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Inhibition of lipopolysaccharide-stimulated nitric oxide production in RAW 264.7 macrophages by a synthetic carbazole, LCY-2-CHO

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Abstract

In activated macrophages, large amounts of nitric oxide (NO) are generated by inducible nitric oxide synthase (iNOS). This is an important mechanism in macrophage-induced cytotoxicity and inflammation. In the present study, a synthetic carbazole compound, 9-(2-chlorobenzyl)-9*H*-carbazole-3-carbaldehyde (LCY-2-CHO), was found to have an inhibitory effect on lipopolysaccharide (LPS)-stimulated NO generation in RAW 264.7 macrophages (IC_{50} value of 1.3 ± 0.4 μM). LCY-2-CHO did not induce cytotoxicity and had a negligible effect on iNOS activity. To explore the mechanism of inhibition of NO generation by LCY-2-CHO, the expression of the *iNOS* gene was examined. LCY-2-CHO abolished the LPS-induced expression of both iNOS protein and mRNA in a parallel concentration-dependent manner with IC_{50} values similar to those required for inhibition of NO generation. LCY-2-CHO did not enhance the degradation of iNOS mRNA. In cells transiently transfected with an iNOS promoter-chloramphenicol acetyltransferase (CAT) reporter construct, LCY-2-CHO attenuated the LPS-induced iNOS promoter activity. However, LCY-2-CHO had no effect on the degradation of IκB-α or IκB-β, DNA binding activity, or transcriptional activity of nuclear factor-κB (NF-κB). These results indicate that LCY-2-CHO inhibits NO generation via a decrease in the transcription of iNOS mRNA through a signaling pathway that does not involve NF-κB activation.

Keywords: RAW 264.7; LCY-2-CHO; Nitric oxide; Inducible nitric oxide synthase; NF-κB

1. Introduction

NO plays an important role in the regulation of many physiological functions, such as host defense, neurotransmission, neurotoxicity, and vasodilation [1,2]. Three major types of NOS isoenzymes that catalyze the conversion of L-arginine to NO and L-citrulline have been identified [2,3]. The neural (nNOS) and endothelial (eNOS) isoenzymes are constitutively expressed, and their activity is regulated by intracellular free Ca^{2+} concentrations. Activated macrophages transcriptionally express the inducible (iNOS) isoenzyme [4,5], which is independent of $[Ca^{2+}]_i$ regulation and is responsible for the prolonged and profound production of NO. The physiological or normal generation of NO mediates the bactericidal and tumoricidal actions of macrophages. However, the aberrant release of NO can lead to amplification of inflammation, as well as tissue injury [6–8]. Therefore, inhibition of NO production is a very important therapeutic target in the development of anti-inflammatory agents.

Expression of the *iNOS* gene is regulated at different levels, including transcriptional, posttranscriptional, translational, and posttranslational steps [9,10]. The transcriptional regulation of the *iNOS* gene has been investigated by cloning the 5′-flanking region of the murine *iNOS* gene [11,12]. The mouse iNOS promoter contains two transcriptional regulatory regions, an enhancer and a basal promoter. There are a number of binding sites for transcription factors, including κB sites, located in both the enhancer and the basal promoter, two juxtaposed interferon-stimulated response elements in the enhancer, an octamer element in the basal promoter, and a γ-interferon-activated site in the enhancer [11–14]. Many reports have shown that activation of NF-κB is the major mechanism involved in

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Abbreviations: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide; L-NAME, *N*-nitro-L-arginine methyl ester; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; PDTC, pyrrolidine dithiocarbamate; and STAT, signal transducer and activator of transcription.

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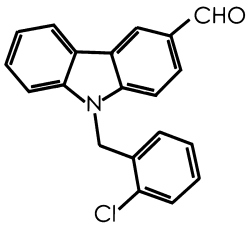

Fig. 1. Chemical structure of LCY-2-CHO.

LPS-induced *iNOS* gene expression [11,12,15]. Normally, NF-κB is present constitutively in the cytosol as homo- or heterodimers and is linked to inhibitory IκB proteins. The activation of NF-κB results in phosphorylation, ubiquitination, and proteasome-mediated degradation of the IκB proteins, followed by the translocation of NF-κB to the nucleus and induction of gene transcription through binding to the *cis*-acting κB element [16,17].

In the study of the anti-inflammatory activities of chemically synthetic carbazoles, 9-(2-chlorobenzyl)-9*H*-carbazole-3-carbaldehyde (LCY-2-CHO) (Fig. 1) was found to inhibit LPS-induced NO generation in RAW 264.7 macrophages in a preliminary *in vitro* test. The aim of the present study was to evaluate the mechanism by which LCY-2-CHO inhibits NO generation in macrophages. The results demonstrated that treatment with LCY-2-CHO decreases LPS-stimulated iNOS protein expression, iNOS mRNA steady-state levels, and iNOS promoter activity. However, LCY-2-CHO does not affect NF-κB transcriptional activity.

