J. Toxicol. Pub. Health
Vol. 21, No. 4, pp. 285-290 (2005)

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Inhibition of p65 Nuclear Translocation by Radicicol, Heat Shock Protein Inhibitor

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Received September 21, 2005; Accepted October 19, 2005

ABSTRACT. We demonstrate that radicicol, a macrocyclic antifungal antibiotic originally isolated from *Monosporium bonorden*, inhibits LPS-induced expression of iNOS gene in RAW 264.7 cells. Treatment of peritoneal macrophages and RAW 264.7 cells with radicicol inhibited LPS-stimulated nitric oxide production in a dose-related manner. Immunohistochemical staining of iNOS and RT-PCR analysis showed that the decrease of NO was due to the inhibition of iNOS gene expression in RAW 264.7 cells. Immunostaining of p65, EMSA, and reporter gene assay showed that radicicol inhibited NF-κ/Rel nuclear translocation, DNA binding, and transcriptional activation, respectively. Collectively, these series of experiments indicate that radicicol inhibits iNOS gene expression by blocking NF-κ/Rel nuclear translocation. Due to the critical role that NO release plays in mediating inflammatory responses, the inhibitory effects of radicicol on iNOS suggest that radicicol may represent a useful anti-inflammatory agent.

Keywords: Radicicol, Macrophages, iNOS, NF-κB/Rel.

INTRODUCTION

Radicicol, a macrocyclic antifungal antibiotic originally isolated from the fungus *Monosporium bonorden* (Delmottte and Delmotte-Plaquee, 1953), is a potent tranquilizer of low toxicity (McCapra *et al.*, 1964) and an inhibitor of *in vivo* angiogenesis (Oikawa *et al.*, 1993). Radicicol blocks the activation of mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) pathway by destabilization of Raf kinase (MAPKKK) resulting from inhibiting chaperone function of HSP90 (Soga *et al.*, 1998). It has been reported that a radicicol-related macrocyclic nonaketide compound inhibits the p38 pathways (Takehana *et al.*, 1999) in anisomycin-induced HeLa cells. However, the p38 pathways are not inhibited by radicicol. Bacterial lipopolysaccharide (LPS) is a potent the immune system activator which induces local inflammation, antibody production, and, in severe infections, septic shock (Rietschel and Brade, 1992). Macrophages play a central role in a host's defense against bacterial infection and are major cellular targets for LPS action. Stimulation of murine macrophages by LPS results in the expression of an iNOS, which catalyzes the production of large amounts of nitric oxide (NO) from L-arginine and molecular oxygen (Palmer *et al.*, 1988). NO, in turn, participates in the inflammatory response of macrophages (Hibbs *et al.*, 1987). The promoter of the murine gene encoding iNOS contains two κB binding sites, located at 55 and 971 bp upstream of the TATA box, respectively (Lowenstein *et al.*, 1993). It has been reported that protein binding to the κB site is necessary to confer inducibility by LPS (Xie *et al.*, 1994).

Since the NO production is very important in the inflammatory response of macrophages, we investigated the effect of radicicol on the production of NO. To further investigate the mechanism by which radicicol inhibits the expression of iNOS gene, we assessed the effects of radicicol on the activation of NF-κB/Rel. Since NF-κB/Rel activation requires nuclear translocation of NF-κB/Rel component p65, we focused the effect of radicicol on the nuclear translocation of p65. The present studies demonstrate that radicicol inhibits iNOS

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gene expression through the inhibition of NF-κB/Rel nuclear translocation.

MATERIALS AND METHODS

Materials

Radicicol was purchased from CalBiochem (San Diego, CA, USA). LPS from *Salmonella thyposa* was purchased from Sigma (St. Louis, MO, USA). Reagents used for cell culture were purchased from Gibco BRL (Grand Island, NY, USA). Anti-iNOS and anti-p65 antibodies were purchased from Ustate Biotechnology (Lake Placid, NY, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

Cell culture

Peritoneal macrophages and RAW 264.7 cells (murine macrophage line) were purchased from American Type Culture Collection (Bethesda, MD). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were then cultured in the presence of 5% CO_2 at 37°C. Peritoneal cells were harvested by sterile peritoneal lavage with Hanks' balanced salt solution, washed, resuspended in culture medium, and plate at 5×10^5 cells/ml. Nonadherent cells were removed by pepeated washing after a 2-h incubation at 37°C.

