGNALING PATHWAY

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Nuclear factor (NF)-κB transcription factors play an evolutionarily conserved and critical role in the triggering and coordination of both innate and adaptive immune responses. Therefore, there is intense interest in understanding the regulation of this transcription factor in the context of various diseases. Studies investigated the suppression mechanism of NF-κB signaling pathways by a β-carboline alkaloid (C-1) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. β-Carboline alkaloid decreased the level of inducible nitric oxide sythase (iNOS) protein and NOS promoter activities in a concentration-dependent manner. This effect was accompanied by the reduction of NF-κB DNA binding activity as well as NF-κB nuclear translocation. In addition, β-carboline alkaloid reduced the degradation and phosphorylation of IκB, and attenuated IKK activity in LPS-stimulated RAW 264.7 cells. Taken together, these results indicate that β-carboline alkaloid has the capability to suppress NF-κB signaling pathway through inhibition of IKK activity in LPS-stimulated RAW 264.7 cells.

The inflammatory response involves the sequential release of mediators and the recruitment of circulating leukocytes, which become activated at the inflammatory site and release further mediators. This response is self-limiting and resolves through the release of endogenous anti-inflammatory mediators and the clearance of inflammatory cells. Current approaches to the treatment of inflammation rely on the inhibition of proinflammatory mediator production and mechanisms that initiate the inflammatory response. Nuclear factor (NF)-κB is transcription factor that acts as a central mediator for the human immune response. It has been reported that NF-κB plays an important role in immune and inflammatory responses through the regulation of genes encoding proinflammatory cytokines, adhesion molecules, chemokines, growth factors,

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and inducible enzymes such as cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) (Karin & Ben-Neriah, 2000; Vane et al., 1994; Tak & Firestein, 2001). Since NF-κB has been known to be involved in inflammation, NF-κB activation has been used as a molecular target for the screening of anti-inflammatory activity of compounds (Kang et al., 2000). In this regard, among the natural products assayed, various compounds isolated form natural products have been described as potent inhibitors of NF-κB activation in response to proinflammatroy stimulation (Kang et al., 2001; Wong et al., 2002).

In previous study, β-carboline alkaloids, 4,8-dimethoxy-1-vinyl-β-carboline, and 4-methoxy-1-vinyl-β-carboline, isolated from *Melia azedarach*, were found to inhibit iNOS activity and the levels of iNOS protein and mRNA in a dose-dependent manner. In addition, production of prostaglandin E_2, levels of COX-2 protein, and NF-κB DNA binding activity were reduced by these compounds (Lee et al., 2000). However, the inhibitory mechanism of β-carboline alkaloid on the signaling pathway for NF-κB activation is not known. The activation of NF-κB is regulated by inhibitory proteins of the IκB family, which retains NF-κB in the cytoplasm. The activation of NF-κB requires sequential phosphorylation, ubiquitination, and degradation of IκB, as well as the consequent exposure of a nuclear localization signal on the NF-κB molecule (Hatada et al., 2000; Je et al., 2004). Multiple kinases were found to phosphorylate IκB at specific amino-terminal serine residues (Karin, 1999; Karin & Delhase, 2000). The most studied kinases are IκB kinase, IKKα and IKKβ (Karin et al., 2004). Evidence also supports the fact that IKKα and IKKβ are themselves phosphorylated and activated by one or more upstream activating kinase. One such upstream kinase, NF-κB inducing kinase (NIK), was identified (Woronicz et al., 1997). Phosphorylation of IκB by the IKK pathway eventually leads to the nuclear translocation of NF-κB, which activates the expression of target genes in the nucleus (Delhase et al., 1999). Although the regulatory mechanism of NF-κB activation has been recently studied, it is still a matter of debate.