2. Materials and methods

2.1. Materials

LCY-2-CHO (purity > 99%) was synthesized [18] and dissolved in DMSO. The RAW 264.7 mouse macrophage-like cell line was obtained from the American Type Culture Collection. DMEM, penicillin, streptomycin, and fetal bovine serum were purchased from Gibco Life Technologies. Polyvinylidene difluoride membrane was obtained from the Millipore Co. Anti-mouse iNOS mAb was obtained from BD Transduction Laboratories. ECL western blotting reagent and Hybond-N nylon membranes were purchased from Amersham Pharmacia Biotech. Rezol™ C&T reagent was purchased from Protech Technology. Express Hyb hybridization solution and pNFκB-LUC were obtained from Clontech Laboratories. Nucleic acid chemiluminescence reagent and a random primer fluorescein labeling kit were obtained from NEN Life Science Products. DOSPER liposomal transfection reagent, a DIG gel shift kit, and a CAT enzyme-linked immunosorbent assay were purchased from Roche Molecular Biochemicals. pRL-TK and a Dual-luciferase reporter assay system were obtained from the Promega Co. p1iNOS-CAT plasmid was obtained from Oxford Biomedical Research. Antibodies against p50, p65, and c-Rel were obtained from Santa Cruz Biotechnology. All other reagents and chemicals were purchased from the Sigma Chemical Co.

2.2. Cell culture

RAW 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin, and were maintained in a humidified incubator with 5% CO_2.

2.3. Nitrite determination and iNOS activity assay

For nitrite determination, cells were seeded onto 96-well plates at 2×10^5 cells/well and allowed to adhere overnight. Before stimulation with 1 μg/mL of LPS (*Escherichia coli*, serotype 0111:B4) for 24 hr, cells were pretreated with vehicle or the indicated concentrations of drug at 37° for 1 hr in a final volume of 200 μL. For the iNOS activity assay, cells were stimulated with LPS for 12 hr. Then the cells were washed twice with PBS. Drugs were added for an additional 12 hr in the absence of LPS. The cell-free culture medium was collected and stored at −70° until NO determination. The production of NO was determined based on the Griess reaction [19]. Briefly, 40 μL of 5 mM sulfanilamide, 10 μL of 2 M HCl, and 20 μL of 40 mM naphthylethylenediamine were added to 150 μL of culture medium. After a 10-min incubation period at room temperature, absorbance was measured at 550 nm via a microplate reader. A standard nitrite curve was generated in the same fashion using $NaNO_2$.

2.4. Western blot analysis

Cells were washed twice with PBS and harvested in Laemmli SDS sample buffer. Protein extracts were separated by SDS–PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hr at room temperature in TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) containing 5% (w/v) nonfat milk. Membranes were washed with TBST buffer, and then were incubated for 1 hr with the indicated antibodies. After washing again with TBST buffer, a 1:10,000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 hr. The blots were developed using ECL western blotting reagents.

2.5. Northern blot analysis

Total cellular RNA was isolated from RAW 264.7 macrophages using REzol™ C&T reagent, according to the instructions of the manufacturer. For Northern blot analysis, 20 μg of total RNA was denatured in a glyoxal/DMSO mixture at 50° for 1 hr [20], separated by electrophoresis on a 1% (w/v) agarose gel containing 100 mM sodium phosphate buffer (pH 6.8), and transferred to Hybond-N nylon membranes. After UV cross-linking, the membranes

were prehybridized, and then hybridized with a cDNA probe using Express Hyb hybridization solution as described by the manufacturer. The iNOS cDNA probe was made by reverse transcription and polymerase chain reaction amplification using the following sense and antisense primers (5′-TCATTGTACTCTGAGGGCTGACACA-3′ and 5′-GCCT-TCAACACCAAGGTTGTCTGCA-3′) [21] and labeled with a random primer fluorescein labeling kit. After hybridization, the membranes were washed and subsequently probed with antifluorescein-horseradish peroxidase conjugated antibody, before visualization with the nucleic acid chemiluminescence reagent. To ensure equal loading of RNA, a GAPDH probe was used as an internal control to normalize iNOS mRNA expression. The densities of the bands were quantitated with an imaging densitometer. In the mRNA degradation assay, cells were stimulated with LPS for 12 hr, and then washed twice with PBS, before the addition of vehicle or drugs in the presence of 10 μg/mL of actinomycin D. The degraded RNA of the drug-treated groups was compared with RNA from cells treated with vehicle.