Nitrite quantitation

NO_2^- accumulation was used as an indicator of NO production in the medium as previously described (Green *et al.*, 1982). Cells were plated at 5×10^5 cells/ml in 96-well culture plates and treated with radicicol for 24 hr. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. Using $NaNO_2$ to generate a standard curve, nitrite production was measured by an O.D. reading at 550 nm.

Western immunoblot analysis

Whole cell lysates (20 μg) were separated by 10% SDS-PAGE and then electro-transferred to nitrocellulose membranes (Amersham International, Buckinghamshire, UK). The membranes were preincubated for 1 hr at room temperature in Tris-buffered saline (TBS), pH 7.6 containing 0.05% Tween-20 and 3% bovine serum albumin. The nitrocellulose membranes were incubated with iNOS-specific antibodies. Immunoreactive bands were then detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham).

RT-PCR

Total RNA was isolated using Tri Reagent (Molecular Researh Center, Cincinnati, OH, USA) as described previously (Chomczynski and Mackey, 1995). The forward and reverse primer sequences are: iNOS: 5'-CTG CAG CAC TTG GAT CAG GAA CCT G-3', 5'-GGG AGT AGC CTG TGT GCA CCT GGA A-3' and b-actin: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. Equal amounts of RNA were reverse-transcribed into cDNA using oligo(dT)15 primers. PCR was performed with cDNA and each primer. Samples were heated to 94°C for 5 min and cycled 30 times at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min, after which an additional extension step at 72°C for 5 min was included. PCR products were electrophoresed in 3% NuSieve 3 : 1 gels (FMC Bioproducts, Rockland, ME) followed by staining in ethidium bromide. The iNOS and β-actin primers produce amplified products at 311 bp and 349 bp, respectively.

Transient transfection of RAW 264.7 cells

Vector constructions were performed as previously described (Jeon *et al.*, 1998). RAW 264.7 cells were transfected using the DEAE-dextran method (Xie *et al.*, 1993b), diluted to 5×10^5 cells per 1 ml of complete media, plated on 24 well plates, and then incubated in the presence of 5% CO_2 at 37°C for 24 hr. The transfectants were treated with LPS and radicicol. Eighteen hours later the cells were lysed with lysis buffer (250 μl). The lysates were centrifuged (12,000 ×g for 10 min at 4°C), and the supernatant was assayed for the expression of CAT enzyme using CAT ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was performed as previously described (Jeon *et al.*, 1996). Nuclear extracts were prepared as previously described (Xie *et al.*, 1993a). Treated and untreated RAW 264.7 cell line was lysed with hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, pH 7.5) and nuclei were pelleted by centrifugation at 3,000 ×g for 5 min. Nuclear lysis was performed using a hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml of aprotinin, and 1 μg/ml of leupeptin). Following lysis, the samples were centrifuged at 14,500 ×g for 15 min, and

supernatant was retained for use in the DNA binding assay. The double-stranded oligonucleotides were end-labeled with [γ-^{32}P]-ATP. Nuclear extracts (5 μg) were incubated with poly (dI-dC) and the [^{32}P]-labeled DNA probe in binding buffer (100 mM KCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml of aprotinin, and 1 mg/ml of leupeptin) for 10 min. DNA binding activity was separated from free probe using a 4% polyacrylamide gel in 0.5× TBE buffer. Following electrophoresis, the gel was dried and subjected to autoradiography.

Statistical analysis

The mean ± SD was determined for each treatment group in a given experiment. When significant differences occurred, treatment groups were compared to the vehicle control using a Dunnett's two-tailed *t* test (Dunnett, 1955).

RESULTS

Effect of radicicol on nitrite production in macrophages

To investigate the effects of radicicol on NO production, we measured the accumulation of nitrite, the stable end product of NO, in the culture media using Griess reagent. Peritoneal RAW 264.7 cells, mouse macrophage cell line, were treated with radicicol in the presence of LPS for 24 h. LPS (200 ng/ml) increased the production of nitrite ≥ 9- and 15-fold over basal levels in peritoneal macrophages and RAW 264.7 cells, respectively (Table 1). This induction in nitrite generation by LPS was inhibited by radicicol in a dose-dependent manner.

