Radicicol Suppresses Expression of Inducible Nitric-Oxide Synthase by Blocking p38 Kinase and Nuclear Factor-κB/Rel in Lipopolysaccharide-Stimulated Macrophages

YOUNG J. JEON, YOUNG K. KIM, MICHAEL LEE, SUN M. PARK, SANG B. HAN, and HWAN M. KIM
Korea Research Institute of Bioscience and Biotechnology, Yusong, Taejon, Korea
Accepted for publication April 13, 2000 This paper is available online at http://www.jpet.org

ABSTRACT
We show that radicicol, a fungal antibiotic, produces a marked inhibition of p38 kinase, nuclear factor-κB/Rel (NF-κB/Rel), and inducible nitric-oxide synthase (iNOS) transcription by the macrophage line RAW 264.7 in response to lipopolysaccharide (LPS). Treatment of RAW 264.7 with radicicol inhibited LPS-stimulated p38 kinase phosphorylation in a dose-related manner. iNOS transcription, which is regulated in part by the NF-κB/Rel family of transcription factors, has been shown to be under the control of the p38 kinase signaling cascade. Our data also show that the p38 kinase pathway is specifically involved in LPS-induced NF-κB/Rel activation and iNOS expression because NF-κB/Rel DNA binding and iNOS mRNA production in the presence of a specific inhibitor of p38 kinase, SB203580, were dramatically diminished. In contrast, PD98059, a specific inhibitor of mitogen-activated protein kinase/extracellular signal-regulated protein kinase 1 had no effect on NF-κB/Rel activation and iNOS expression. LPS-induced loss of inhibitory proteins IκB-α and IκB-β and translocation of p65, c-Rel, and p50 was inhibited by radicicol. Collectively, this series of experiments indicates that radicicol inhibits iNOS gene expression by blocking p38 kinase signaling. Due to the critical role that NO release plays in mediating inflammatory responses, the inhibitory effects of radicicol on iNOS suggest that this potent antifungal agent may represent a useful anti-inflammatory agent.

Radicicol, a macrocyclic antifungal antibiotic originally isolated from the fungus Monosporium bonorden (Delmotte and Delmotte-Plaigue, 1953), is a potent tranquilizer of low toxicity (McCrapa et al., 1964; Mirrington et al., 1964) and an inhibitor of in vivo angiogenesis (Oikawa et al., 1993). Radicicol induces reversal of the transformed phenotype of src-transformed cells (Kwon et al., 1992a) and has been reported to inhibit the phosphorylation and protein kinase activity of pp60v-src (Kwon et al., 1992b). Radicicol blocks the activation of mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/extracellular signal-regulated protein kinase) pathway by destabilization of Raf kinase, resulting from inhibiting chaperone function of HSP90 (Soga et al., 1998; Roe et al., 1999). It has recently 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.

We investigated the effect of radicicol on the lipopolysaccharide (LPS)-induced nitric oxide (NO) response in macrophages, an important aspect of inflammation. Stimulation of murine macrophages by LPS results in the expression of an inducible NO synthase (iNOS), which catalyzes the production of large amounts of 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). Therefore, inhibiting high-output NO production by blocking iNOS production or activity may be a useful strategy for treatment of inflammatory disorders. Nonselective inhibitors of NOS activity, such as Nω-monomethyl-L-arginine, can cause sustained increases in mean arterial pressure in septic animals and humans (Thiemermann, 1997; Avontuur et al., 1998), but the untoward effects of nonselective vasoconstriction may outweigh this benefit (Avontuur et al., 1998). Selective iNOS inhibition may be more effective, with less end-organ injury (Liaudet et al., 1998). Badger et al. (1996) reported that infusion of the p38 inhibitor SB203580 reduced mortality in LPS-treated mice. The p38 kinase is an important mediator of stress-induced gene expression (Raineggad

Received for publication January 4, 2000.