Studies were thus undertaken to investigate the inhibitory mechanism of NF-κB activation by β-carboline alkaloid by examining its effects on NF-κB DNA binding activity, NF-κB nuclear translocation, IκB degradation/phosphorylation, and IKK activity in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

MATERIALS AND METHODS

Reagents

β-Carboline alkaloid (4,8-dimethoxy-1-vinyl-β-carboline; C-1) was isolated from *Melia azedarach* L. var *japonica* (Lee et al., 2000). 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). Both α- and γ-^{32}P-labeled dATP (3000 Ci/mmol) were obtained from Amersham International (Amersham, Bucks., UK) and [^{14}C]chloramphenicol (50 mCi/mmol) was from NEN Life Science Products (Boston). Klenow DNA polymerase was

obtained from Gibco BRL Life Biotechnologies (Grand Island, NY). p65 NF-κB and IKK α/β antibodies were purchased from Santa Cruz (Santa Cruz, CA). IκB, phospho-IκB, phospho-IKK, phospho-Erk, and phospho-p38 antibodies were purchased from Cell Signaling Technology (USA). Horseradish peroxidase-conjugated anti-rabbit antibody was obtained from Zymed Laboratories (USA).

Cell Culture and Cell Stimulation

The murine macrophage/monocyte cell line RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC) and maintained at 37°C in humidified 5% CO_2/95% air in DMEM containing 10% fetal bovine sarum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated at a density of 60% in culture dish and then cultured in DMEM for 1 or 2 d before experiment. For stimulation, the medium were replaced by DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and LPS was added at a final concentration of 10 μg/ml in the presence or absence of β-carboline alkaloid for the indicated periods.

Western Blot Analysis

Forty microliters of cell-free cytosolic extracts containing an equal amount of protein (40 μg) were loaded and electrophoresed onto sodium dodecyl sulfate (SDS)–polyacrylamide gel. Proteins were separated electrophoretically and transferred to a polyvinyldifluoride (PVDF) membrane using a Semi-Dry transfer system. Membrane was blocked for 30 min in TBS containing 0.1% Tween 20 (TBS-T) and 5% (w/v) dry skim milk powder, washed with TBS-T, and incubated overnight with primary antibodies (1:2000 dilution in TBS-T) at 4°C. The membrane was then washed with TBS-T and incubated for 2 h with HRP-conjugated secondary antibody (1:5000 dilution in TBS-T) at room temperature. The immunoblot was visualized using Amersham ECL-film.

Electrophoretic Mobility Shift Assay (EMSA) of NF-κB

Nuclear proteins were extracted by the modification of Andrews and Faller (1991). All the procedures for nuclear protein extraction were performed at 0 to 4°C with ice-cold reagents. Cells were washed twice with phosphate-buffered saline (PBS) and harvested by scraping into 1 ml PBS, and pelleted at 400 × g for 5 min. The pellet was resuspended in 1 ml of ice-cold hypotonic buffer containing 10 m*M* Tris-HCl (pH 7.4), 3 m*M* $CaCl_2$, and 2 m*M* $MgCl_2$. After centrifugation at 1200 × g for 10 min, the pelleted nuclei were resuspended with 50 μl ice-cold hypertonic extraction buffer containing 20 m*M* HEPES-KOH (pH 7.9), 25% (w/v) glycerol, 420 m*M* NaCl, 0.2 m*M* ethylenediamine tetraacetic acid (EDTA), 1.5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), and protease inhibitors and incubated at 4°C for 40 min with constant shaking. Nuclear extracts were isolated by centrifugation at 20,000 × g for 30 min. Protein concentrations were determined by Bradford (1976) assay and then aliquots of

nuclear extracts were frozen at −70°C. The oligonucleotide probe used for EMSA contained the NF-κB consensus sequence. Double-stranded NF-κB consensus sequence was obtained from Bioneer Corp. and used for radioactive labeling after annealing. The sequences of probe 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-GGG-5′**