Table 1. Inhibition of nitrite production in macrophages by radicicol

	Treatment	Nitrite (nmole/10^6 cells)
Peritoneal cells	Control	3.5 ± 1.1
	LPS (200 ng/ml)	32.4 ± 2.3
	LPS + Rad (10 ng/ml)	34.2 ± 4.7
	LPS + Rad (50 ng/ml)	24.3 ± 5.2*
	LPS + Rad (100 ng/ml)	18.7 ± 4.8*
	LPS + Rad (200 ng/ml)	10.3 ± 2.2*
RAW 264.7	Control	4.3 ± 1.7
	LPS (200 ng/ml)	64.7 ± 2.4
	LPS + Rad (10 ng/ml)	58.4 ± 5.7
	LPS + Rad (50 ng/ml)	51.6 ± 4.8*
	LPS + Rad (100 ng/ml)	27.9 ± 2.1*
	LPS + Rad (200 ng/ml)	13.2 ± 3.8*

Each value shows the mean ± S.D. of triplicate determinations. *, response that is significantly different from the control group as determined by Dunnett's two-tailed *t* test at $P < 0.05$.

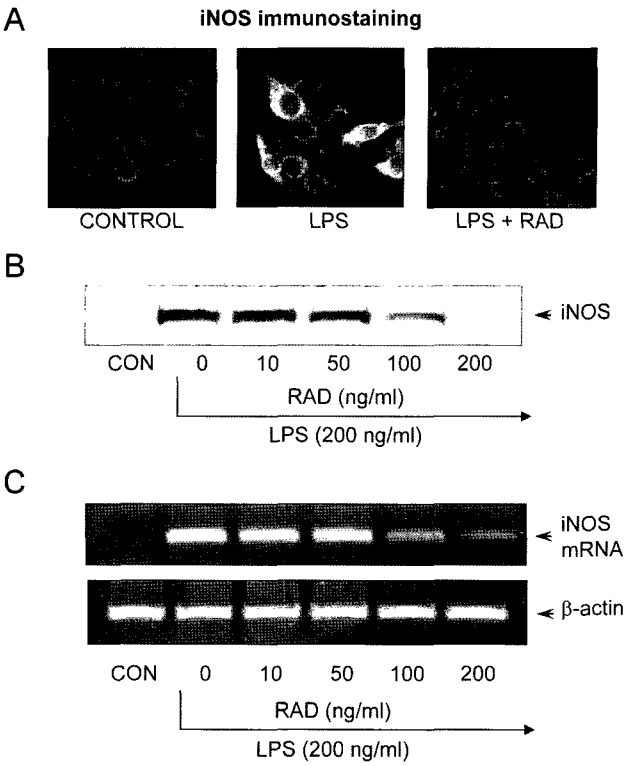

Fig. 1. Inhibition of iNOS gene expression production by radicicol in LPS-stimulated RAW 264.7 cells. (A) RAW 264.7 cells (5 × 10^5 cells/ml) were incubated with radicicol (100 ng/ml) in the presence of LPS (200 ng/ml) for 24 hr on cover slide in 12 well plates. Cells were subjected to immunohistochemical staining using an antibody specific for murine iNOS. Immunoreactivity of iNOS was localized along the margin of the cytoplasm of in control. (B) RAW 264.7 cells were treated with radicicol (10, 50, 100, and 200 ng/ml) in the presence of LPS (200 ng/ml) for 24 hr. Cell lysates were then prepared and subjected to Western immunoblotting. (C) Cells were incubated with radicicol in the presence of LPS for 8 hr. Total RNA was isolated and analyzed for the magnitude of mRNA expression of iNOS using RT-PCR.

Immunohistochemical staining of iNOS confirmed that the decrease of NO was due to the inhibition of iNOS production (Fig. 1A). No effect on cell viability was observed at any treatment groups and always exceeded 90% as determined by trypan blue staining (data not shown). After RAW 264.7 cells were exposed to radicicol in the presence of LPS, the expression level of iNOS gene was monitored by Western immunoblot analysis and RT-PCR. As shown in Fig. 1B, iNOS protein production was inhibited by radicicol treatment in a dose-dependent manner. Consistent with this finding the transcription of iNOS mRNA was inhibited by radicicol (Fig. 1C). The result reflected that the decreased production of NO in macrophage was mediated by the inhi-

bition of iNOS gene expression. Control β-actin was constitutively expressed and was not affected by the treatment of radicicol. These results indicate that radicicol decreases the gene expression of iNOS, which is involved in inflammation (Hibbs *et al.*, 1987).