1 This study was supported by a research grant from the Ministry of Science and Technology (BSHS 1840-98030-3II).

ABBREVIATIONS: MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric-oxide synthase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; bp, base pair; NF-κB/Rel, nuclear factor-κB/Rel; RT-PCR, reverse-transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ATF-2, activating transcription factor-2; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol.
et al., 1995). In particular, the p38 kinase is known to play a key role in LPS-induced signal transduction pathways leading to cytokine synthesis (Lee et al., 1994; Lee and Young, 1996). Recently, it was demonstrated that p38 MAPK activation is involved in iNOS expression in tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-1 (IL-1)-stimulated mouse astrocytes, as well as in LPS-stimulated mouse macrophages (Da Silva et al., 1997; Chen and Wang, 1999). The promoter of the murine gene encoding iNOS contains two \(\kappa\)B-binding sites, located at 55 and 971 base pairs (bp) upstream of the TATA box, respectively (Lowenstein et al., 1993). It has been reported that protein binding to the \(\kappa\)B site is necessary to confer inducibility by LPS (Xie et al., 1994). The nuclear factor-\(\kappa\)B/Rel (NF-\(\kappa\)B/Rel) family of transcription factors is composed of pleiotropic regulators of many genes involved in immune and inflammatory responses, including iNOS (Xie et al., 1994). In this study, we investigate the role of radicicol on the regulation of LPS-induced iNOS activity, NO formation, NF-\(\kappa\)B/Rel activity, and p38 kinase activity in the macrophage cell line RAW 264.7.

**Materials and Methods**

**Cell Culture.** The peritoneal macrophages and RAW 264.7 cells (ATCC TIB71) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Peritoneal cells were harvested by sterile peritoneal lavage with Hanks’ balanced salt solution, washed, resuspended in culture medium, and plated at 5 \(\times\) 10\(^5\) cells/ml. Nonadherent cells were removed by repeated washing after a 2-h incubation at 37°C.

**Nitrite Quantification.** NO\(_3\) accumulation was used as an indicator of NO production in the medium as previously described (Green et al., 1982). Cells were plated at 5 \(\times\) 10\(^5\) cells/ml in 24-well culture plates and stimulated with LPS (200 ng/ml) in the presence or absence of radicicol (0.03, 0.1, or 0.3 \(\mu\)g/ml) for 24 h. 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. With NaNO\(_2\) to generate a standard curve, nitrite production was measured at 550 nm.

**Quantitative Reverse-Transcription-Polymerase Chain Reaction (RT-PCR).** Competitive RT-PCR was performed as previously described (Jeon et al., 1999). Briefly, total RNA was isolated with TriReagent (Molecular Research Center, Cincinnati, OH). The forward and reverse primer sequences are as follows: iNOS, 5'-CTCGACCATCGTGATACGGAAACCTG-3', 5'-GGGAGTACCTCTGTGACCTGGAA-3'; IL-\(\beta\), 5'-TGCGAGGTCCTCAGAATCT-3', 5'-CTGCGGCTGAAATGGCTCCCTTGAAC-3'; TNF-\(\alpha\), 5'-CCTGTAGCCCGTGCTGAGCT-3', 5'-CTGCGGCTGAAATGGCTCCCTTGAAC-3'; -actin, 5'-TGGACCTCGTCTGATAGCTC-3', 5'-GGGAGTACCTCTGTGACCTGGAA-3'. The amplified products were electrophoresed in 8% polyacrylamide gel followed by staining in ethidium bromide. The IL-\(\beta\), TNF-\(\alpha\), and -beta-actin primers produce amplified products at 387, 374, and 349 bp, respectively. The iNOS primers produce a 311- and a 231-bp product from the RNA and the internal standard, respectively.

**Western Immunoblot Analysis.** Whole-cell lysates (20 \(\mu\)g) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), then electrophoretically transferred to nitrocellulose membranes (Amersham International, Buckinghamshire, UK). The membranes were preincubated for 1 h at room temperature in Tris-buffered saline, pH 7.6, containing 0.05% Tween 20 and 3% fatty acid-free BSA. The nitrocellulose membranes were incubated with phosphorylated p38 or p38-specific antibodies purchased from New England Biolabs (Beverly, MA). Immunoreactive bands were then detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham International).

**Immunoprecipitation.** Immunoprecipitation was performed on the whole cell lysates with anti-p38 (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A agarose beads. After incubation for 2 h at 4°C, immunoprecipitates were washed twice with ice-cold lysis buffer. For immunoblotting, immunoprecipitates were denatured, separated by 10% SDS-PAGE, and electrotransferred to nitrocellulose membranes, and immunoblot analysis was performed.