One nanomole of each oligonucleotide in annealing buffer containing 10 m*M* Tris-HCl (pH 8), 50 m*M* NaCl, 10 m*M* $MgCl_2$, and 0.1 m*M* EDTA was heated at 95°C for 10 min in water bath, cooled slowly to 30°C for 2 h and diluted to 2 pmol/μl. The oligonucleotide probe was labeled with α-[^{32}P]dATP using Klenow fragment. Total volume of labeling mixture was 25 μl as follows: 2 pmol oligomer (DNA probe), 0.4 m*M* dNTPs (without dATP), labeling buffer containing 50 m*M* Tris-HCl (pH 8), 50 m*M* NaCl, and 10 m*M* $MgCl_2$, 4 μl α-[^{32}P]dATP (>3000 Ci/mmol, 250 Ci/25 μl), 1 μl Klenow fragment (1 U/μl). 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 for 30 min at room temperature with 5 μg nuclear protein in 20 μl binding buffer containing 10 m*M* HEPES-KOH (pH 7.7), 2.5 m*M* $MgCl_2$, 1 m*M* DTT, 10% glycerol (w/v), protease inhibitors, 1 μg/ml Poly dI-dC, and 100,000 cpm ^{32}P-labeled probe. DNA–protein complex was separated from unbound probe on native 6% polyacrylamide gels at 150 V constant in 0.25× TBE running buffer. After electrophoresis, the gel was vacuum-dried and autoradiographed by exposure to x-ray film at −70°C for 24 h.

Immunofluorescence Microscopy of NF-κB

RAW 264.7 cells were plated on 22 × 22 mm round glass coverslips at 1×10^4 cells/12-well culture plate in DMEM medium and incubated at 37°C until cells were 60% confluent. After being pretreated with various concentrations of β-carboline alkaloid for 1 h, the cells were stimulated with 10 μg/ml LPS for indicated times. After cells were washed twice with PBS, cells were permeabilized in PBS containing 0.2% Trixton X-100 and 5% bovine serum albumin (BSA) for 5 min on ice. The coverslips were washed with blocking solution (PBS containing 5% BSA), and 30 μl NF-κB primary antibody (1:200 dilution in blocking solution) was placed on each coverslip. The slide was then placed in the humidified chamber and incubated at room temperature for 2 h. Coverslips were washed with blocking solution and then incubated in FITC-conjugated secondary antibody (1: 200 dilution in blocking solution) at room temperature for 1 h in a dark humidified chamber. On coverslips washed with blocking solution, bound antibodies were detected using fluorescence microscope.

Transfection of iNOS-CAT Reporter Gene and Chloramphenical Acetyltransferase (CAT) Assay

RAW 264.7 cells were plated at 1×10^6 cell/60-mm culture dish in DMEM medium and incubated at 37°C until cells were 60% confluent. For iNOS promoter analysis, cells were transfected with 2 μg/culture dish of iNOS-CAT reporter plasmid DNA using superfect reagent according to the manufacturer's recommendations. Twenty-four hours after the transfection, the medium was changed to a fresh medium and then cells were treated with β-carboline alkaloid and 10 μg/ml LPS for 24 h. The CAT activity was measured according to the CAT enzyme assay kit (Promega, Madison, WI). Briefly, the cells were washed twice with Mg^{2+}- and Ca^{2+}-free PBS buffer (pH 7.1) and then lysed by 1× lysis buffer. After centrifugation at 15,000 × g for 10 min, supernatant was used for CAT enzyme sources. Enzyme reaction was performed for 8 h at 37°C with 100 μg enzyme source in 125 μl reaction mixture containing 3 μl [^{14}C]chloramphenicol (50 mCi/mmol), 5 μl *n*-butyryl CoA. Enzyme reaction were terminated with 500 μl ethyl acetate and then samples were vortexed for 1 min before centrifugation at top speed in a microcentrifuge for 3 min to achieve good phase separation. The upper organic phase was transferred to a fresh tube and evaporated to dryness and then resuspended with 30 μl ethyl acetate. Ten microliters of sample was spotted onto a silica-gel thin-layer chromatography (TLC) plate and dried. The TLC plate was placed in a developing chamber and pre-equilibrated for 1 h with chloroform/methanol (97:3). After developing, the TLC plate was dried at room temperature and then autoradiographed by exposure to x-ray film at −70°C for 3 d.