2.6. Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared as described previously [22]. Briefly, cells were washed twice with ice-cold PBS and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM NaF, and 1 mM Na_3VO_4) and incubated on ice for 15 min. Then the cells were lysed by the addition of 0.5% Nonidet P-40 and vigorous vortexing for 10 sec. The nuclei were pelleted by centrifugation at 12,000 *g* for 30 sec at 4° and resuspended in extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, and 1 mM Na_3VO_4). After 15 min on ice, lysates were centrifuged at 12,000 *g* for 10 min at 4°. Supernatants were obtained and stored at −70°. The oligonucleotide sequence 5′-tcgaCCAACTGGGGACTCT-CCCTTTGGGAACA-3′, corresponding to the consensus κB site (−92 to −65) of the iNOS promoter, was synthesized [14,23]. Nuclear extract (5 μg) was used for EMSA with a DIG gel shift kit, according to the protocol of the manufacturer. The specificity of binding was determined by the addition of 50-fold unlabeled oligonucleotide. Before the binding reaction, supershift assays were carried out by the addition of 0.5 μg of anti-p50, anti-p65, or anti-c-Rel antibody to the nuclear extracts at 4° for 30 min.

2.7. Plasmids, transient transfection, and reporter assay

CAT under the control of the iNOS promoter, p1iNOS-CAT, was used to quantify iNOS promoter activity. An NF-κB reporter construct, consisting of the firefly *luciferase* gene under the control of four tandem copies of the consensus NF-κB site pNFκB-LUC, was used to quantify NF-κB transcriptional activity. A *Renilla* luciferase reporter under the control of the herpes simplex virus thymidine kinase promoter, pRL-TK, was used as an internal control to normalize the reporter gene activity. RAW 264.7 macrophages were transfected by DOSPER liposomal transfection reagent according to the instructions of the manufacturer. Forty hours later, the culture medium was replaced, and cells were pretreated with vehicle or LCY-2-CHO for 1 hr followed by stimulation with LPS. After 6 or 24 hr, the cells were lysed, and the luciferase activity and CAT protein levels were determined, respectively. The induction of CAT protein was quantified using a CAT ELISA kit according to the instructions of the manufacturer. Luciferase activities were determined by a luminometer using a Dual-luciferase reporter assay according to the instructions of the manufacturer.

2.8. Statistical analysis

Statistical analyses were performed using a Bonferroni *t*-test after one-way analysis of variance. $P \leq 0.05$ was considered significant for all tests. Analysis of the regression line test was used to calculate IC_{50} values. Data are presented as means ± SD for the indicated number of independent experiments.

3. Results

3.1. Effect of LCY-2-CHO on NO production

To assess the effect of LCY-2-CHO on LPS-induced NO production in RAW 264.7 macrophages, cell culture medium was harvested, and the concentration of accumulated nitrite, the oxidative product of NO, was determined by the Griess method. LCY-2-CHO inhibited LPS-induced NO release in RAW 264.7 cells in a concentration-dependent manner with an IC_{50} value of 1.3 ± 0.4 μM (Fig. 2A). Cell viability was >90% at the concentrations tested as assessed by trypan blue exclusion and the lactate dehydrogenase release assay (compared with the 0.1% Triton X-100-treated value). The addition of LCY-2-CHO to cells that had been stimulated with LPS for 12 hr to induce iNOS activity did not affect the induced iNOS activity as evidenced by nitrite formation (26.9 ± 0.5 μM in the control vs 24.5 ± 1.7 μM at 10 μM LCY-2-CHO; 8.8 ± 5.8% inhibition, $P > 0.05$). The NOS inhibitor L-NAME (1 mM) suppressed iNOS activity to 33.2 ± 4.9% of the control value.

3.2. Effects of LCY-2-CHO on iNOS protein and mRNA expressions

Western blot and Northern blot analyses were performed to examine whether the inhibition of NO generation by LCY-2-CHO influences the expression of iNOS protein and mRNA, respectively. In unstimulated RAW 264.7