**In Vitro p38 Kinase Assay.** p38 Kinase activity was assayed by phosphorylation of activating transcription factor-2 (ATF-2; Santa Cruz Biotechnology). Immunoprecipitated p38 were washed twice in kinase buffer containing 25 mM HEPES, pH 7.2, 20 mM MgCl\(_2\), 0.1 mM sodium orthovanadate, and 2 mM dithiothreitol (DTT), and incubated in kinase buffer containing ATP-2 (3 \(\mu\)g), 20 \(\mu\)M ATP, and 5 \(\mu\)Ci of \([\gamma^{32P}]\)ATP for 30 min at 30°C. Reactions were terminated by the addition of gel-loading buffer, the samples were resolved by SDS-PAGE, and phosphoproteins visualized by autoradiography.

**Electrophoretic Mobility Shift Assay (EMSA).** EMSA was performed as previously described (Jeon et al., 1996). Nuclear extracts were prepared as previously described (Xie et al., 1993). Treated and untreated RAW 264.7 cells were lysed with hypotonic buffer (10 mM HEPES, 1.5 mM MgCl\(_2\), pH 7.5) and the nuclei were pelleted by centrifugation at 3000g for 5 min. Nuclear lysis was performed with 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 phenylmethylsulfonyl fluoride, 1 \(\mu\)g/ml apronin, and 1 \(\mu\)g/ml leupeptin. After lysis, the samples were centrifuged at 14,500g for 15 min, and the supernatant was retained for use in the DNA-binding assay. The oligonucleotide sequences for NF-\(\kappa\)B/Rel (Pierce et al., 1988; Jeon et al., 1996) and octamer-binding transcription factor (Annweiler et al., 1993) were as follows: 5'-GATCCTCAGAGGGGACCTTCCGAGAAGA-3' and 5'-GATCCTTCTAGAGGATCATCGAACATCGA-3', respectively. The double-stranded deoxyoligonucleotides were end-labeled with \([\gamma^{32P}]\)ATP. Nuclear extracts (5 \(\mu\)g) were incubated with poly(dI-dC) and the \([\gamma^{32P}]\)P-labeled DNA probe in binding buffer (100 mM KCl, 30 mM HEPES, 1.5 mM MgCl\(_2\), pH 7.5) and the nuclei were pelleted by centrifugation at 3000g for 5 min. Nuclear lysis was performed with 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 phenylmethylsulfonyl fluoride, 1 \(\mu\)g/ml apronin, and 1 \(\mu\)g/ml leupeptin for 10 min. DNA-binding activity was separated from the free probe with a 4.8% polyacrylamide gel in 0.5 \(\times\) TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA). After electrophoresis, the gel was dried and subjected to autoradiography.

**Statistical Analysis.** The mean \(\pm\) S.D. was determined for each treatment group in a given experiment. When significant differences occurred, treatment groups were compared with the vehicle controls with a Dunnett’s two-tailed t test (Dunnett, 1955).

**Results**

**Effect of Radicicol on Nitrite Production and iNOS Gene Expression in Macrophages.** LPS (200 ng/ml) alone increased the production of nitrite \(\geq\)8-fold over basal levels in peritoneal macrophages (Fig. 1A) and RAW 264.7 cells (Fig. 1B). This induction in nitrite generation by LPS was inhibited by radicicol in a dose-dependent manner. Consistent with these findings, radicicol inhibited the production of the iNOS mRNA in LPS-stimulated RAW 264.7 cells (Fig. 2). Under the same treatment condition the effect of radicicol on the expression of TNF-\(\alpha\) and IL-1\(\beta\), cytokine markers of
macrophage activation, also were examined. Radicicol inhibited TNF-α and IL-1β mRNA expression in a dose-related manner. The expression of iNOS mRNA was more sensitively inhibited rather than TNF-α and IL-1β mRNA expression by radicicol. Competitive RT-PCR analysis was used to determine more precisely whether radicicol inhibited the expression of iNOS mRNA. iNOS mRNA was not detectable in unstimulated RAW 264.7 cells. Conversely, RNA isolated from RAW 264.7 cells treated for 6 h with LPS showed active transcription of the iNOS gene. Furthermore, radicicol inhibited LPS-induced iNOS mRNA production in a dose-dependent manner (Fig. 3). No effect on cell viability was observed in any of the treatment groups and always exceeded 90% as determined by trypan blue staining (data not shown).