Transfection of NF-κB-Luc Reporter Gene and Luciferase Assay

For NF-κB promoter analysis, cells were transfected with 1 μg/p well of NF-κB-Luc reporter plasmid DNA using superfect reagent according to the maufacturer's recommendations. The medium was replaced with fresh medium 24 h after the transfection, and then cells were treated with the variety of concentrations of β-carboline alkaloid and 10 μg/ml LPS for 24 h. Cell lysates were collected for the luciferase assay. The luciferase activities of the cell lysates were measured according to the luciferase assay kit manual (Promega, Madison, WI.). Luciferase activities were normalized for the amount of the protein in cell lysates.

Immunoprecipitation and IKK Assay

After pretreatment with β-carboline alkaloid for 1 h, the cells were stimulated with 10 μg/ml LPS for indicated times. The cells were rinsed twice with an ice-cold wash buffer solution containing 50 m*M* Tris-HCl (pH 7.5), 120 m*M* NaCl, 20 m*M* NaF, 1 m*M* EDTA, 5 m*M* ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 m*M* sodium pyrophosphate, 30 m*M* *p*-nitrophenyl phosphate, 1 m*M* benzylamidine, and 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF), and then harvested with 500 μl buffer containing

20 m*M* Tris-HCl (pH 7.5), 200 m*M* NaCl, 1% Triton X-100 (w/v), 1 m*M* DTT, 0.5 μ*M* PMSF, and protease inhibitors. After incubating for 10 min on ice, samples were vigorously vortexed before centrifuging at 15,000 × g for 15 min. The supernatants were used as an enzyme source and then aliquots of cell extracts were frozen in a deep freezer at −70°C. One milligram of cell extract was incubated with 2 μg IKKα antibody on a rotating platform plate at 4°C for 2 h. Immunoprecipitation was facilitated by the addition of 50 μl of 6% protein A-Sepharose at 4°C for 1 h on a rotating platform plate. The beads were washed twice at 4°C with an TNT buffer and kinase buffer containing 20 m*M* HEPES-KOH (pH 8), 10 mM $MgCl_2$, 100 μ*M* sodium *ortho*-vanadate, 20 m*M* β-glycerophosphatate, 50 m*M* DTT, 0.5 μ*M* PMSF, and protease inhibitors. Total volume of reaction mixture was 25 μl as follows: immunoprecipitate, 1 μg GST-IκB, 1 μl γ-[^{32}P]-ATP (> 3000 Ci/mmol, 250 Ci/25 μl), 3 μl of 1 m*M* ATP in kinase buffer. Enzyme reaction was performed for 40 min at 30°C and then reaction mixtures were loaded on 15% SDS–PAGE. After electrophoresis, the gel was vacuum-dried and autoradiographed by exposure to x-ray film at −70°C for 2 d.

RESULTS AND DISCUSSION

β-Carboline Alkaloid Inhibits the NF-κB DNA-Binding Activity in LPS-Stimulated RAW 264.7 Cells

In previous study, β-carboline alkaloid was found to reduce levels of proteins and mRNA of iNOS and COX-2 (Lee et al., 2000). NF-κB is a key component of innate immunity (Hoffmann et al., 1999), promoting the expression by macrophages of a set of genes involved in host defense, such as proinflammatory cytokines (Ghosh et al., 1998; Baeuerle, 1998), iNOS, COX-2, cell adhesion molecules, and various matrix metalloproteinases (MacMicking et al., 1997; Li et al., 1997; Rao, 2000). To better characterize the mechanisms involved in the inhibition exerted by β-carboline alkaloid on iNOS and COX-2 expression, NF-κB DNA binding activity was analyzed by EMSA. While stimulation of RAW 264.7 cells with LPS for 1 h markedly increased the DNA binding activity of NF-κB, pretreatment with β-carboline alkaloid before treatment with LPS blocked the NF-κB DNA binding activity in a concentration-dependent manner (Figure 1A).