Inhibition of NF-κB/Rel in response to radicicol in LPS-stimulated RAW 264.7 cells

To further investigate the molecular mechanism of radicicol-mediated inhibition of macrophage, we focused on the transcription factors whose binding sites are in the promoter of iNOS gene. Since it has been reported that protein binding at the κB binding site is necessary to confer inducibility by LPS of iNOS (Xie *et al.*, 1994), we assessed the effect of radicicol on NF-κB/Rel using a transient transfection assay. When RAW 264.7 cells were transiently transfected with p(NF-κB/Rel)$_3$-CAT, the CAT gene expressions were found to be inhibited by radicicol in the presence of LPS (Fig. 2A). Basal levels of CAT expression in unstimulated RAW 264.7 cells were $< 8 \pm 3.1$ pg/ml (mean ± standard deviation, two experiment). On LPS-stimulation, CAT expression by RAW 264.7 cells increased by 10.3-fold. Radicicol treatment inhibited LPS-induced CAT expression in a dose-dependent manner. RAW 264.7 cells expressed very strong basal Oct activity, and the activity was not influenced by either LPS or radicicol (Fig. 2B).

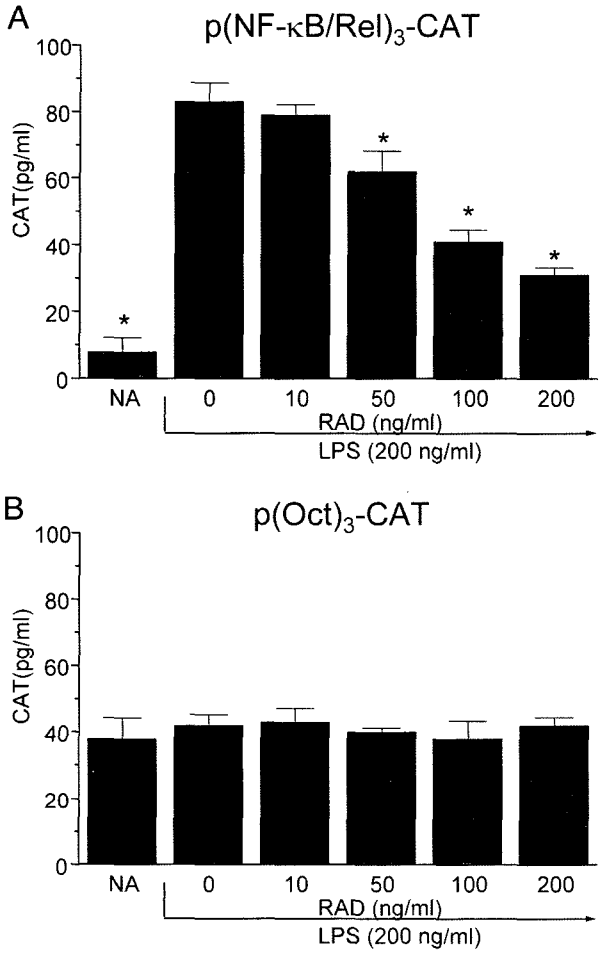

Fig. 2. Inhibition of NF-κB/Rel transcriptional activation by radicicol in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were transfected with p(NF-κB/Rel)$_3$-CAT (A) or p(Oct)$_3$-CAT (B) by DEAE dextran method. Twenty-four hours after transfection, cells were treated with the indicated concentrations of radicicol in the presence of LPS (200 ng/ml) for 18 hr. Cell extracts were then prepared and analyzed for the expression of CAT using CAT ELISA kit. *, response that is significantly different from the control group as determined by Dunnett's two-tailed *t* test at $P < 0.05$.