Effects of Radicicol on Activation of p38. Because p38 MAPK has been shown to be required for iNOS induction mediated by LPS in RAW 264.7 macrophages (Chen and Wang, 1999), we investigated the effect of radicicol on the activation of p38 in LPS-stimulated RAW 264.7 cells. Activation of MAPK requires phosphorylation at threonine and tyrosine residues. Immunoblot analysis with antiphosphospecific p38 antibody was performed. Time course experiment showed the activation of p38 was peak after 10- or 30-min treatment and declined to basal level after 60-min treatment (data not shown). When cells were pretreated with radicicol (0.1, 0.03, 0.1, or 0.3 μg/ml) for 30 min before incubation with LPS (200 ng/ml) for 20 min, LPS-induced activation of p38 MAPK was attenuated in a dose-dependent manner (Fig. 4). To further confirm the inhibition of p38 enzyme activation by radicicol and the correlation between phosphorylation of p38 and kinase activity, we performed in vitro p38 kinase assay. When we assayed the immunoprecipitated p38 kinase activity by phosphorylation of ATF-2, p38 kinase activity was found to be inhibited by radicicol (200 ng/ml) treatment (Fig. 5). SB203580, a potent inhibitor of p38 kinase, inhibited LPS-induced p38 kinase activity, whereas PD98059, an inhibitor of extracellular signal-regulated protein kinase-1/2, did not inhibit the kinase activation.
Effects of MAPK Inhibitors on iNOS Gene Expression in Macrophages. The effect of MAPK inhibitors on iNOS gene expression was analyzed by competitive RT-PCR. RNA isolated from RAW 264.7 cells treated for 6 h with LPS showed active transcription of the iNOS gene. Furthermore, radicicol inhibited LPS-induced iNOS mRNA production (Fig. 6). The LPS-induced iNOS mRNA expression also was inhibited by SB203580 treatment, whereas PD98059 could not influence the expression of iNOS mRNA. These results suggested that p38 kinase pathway plays an important role in the LPS-induced iNOS gene expression.

Inhibition of NF-κB/Rel-Binding Activity by Radicicol Treatment in LPS-Stimulated RAW 264.7 Cells. It has been reported that protein binding at the κB binding site is necessary to confer inducibility of iNOS by LPS (Xie et al., 1994). In these experiments, we investigated the role of p38 kinase inhibition by radicicol on regulation of NF-κB/Rel and the relationship between NF-κB/Rel and LPS-induced iNOS gene expression. Our initial studies demonstrated that LPS (200 ng/ml) treatment of RAW 264.7 cells induced a marked increase in NF-κB/Rel binding to its cognate site at 2 h, which could be visualized as two distinct bands (Fig. 7A). In the presence of radicicol, LPS-induced NF-κB/Rel binding was noticeably inhibited in a dose-related manner (Fig. 7A).
The NF-κB/Rel binding complex was identified by gel supershift assay (Fig. 7B). Both upper and lower bands were supershifted dramatically when the nuclear extract was preincubated with antibodies against p50 and c-rel, respectively. Thus, the upper band appears to be composed of p50/p65 and p50/c-rel heterodimers, whereas the lower band appears to consist of p50 homodimers. The specificity of the bindings was demonstrated by competition assays with 32P-unlabeled κB (Fig. 7C).

**Involvement of p38 Kinase Pathway in Activation of NF-κB/Rel.** Because our results (Fig. 6) and previous reports (Da Silva et al., 1997; Chen and Wang, 1999) showed that the p38 kinase pathway is important in the induction of iNOS, we investigated the effect of p38 kinase inhibitor on the activation of NF-κB/Rel. Treatment of RAW 264.7 cells with p38 kinase inhibitor SB203580 inhibited the activation of NF-κB/Rel in LPS-stimulated RAW 264.7 cells, whereas PD98059 did not affect NF-κB/Rel activation (Fig. 8). Another transcription factor, octamer-binding transcription factor, whose binding site is located in the promoter of iNOS gene, was high in activity and not affected by MAPK inhibitors (Fig. 8).