It was reported that DNA binding activity of active NF-κB can be directly modified by sesquiterpene lactones (Lyss et al., 1998; Han et al., 2001). Therefore, studies determined whether β-carboline alkaloid could also directly modify DNA binding activity of active NF-κB. Nuclear extracts of RAW 264.7 cells stimulated with LPS for 1 h were pooled and incubated with various concentrations of test compound. As shown in Figure 1B, DNA binding activity of active NF-κB was not affected by β-carboline alkaloid, indicating that the in vivo inhibitory effect might be attributed to the interruption of upstream events rather than direct inhibition of DNA binding activity of NF-κB.

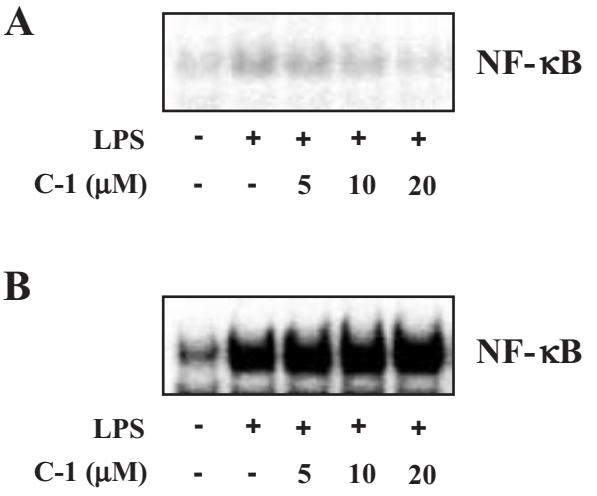

FIGURE 1. Effects of β-carboline alkaloid on DNA binding activity of NF-κB in LPS-stimulated RAW 264.7 cells. (A) NF-κB DNA binding activity was analyzed by EMSA. RAW 264.7 cells were plated at a density of 1×10^7 cells/100-mm culture dish in DMEM and incubated at 37°C CO_2 incubator. After 1-h pretreatment of β-carboline alkaloid (C-1), the cells were stimulated with 10 μg/ml of LPS for 1 h. The nuclear extracts were subjected to EMSA as described in materials and methods. (B) Cells were pretreated with LPS for 1 h and nuclear extract of the stimulated cells were pooled. These extracts were incubated for 30 min with various concentrations of β-carboline alkaloid. Nuclear proteins were prepared and analyzed for NF-κB DNA binding by EMSA.

iNOS Promoter Activity was Inhibited by β-Carboline Alkaloid in LPS-Stimulated RAW 264.7 Cells

Although β-carboline alkaloid blocked NF-κB DNA binding activity, DNA binding alone is not always sufficient for NF-κB-dependent gene transcription (Rossi et al., 2000). To obtain more direct evidence on the inhibition of NOS gene transcription by β-carboline alkaloid, studies examined the effect of β-carboline alkaloid on the NOS promoter activity using a reporter gene assay. Thus, cells were transiently transfected with the iNOS-CAT plasmid, and CAT activity in cell extract was measured. An almost 20-fold increase in CAT enzyme activity over the control was observed upon stimulation with LPS. LPS stimulation of CAT activity was blocked in a dose-dependent manner by β-carboline alkaloid (Figure 2A). Consistent with the result, expression of luciferase reporter gene with promoter containing NF-κB binding sites was completely inhibited by pretreatment with β-carboline alkaloid (Figure 2B).