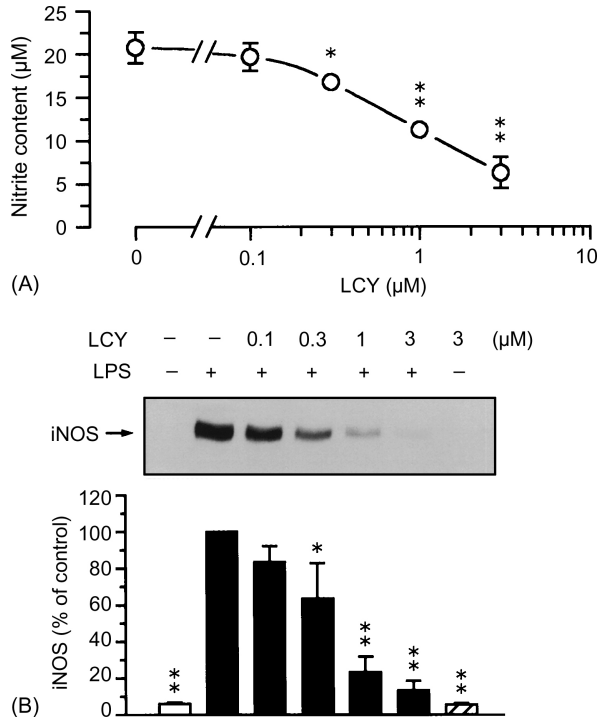

Fig. 2. Inhibition of LPS-stimulated nitrite accumulation and iNOS protein expression in RAW 264.7 macrophages by LCY-2-CHO. (A) Cells were pretreated with DMSO (as control) or the indicated concentrations of LCY-2-CHO (LCY) for 1 hr before stimulation with 1 μg/mL of LPS for 24 hr. The culture medium was collected, and the nitrite release was determined. Values are expressed as means ± SD of three independent experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with the control value. (B) Cells were pretreated with DMSO or the indicated concentrations of LCY-2-CHO for 1 hr before stimulation with 1 μg/mL of LPS for 24 hr. Stimulated and unstimulated cells were then lysed, and the lysates were subjected to SDS–PAGE and immunoblot analysis using anti-iNOS antibody. The upper panel represents the results of Western blot analysis, and the lower panel shows the percent response of the control (100% = 13.9 ± 1.62 densitometer units) as means ± SD from three independent experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with the control value (second column).

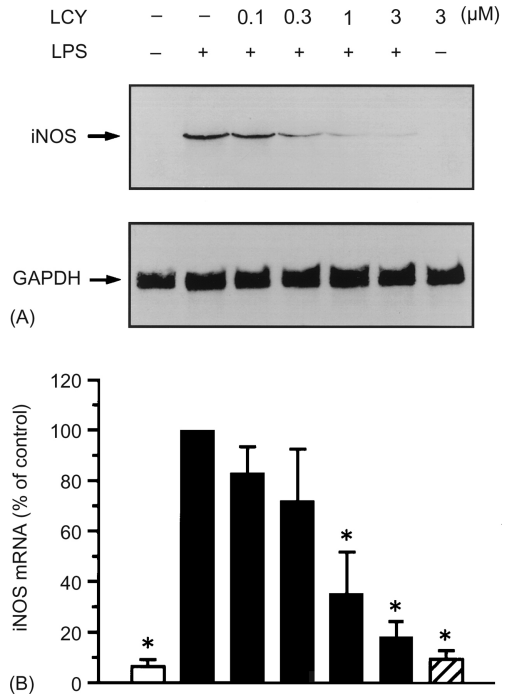

Fig. 3. Inhibitory effect of LCY-2-CHO on iNOS mRNA steady-state levels in RAW 264.7 macrophages stimulated with LPS. Cells were pretreated with DMSO or the indicated concentrations of LCY-2-CHO (LCY) for 1 hr before stimulation with 1 μg/mL of LPS for 16 hr. Expression of iNOS mRNA in stimulated and unstimulated cells was analyzed by Northern blot. GAPDH was used as an internal control. (A) Represents the results of Northern blot analysis, and (B) shows the percent response of the control (100% = 12.0 ± 1.36 densitometer units) as means ± SD from three independent experiments. The iNOS mRNA densitometry values were normalized to their respective GAPDH densitometry values. Key: (*) $P < 0.01$ compared with the control value (second column).

macrophages, iNOS protein was undetectable. However, pronounced expression of iNOS protein was demonstrated upon exposure to LPS for 24 hr (Fig. 2B). Pretreatment of cells with LCY-2-CHO decreased iNOS protein levels in a concentration-dependent manner with an IC_{50} value of 0.4 ± 0.1 μM. LCY-2-CHO had a similar inhibitory effect upon the expression of iNOS mRNA (IC_{50} value of 0.6 ± 0.2 μM) (Fig. 3, A and B). LCY-2-CHO did not affect the expression of the housekeeping gene *GAPDH*. In addition, LCY-2-CHO did not enhance the degradation of iNOS mRNA as assessed by measuring the half-life of the expressed iNOS mRNA induced by LPS ($T_{1/2}$ 6.5 ± 1.8 vs 4.8 ± 1.3 hr for control, $P > 0.05$) (Fig. 4, A and B).