Inhibition of NF-κB/Rel nuclear translocation by radicicol in LPS-stimulated RAW 264.7 cells

We further assessed the effect of radicicol on the NF-κB/Rel whose binding motif is in the promoter of iNOS gene using EMSA. LPS treatment of RAW 264.7 cells induced a marked increase in NF-κB/Rel binding to its cognate site. And the induction of NF-κB/Rel binding

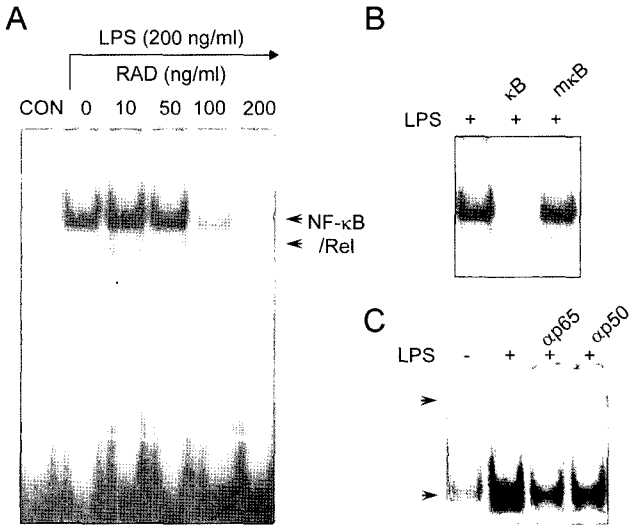

Fig. 3. Inhibition of NF-κB/Rel DNA binding by radicicol in LPS-stimulated RAW 264.7 cells. Cells (5×10^5 cells/ml) were incubated with radicicol (10, 50, 100, and 200 ng/ml) in the presence of LPS (200 ng/ml) for 2 hr. Nuclear extracts (5 μg/ml) were then isolated and analyzed for the activity of NF-κB/Rel (A). In competition studies, 1 pmole of unlabeled κB or mutant κB was added to the reaction mixture (B). For supershift assays, nuclear extracts (5 μg) were incubated with poly (dI-dC), antibodies specific for p65 or p50, and ^{32}P-labeled κB probe for 25 min (C). Reaction products were electrophoresed, and the gels were dried and autoradiographed.

was inhibited by radicicol in a dose-related manner (Fig. 3A). The specificity of the retarded bands was confirmed by the addition of an excess of ^{32}P-unlabeled double-stranded κB that competed for protein binding (Fig. 3B). Mutated cold κB could not compete for the binding. The NF-κB/Rel binding complex was identified by gel supershift assay (Fig. 3C). The band was supershifted dramatically when the nuclear extract was preinducbated with antibodies against p50 and p65.

The DNA binding of the NF-κB/Rel transcription factor is preceded by the nulear translocation of NF-κB/Rel. To further investigate whether radicicol inhibits the nuclear translocation of p65, which is a component of NF-κB/Rel and has a transcriptional activation activity, we analyzed the activity using immunohistochemical staining. LPS-stimulated RAW 264.7 cells showed marked p65 staining in the nuclei, while unstimulated cells showed weaker nuclear NF-κB/Rel expression, but stronger staining in the cytoplasm. Radicicol treatment significantly inhibited LPS-induced nuclear translocation of p65 (Fig. 4). These results indicate that radicicol decreases the nuclear translocation and DNA binding of NF-κB/Rel, which is important in the regulation of iNOS gene expression.

We further confirmed the inhibition of nuclear translocation of p65 and p50 by Western immunoblot assay. The amounts of nuclear p65 and p50 were increased at 2 hr after LPS treatment, as shown in Fig. 5A. However, stimulation of cells with LPS in the presence of radicicol (100 ng/ml) resulted in the reduction of nuclear contents of p65 and p50.