β-Carboline Alkaloid Inhibits NF-κB Nuclear Translocation in LPS-Stimulated RAW 264.7 Cells

The results already described led to the assumption that the inhibitory effect of β-carboline alkaloid on NF-κB DNA binding activity might be due

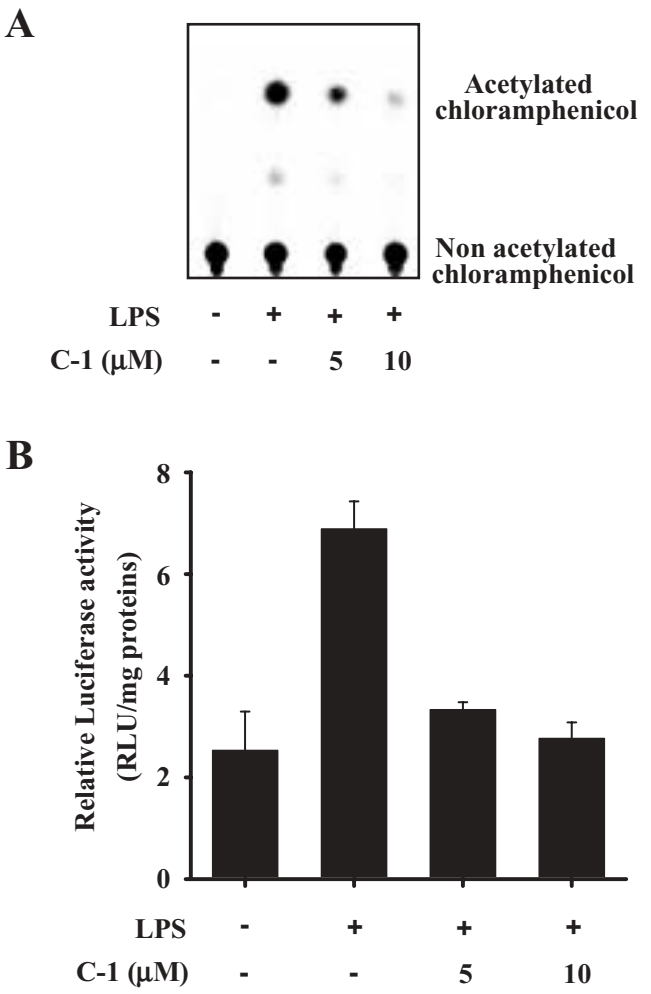

FIGURE 2. Effects of β-carboline alkaloid on iNOS promoter activity in LPS-stimulated RAW 264.7 cells. Cells were transfected with (A) 2 μg/p well of iNOS-CAT reporter plasmid DNA and (B) 1 μg/p well of NF-κB-Luc reporter plasmid DNA, respectively. Cells were treated with β-carboline alkaloid and 10 μg/ml of LPS for 24 h. Cell lysates were collected for the CAT enzyme assay and the luciferase assay. (A) The CAT activity was measured described as materials and methods. (B) Luciferase activities were normalized for the amount of the protein in cell lysates. Data are shown as means (bars, SE) (n = 3).

to inhibition of upstream events resulting in NF-κB activation, such as the release of the inhibitory IκB subunit and the NF-κB nuclear translocation. Thus, the effect of β-carboline alkaloid was determined on the translocation of the subunit p65 of heterodimer of NF-κB using fluorescence microscopy analysis. RAW 264.7 cells were preincubated for 1 h with β-carboline alkaloid and then treated with 10 μg/ml LPS for 1 h at 37°C. Stimulated cells

were fixed and stained with two agents: (1) propidium iodide, which stains the cell nuclei, and (2) FITC-conjugated secondary antibody, which binds to anti-NF-κB p65 antibody. As shown in Figure 3, LPS stimulation resulted in translocation of the p65 NF-κB subunit from the cytosol into the nucleus. However, treatment with β-carboline alkaloid resulted in the reduction of accumulation of p65 NF-κB subunit in the nucleus, indicating that the inhibition of DNA binding activity by β-carboline alkaloid might be attributed to prevention of p65 NF-κB subunit translocation from the cytosol into the nucleus.