3.3. Effect of LCY-2-CHO on iNOS promoter activity

To understand the effect of LCY-2-CHO on the expression of the *iNOS* gene, RAW 264.7 macrophages were transiently transfected with a p1iNOS-CAT reporter construct containing the iNOS promoter sequence linked to the CAT gene. Exposure of the cells to LPS significantly increased the expression of CAT protein as assessed by CAT ELISA (Fig. 5). The LPS-stimulated iNOS promoter activity was attenuated by LCY-2-CHO in a concentration-dependent manner (about 66 and 55% of control value at 1 and 3 μM LCY-2-CHO, respectively). LCY-2-CHO alone had no effect on iNOS promoter activity.

3.4. Effect of LCY-2-CHO on the degradation of IκB-α and IκB-β

The activation of NF-κB is critical for the induction of *iNOS* gene expression in cells stimulated with LPS [11,12,15], and the degradation of IκB-α and -β proteins plays a critical role in NF-κB activation [16,17]. Based on Western blot analysis, the cellular IκB-α level was eliminated within 30 min, and then rebounded to near normal levels within 1 hr, in cells exposed to LPS (Fig. 6). Under the same conditions, the cellular level of IκB-β was also abrogated within 30 min; however, recovery of the IκB-β level was not observed over the 2-hr study period. The transcriptional regulation of IκB-α is via the NF-κB

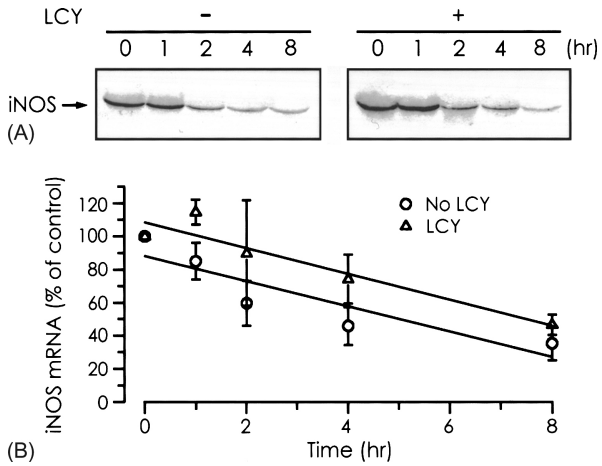

Fig. 4. Effect of LCY-2-CHO on the stability of iNOS mRNA. Cells were stimulated with LPS for 12 hr before the addition of vehicle or 3 μM LCY-2-CHO (LCY) in the presence of 10 μg/mL of actinomycin D. At the indicated time after transcriptional blockade, iNOS mRNA was analyzed by Northern blot. (A) Represents the results of Northern blot analysis, and (B) shows the percent response of the corresponding control values (100% = 16.4 ± 2.19 densitometer units for vehicle and 14.5 ± 2.08 densitometer units for LCY-2-CHO) as means ± SD from four independent experiments.

protein. This regulation can prevent excessive activation of NF-κB. When IκB-α protein is newly synthesized, it can interact directly with transcriptionally active NF-κB proteins in the nucleus and cytosol [24]. Pretreatment of cells

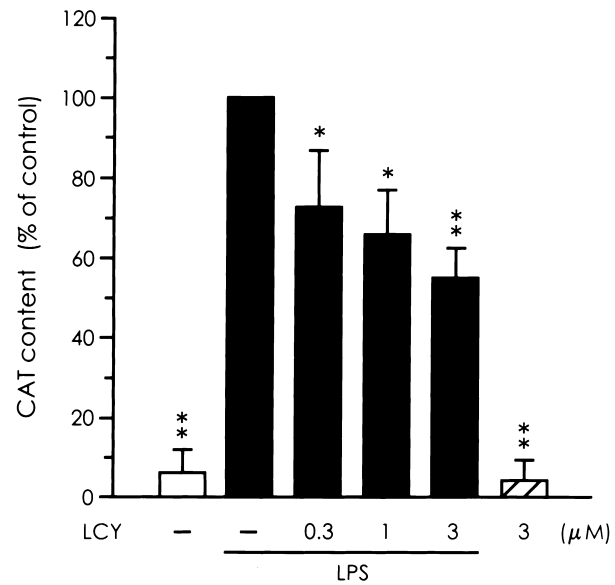

Fig. 5. Effect of LCY-2-CHO on iNOS promoter activity in RAW 264.7 macrophages stimulated with LPS. Cells were transiently co-transfected with the p1iNOS-CAT reporter gene and *Renilla* luciferase reporter gene. Forty hours later, cells were pretreated with DMSO or the indicated concentrations of LCY-2-CHO (LCY) for 1 hr before stimulation with 1 μg/mL of LPS for 24 hr. Cells were then lysed, and the lysates were harvested. The amount of CAT protein was quantified by an ELISA kit, and *Renilla* luciferase activity was detected by the Dual-luciferase reporter assay system. The amount of CAT protein was normalized to the respective *Renilla* luciferase activity. Values (means ± SD) are expressed as the percent response of the control from four independent experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with the control value (second column).