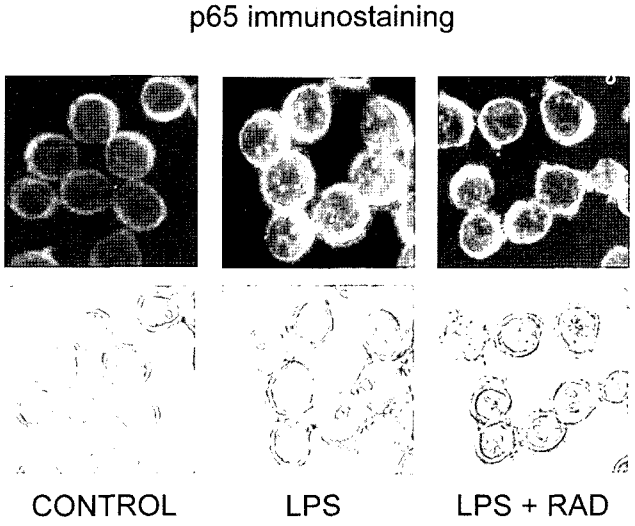

Fig. 4. Inhibition of p65 nuclear translocation by radicicol in LPS-stimulated RAW 264.7 cells. Cells (5×10^5 cells/ml) were incubated with radicicol (100 ng/ml) in the presence of LPS (200 ng/ml) for 2 hr on cover slide in 12 well plates. Cells were subjected to immunohistochemical staining using an antibody specific for murine p65.

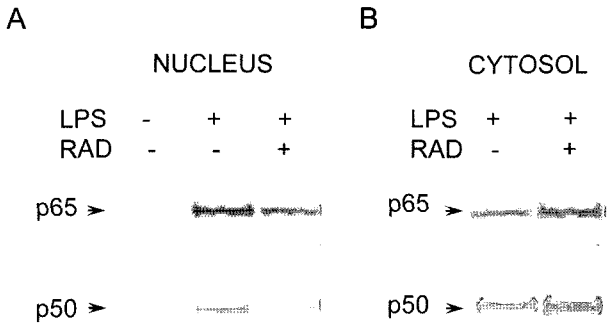

Fig. 5. Inhibition of nuclear translocation of NF-κB/Rel family members by radicicol in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with radicicol (100 ng/ml) in the presence of LPS (200 ng/ml) for 2 hr. Nuclear extracts (A) and cytosolic extracts (B) were then prepared and subjected to Western blot analysis.

DISCUSSION

We demonstrate that radicicol treatment significantly attenuates LPS-induced NO production and iNOS transcription through the blocking of p65 nuclear translocation in the macrophage line RAW 264.7. Our study showed that NF-κB/Rel is positively regulated by LPS for iNOS gene expression, and radicicol treatment of RAW 264.7 cell significantly inhibited LPS-induced NF-κB/Rel activity. The NF-κB/Rel is a pleiotropic regulator of many genes involved in immune and inflammatory responses, including iNOS (Xie *et al.*, 1994). NF-κB/Rel exists in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor, IκB. Macrophage activation by certain external stimuli results in the phosphorylation of IκB, thus releasing the active DNA-binding form of NF-κB/Rel to translocate to the nucleus to bind κB motifs in the regulatory region of a variety of genes. EMSA studies showed strong induction by LPS of two separate κB binding complexes at 2 hr. Radicicol inhibited activation of both of these κB binding complexes; however, the magnitude of inhibition seemed greater for the protein complex represented by the top of the two bands. The upper band appears to be composed of p50/p65 heterodimers, whereas the lower band appears to consist of p50 homodimers. It has been shown that p50 proteins have DNA binding activity and p65 proteins have transactivation domains in their C termini and thus are able to activate transcription of target genes (Schmitz and Baeuerle, 1991). This finding suggests that radicicol may inhibit the formation

of p50/p65 heterodimers based on the EMSA studies (Fig. 3). The inhibition of nuclear translocation of p65 by radicicol was further confirmed by the immunostaining of p65 and Western immunoblot assay, respectively (Fig. 4, Fig. 5).

In summary, these experiments demonstrate that radicicol, a macrocyclic antifungal antibiotic originally isolated from the fungus *Monosporium bonorden*, inhibits LPS-induced expression of iNOS gene in RAW 264.7 cells. Based on our findings, the most likely mechanism that can account for this biological effect involves the inhibition of p65 nuclear translocation. At least two significant points are brought out by these studies. First, these experiments further confirm the critical role of NF-κ/Rel in the regulation of iNOS. Second, due to the critical role that NO release plays in mediating inflammatory responses, the inhibitory effects of radicicol on iNOS suggest that radicicol may represent a useful anti-inflammatory agent.

ACKNOWLEDGEMENTS

This research was supported by research funds from Chosun University, 2002.

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