β-Carboline Alkaloid Inhibits Degradation and Phosphorylation of IκB-α in LPS-Stimulated RAW 264.7 Cells

In most cell types, NF-κB is sequestered in an inactive, cytoplasmic complex by binding to a group of inhibitory proteins belonging to the IκB family. The active cytoplasmic NF-κB is released from the complex by phosphorylation and proteolytic degradation of IκBα in response to proinflammatory signals, thus unmasking NF-κB nuclear translocation sequences and freeing it to enter the nucleus. To determine whether inhibition of LPS-induced NF-κB activation resulted from the inhibition of IκBα degradation, cells were stimulated

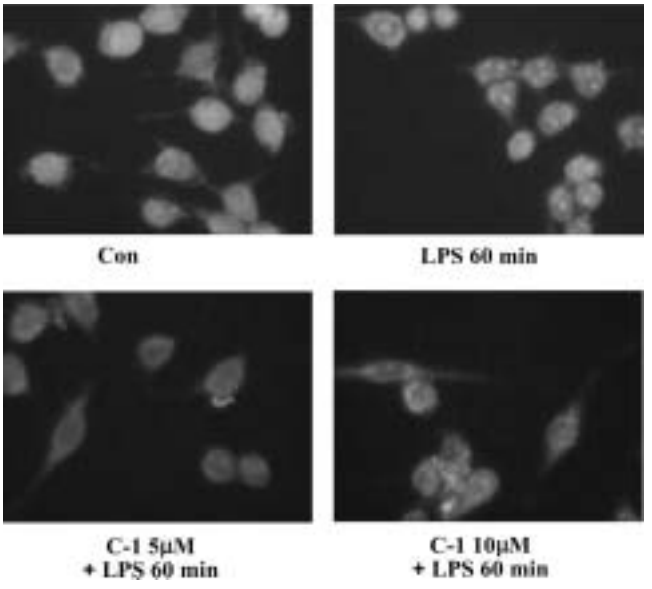

FIGURE 3. Effects of β-carboline alkaloid on NF-κB nuclear translocation in LPS-stimulated RAW 264.7 cells. Distribution of NF-κB proteins was analyzed by immunofluorescence. After 1 h pretreatment with β-carboline alkaloid (C-1), cells were stimulated with 10 μg/ml of LPS for 1 h. Cells were washed and incubated NF-κB primary antibody, and then incubated in FITC-conjugated secondary antibody. After these treatments, bound antibodies were detected using fluorescence microscope.