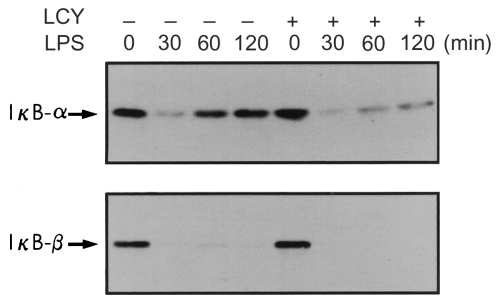

Fig. 6. Effect of LCY-2-CHO on IκB-α and IκB-β degradation in RAW 264.7 cells stimulated with LPS. Cells were pretreated with DMSO or 3 μM LCY-2-CHO (LCY) for 1 hr before stimulation with 1 μg/mL of LPS for the indicated time periods. Cells were then lysed, and the lysates were subjected to SDS–PAGE and immunoblot analysis using anti-IκB-α or anti-IκB-β antibodies. Similar results were obtained from three independent experiments.

with LCY-2-CHO did not suppress the degradation of IκB-α or IκB-β, but the reappearance of IκB-α was delayed for over 2 hr in LPS-stimulated cells (Fig. 6). These results lead us to suspect the involvement of NF-κB inhibition in the mechanism of LCY-2-CHO action.

3.5. Effect of LCY-2-CHO on NF-κB DNA binding activity

We next determined by EMSA whether LCY-2-CHO interferes with the binding of NF-κB to DNA. The nuclear extracts were prepared, and EMSA was performed using an oligonucleotide corresponding to the κB sequence of the iNOS promoter. LPS stimulation induced a marked increase in NF-κB DNA binding. Treatment with LCY-2-CHO did not suppress the LPS-induced response (Fig. 7A). PDTC, an inhibitor of NF-κB activation [25], decreased the nuclear DNA binding activity of NF-κB. LCY-2-CHO alone had no effect on NF-κB binding activity (data not shown). The nature of the NF-κB dimer was characterized by supershift experiments in the presence of antibody against p50, p65, or c-Rel. Incubation of nuclear extract with anti-p50 or anti-p65 antibody, but not anti-c-Rel antibody, prior to EMSA resulted in a supershift (Fig. 7B). These observations suggest the involvement of p65/p50 heterodimer and p50 homodimers, consistent with previous reports [15,23]. It has been reported that the presence of dithiothreitol in the nuclear extract and EMSA masks the decrease in NF-κB binding activity [26]. Fig. 7C shows that, in the absence of dithiothreitol, LCY-2-CHO did not affect LPS-stimulated NF-κB DNA binding activity, and LCY-2-CHO alone had no effect.

3.6. Effect of LCY-2-CHO on NF-κB transcriptional activity

To further investigate the effect of LCY-2-CHO on NF-κB transcriptional activity, RAW 264.7 cells were transiently transfected with a reporter plasmid containing four