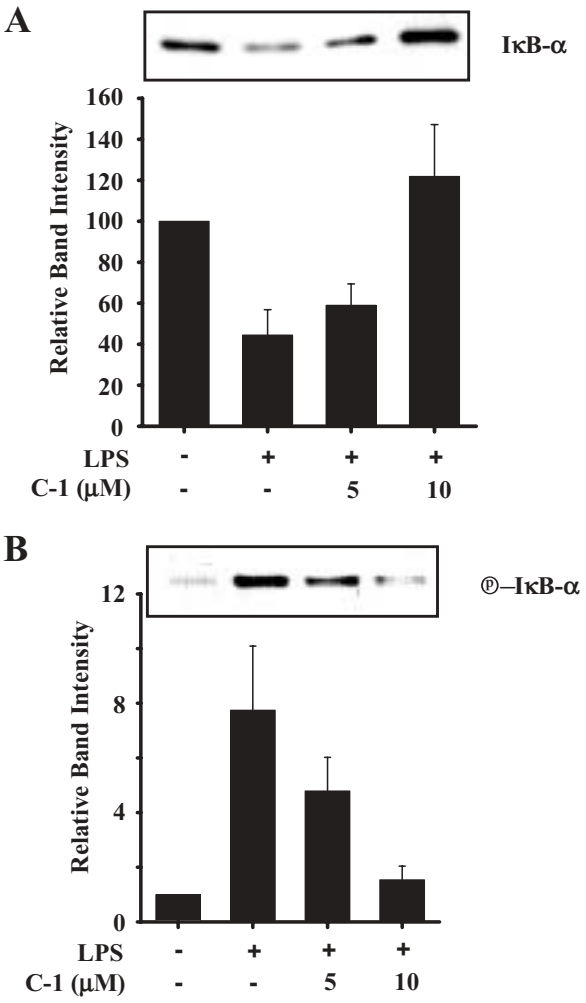

FIGURE 4. Effects of β-carboline alkaloid on degradation and phosphorylation of IκBα in LPS-stimulated RAW 264.7 cells. The RAW 264.7 cells were stimulated with 10 μg/ml of LPS for (A) 45 min or (B) 30 min after pretreatment of (A) β-carboline alkaloid (C-1) or (B) β-carboline alkaloid and MG-132. Cellular extracts were analyzed by Western blotting with (A) anti-IκBα polyclonal antibody and (B) anti-phopho-Ser32-IκB-α antibody. The diagram shows relative band intensity. The data were quantified using densitometry, and the results were based on three independent experiments.

with LPS following pretreatment with the indicated concentrations of β-carboline alkaloid. LPS-induced IκBα degradation was attenuated by pretreatment with β-carboline alkaloid (Figure 4A). Consistent with this result, LPS-induced IκBα phsphorylation was inhibited when cells were pretreated with the indicated concentrations of β-carboline alkaloid (Figure 4B).

β-Carboline Alkaloid Inhibits IKK Activity in LPS-Stimulated RAW 264.7 Cells

To further examine the effect of β-carboline alkaloid on the IKK activity, an immunoprecipitation kinase assay was carried out using GST-IκBα as a substrate. LPS stimulation of IKK activity was attenuated in a concentration-dependent manner by pretreatment with β-carboline alkaloid (Figure 5, upper panel).

It was reported that $15dPGJ_2$ inhibited NF-κB activity as well as IKKβ activity via the formation of Michael adducts involving cysteine 179 (Rossi et al., 2000). To determine whether the inhibitory effect of β-carboline alkaloid on IKK activity was due to direct interaction between β-carboline alkaloid and IKK complex, an in vitro IKK assay was performed. Thus, cell extracts were prepared after stimulation of RAW 264.7 cells with LPS for 10 min and incubated with the indicated concentrations of β-carboline alkaloid. LPS-induced IKK activation was not affected by β-carboline alkaloid, indicating that the effect might be due to the inhibition of upstream molecules of IKK (Figure 5, lower panel).

In conclusion, the study described herein suggests that β-carboline alkaloid suppresses NF-κB in LPS-activated RAW 264.7 cells through the inhibition of NF-κB translocation from the cytosol into the nucleus, resulting from the attenuation of IKK activity and IκBα degradation. Taken together, our studies provide a new insight into molecular mechanisms by which β-carboline alkaloid inhibits NF-κB-dependent expression of genes associated with inflammation, and further suggest that the inhibition of NF-κB may provide a novel approach for the treatment of inflammation.

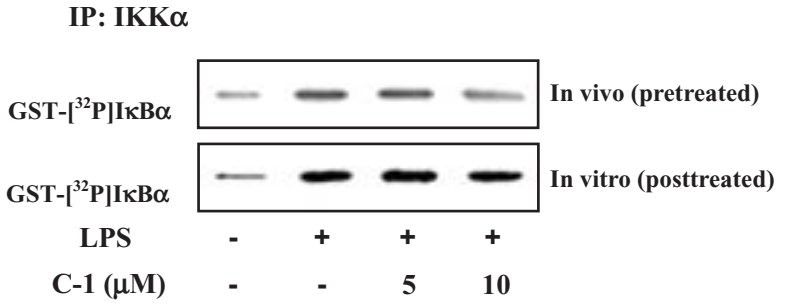

FIGURE 5. Effects of β-carboline alkaloid on IKK activity in LPS-stimulated RAW 264.7 cells. Levels of phosphorylated GST-IκBα were analyzed by immunoprecipitation IKK assay. In upper panel, after 1-h pretreatment of β-carboline alkaloid, the cells were stimulated with 10 μg/ml of LPS for 10 min. In lower panel, cells were stimulated for 10 min with LPS and the cytosol of LPS-simulated cells was pooled. After these treatments, immunoprecipitates were assayed for IKK assays as described in Material and Methods.

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