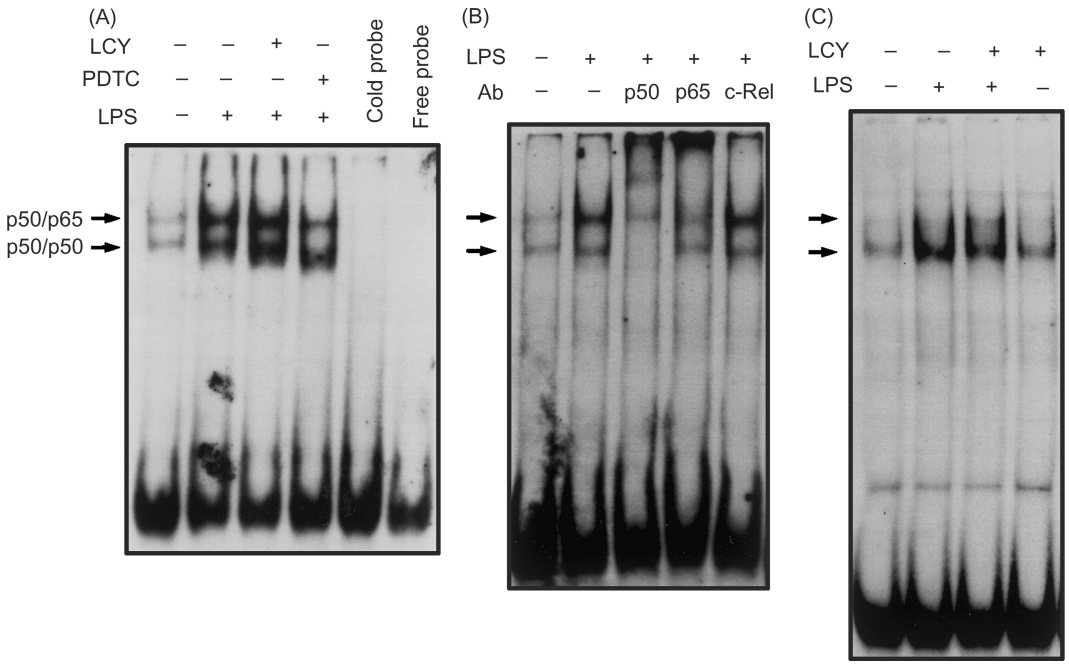

Fig. 7. Effect of LCY-2-CHO on NF-κB DNA binding activity. (A) Cells were pretreated with DMSO, 3 μM LCY-2-CHO (LCY), or 25 μM PDTC for 1 hr before stimulation with 1 μg/mL of LPS for 1 hr. Then the nuclear extracts were prepared, and EMSA was performed. The cold probe lane contained the 1-hr post-stimulation nuclear extract incubated with DIG-labeled NF-κB probe plus 50-fold excess unlabeled probe. (B) The supershift experiments were carried out by incubation of nuclear extract with 0.5 μg of anti-p50, anti-p65, or anti-c-Rel antibody before the binding reaction. (C) Similar to (A) except that the preparation of the nuclear extracts and the DNA binding reactions were carried out in the absence of dithiothreitol. Similar results were obtained from three independent experiments.

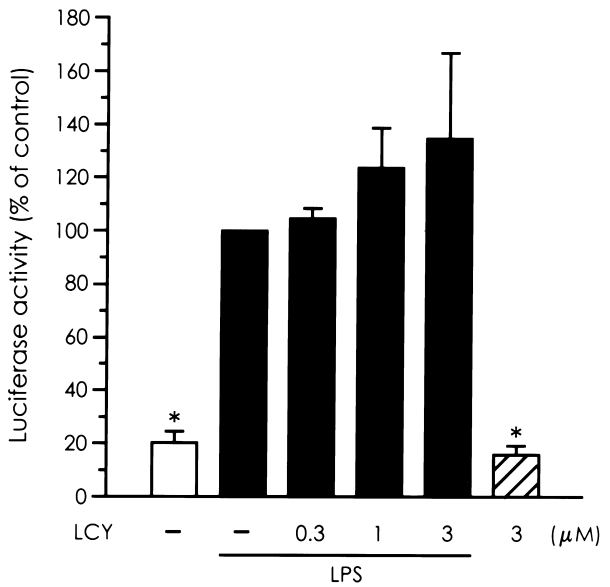

Fig. 8. Effect of LCY-2-CHO on NF-κB transcriptional activity in RAW 264.7 macrophages stimulated with LPS. Cells were transiently co-transfected with NF-κB firefly luciferase reporter gene and *Renilla* luciferase reporter gene. Forty hours later, cells were pretreated with DMSO or the indicated concentrations of LCY-2-CHO (LCY) for 1 hr before stimulation with 1 μg/mL of LPS for 6 hr or without stimulation. Cells were then lysed, and the firefly and *Renilla* luciferase activities were measured sequentially by a luminometer using a Dual-luciferase reporter assay system. The firefly luciferase activities were normalized to respective *Renilla* luciferase activities. Values (means ± SD) are expressed as the percent response of the control from four independent experiments. Key: (*) $P < 0.01$ compared with the control value (second column).

tandem copies of the NF-κB consensus sequence linked to the *luciferase* gene. NF-κB transcriptional activity was measured in terms of the activity of expressed luciferase by a luminometer. LPS stimulation resulted in a significant increase in luciferase activity. The lack of effect of LCY-2-CHO on the LPS-induced response (Fig. 8) is compatible with the findings that both IκB degradation and binding of NF-κB to DNA are unaffected by LCY-2-CHO.

4. Discussion

In the present study, we demonstrate that a synthetic carbazole compound, LCY-2-CHO, inhibits NO generation via a decrease in iNOS transcriptional expression through a signaling pathway other than NF-κB activation. Inhibition of LPS-stimulated NO generation in RAW 264.7 macrophages was not attributable to cytotoxicity as assessed by trypan blue exclusion, a lactate dehydrogenase release assay, and expression of the housekeeping gene *GAPDH*. In addition, the finding that LCY-2-CHO had no inhibitory effect on induced iNOS activity implies upstream inhibition.

To explore the mechanism of inhibition of NO generation by LCY-2-CHO, the expression of iNOS protein and mRNA was examined by using Western and Northern blot analyses, respectively. LCY-2-CHO abolished the LPS-induced expression of both iNOS protein and mRNA in

a parallel concentration-dependent manner with IC_{50} values similar to those required for inhibition of NO generation. In addition, LCY-2-CHO did not affect the degradation of iNOS mRNA as assessed by measuring the half-life of the expressed iNOS mRNA induced by LPS. Thus, the inhibition of NO generation may be attributed to the suppression of iNOS mRNA transcription followed by iNOS protein expression.

To examine the effect of LCY-2-CHO on the expression of the *iNOS* gene, cells transiently transfected with a p1iNOS-CAT reporter construct containing the iNOS promoter sequence linked to the *CAT* gene were used. The finding that LPS-stimulated iNOS promoter activity was attenuated by LCY-2-CHO further supports the hypothesis that LCY-2-CHO inhibition of LPS-stimulated NO production occurs during *iNOS* gene expression. The activation of NF-κB is critical for the induction of *iNOS* gene expression in cells stimulated with LPS [11,12,15]. Degradation of IκB proteins plays a critical role in NF-κB activation [16,17]. However, LCY-2-CHO had no effect on the degradation of IκB-α or IκB-β. Moreover, LCY-2-CHO did not suppress DNA binding activity or the transcriptional activity of NF-κB as assessed in nuclear extracts with EMSA and in cells transiently transfected with a reporter plasmid containing four tandem copies of the NF-κB consensus sequence linked to the *luciferase* gene, respectively. These observations further refute the involvement of NF-κB inhibition in the action of LCY-2-CHO.

The 5′-flanking region of the murine *iNOS* gene is known to contain two transcriptional regulatory regions, an enhancer and a basal promoter [11,12]. The promoter region of the mouse *iNOS* gene contains several consensus sequences for the binding of transcriptional factors, such as NF-κB, signal transducer and activator of transcription (STAT) family, CCAAT/enhancer binding protein (C/EBP), cAMP-responsive element-binding protein (CREB), and octamer binding factors [11–14,27]. All transcription factors are necessary for *iNOS* gene induction, and transcription factors that interact with the iNOS promoter must work cooperatively. Although LCY-2-CHO does not inhibit NF-κB activity, it might interfere with other transcription factors, resulting in decreased iNOS promoter activity and, probably, account for the delayed reappearance of IκB-α in LPS-stimulated cells. The IκB-α promoter is known to also contain an Sp-1 binding sequence [28]. Moreover, in STAT1-deficient peritoneal macrophages, the LPS-induced iNOS expression is abolished almost completely, despite the fact that LPS activates NF-κB [29].

Several lines of evidence indicate that NF-κB is necessary but not sufficient for expression of the *iNOS* gene [29–32]. In murine macrophages, protein phosphatase 1/2A inhibitors reduce LPS-induced iNOS promoter activity but do not suppress the transcriptional activity of NF-κB [32]. This indicates the involvement of a protein phosphatase 1/2A-sensitive signaling pathway other than NF-κB in the regulation of *iNOS* gene expression in macrophages. Whether LCY-2-CHO inhibits protein phosphatase 1/2A activity requires further investigation.

The physiologic or normal production of NO from phagocytes is beneficial for the host defense against microorganisms, parasites, and tumor cells [2]. However, overproduction of NO can be harmful and result in septic shock, neurologic disorders, rheumatoid arthritis, and autoimmune diseases [6–8]. Therefore, a therapeutic agent that inhibits the biosynthesis of NO may be useful for the relief of these inflammatory conditions. In addition to developing selective iNOS enzyme inhibitors, the designing and synthesis of inhibitors of iNOS enzyme expression have emerged as a viable strategy in drug development. Moreover, a combination of inhibitors with these two different modes of action would block the formation of NO more effectively and thus be an attractive pharmacological target.

In summary, we have demonstrated that LCY-2-CHO, a synthetic carbazole compound, inhibits LPS-stimulated NO generation in RAW 264.7 macrophages. This effect did not result from cytotoxicity or the inhibition of iNOS activity. The mechanism by which LCY-2-CHO decreased NO generation involves the suppression of *iNOS* gene expression and iNOS promoter activity. The basis for this conclusion is 2-fold: (a) attenuation of iNOS protein and mRNA levels; and (b) attenuation of CAT protein levels in cells transfected with an iNOS promoter-CAT reporter construct. The decreased iNOS promoter activity was not due to the suppression of NF-κB activation.

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