**Reduction of Nuclear Contents of NF-κB/Rel Family Members by Radicicol Treatment in LPS-Stimulated RAW 264.7 Cells.** To further characterize the mechanism of radicicol in the inhibition of nuclear factor bindings, we investigated the effect of radicicol on the mobilization of NF-κB/Rel into the nucleus of LPS-stimulated RAW 264.7 cells. Nuclear extracts were prepared and subjected to immunoblot analysis. The amounts of nuclear c-rel, p65, and p50 were increased at 30 min after LPS treatment, as shown in Fig. 9A. However, stimulation of cells with LPS in the presence of radicicol (200 ng/ml) resulted in the reduction of nuclear contents of c-rel, p65, and p50. Because the activation of NF-κB/Rel is dependent on the phosphorylation and subsequent degradation of IκB proteins, we investigated the effect of radicicol on the degradation of IκB in LPS-stimulated RAW 264.7 cells. Treatment of RAW 264.7 cells with LPS (200 ng/ml) for 30 min induced significant reduction of IκBα and IκBβ (Fig. 9B). Conversely, stimulation of cells with LPS in the presence of radicicol (200 ng/ml) prevented the loss of IκBα and IκBβ.

**Fig. 8.** Effect of radicicol, PD98059, or SB203580 on NF-κB/Rel activation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with radicicol (200 ng/ml), PD98059 (50 μM), or SB203580 (30 μM) for 30 min before incubation with LPS (200 ng/ml) for 2 h. Nuclear extracts were then prepared and subjected to EMSA. One of two representative experiments is shown.

**Discussion**

We demonstrate that radicicol treatment significantly attenuates LPS-induced NO production and iNOS transcription through the blocking of NF-κB/Rel and negative regulation of p38 kinase pathway in the macrophage line RAW 264.7. The major finding of this study is that radicicol significantly inhibits iNOS expression in the macrophage line RAW 264.7. Because radicicol inhibits NF-κB/Rel, which is critically involved in the transcription of iNOS gene, the mechanism for the inhibition of iNOS may be related to the inhibition of transcription. However, we cannot exclude the possibility that radicicol promotes mRNA instability.

We also showed that radicicol significantly inhibits the p38 kinase pathway in RAW 264.7 cells. Although it has recently been reported that a radicicol-related macrocyclic nonaketide compound inhibits the p38 pathways (Takehana et al., 1999) in anisomycin-induced HeLa cells, the p38 pathways are not inhibited by radicicol or another analog 87-250904-F1. This is the first report showing that radicicol inhibits the p38 kinase pathway in LPS-stimulated macrophage line RAW 264.7 cells. Because radicicol inhibits LPS-induced but not anisomycin-induced p38 kinase pathways, the inhibition mechanism may be signal specific. The p38 kinase is an important mediator of stress-induced gene expression (Rainingaud et al., 1995). In particular, the p38 kinase is known to play a key role in LPS-induced signal transduction pathways leading to cytokine synthesis (Lee et al., 1994; Lee and Young, 1996). The involvement of p38 kinase and iNOS expression is controversial. Paul et al. (1999) described no effect of SB203580 on iNOS expression in LPS-induced RAW 264.7 macrophages. Also Chan et al. (1999) found no effect of SB203580 on interferon-γTNF-α induced iNOS expression in mouse macrophages. However, it was demonstrated that p38 MAPK activation is involved in iNOS expression in TNF-α- and IL-1-stimulated mouse astrocytes, as well as in LPS-stimulated mouse macrophages (Da Silva et al., 1997; Chen and Wang, 1999). Our data also showed that the p38 MAPK pathway is specifically involved in LPS-induced iNOS expression because iNOS mRNA production in the presence of a specific inhibitor of p38 MAPK, SB203580, was dramatically diminished. In contrast, PD98059, a specific inhibitor of MAPK/extracellular signal-regulated protein kinase ki-
nase 1, had no effect on iNOS expression. Thus, radicicol, like to SB203580, inhibits the iNOS gene expression through blocking the p38 kinase pathway.

The p38 MAPK also regulates LPS-induced TNF-α, IL-1, and IL-10 production in monocytes and TNF-induced IL-6 production in fibroblasts (Beyaert et al., 1996; Foey et al., 1998). These findings are consistent with the idea that p38 MAPK can be predominantly activated by LPS and inflammatory cytokines such as TNF and IL-1, and can play an important role in the expression of a number of proinflammatory molecules (Lee and Young, 1996). Our data also showed that LPS-induced production of IL-1β and TNF-α mRNA was inhibited by radicicol. However, the expression of iNOS mRNA was more sensitively inhibited rather than TNF-α and IL-1β mRNA expression by radicicol (Fig. 2). The differences in the sensitivities can possibly be explained by the differential role of p38 kinase in the regulation of the cytokine expression. For TNF-α and IL-1β mRNA expression, p38 kinase functions by regulating processes that control translation of the cytokine mRNA (Young et al., 1993; Prichett et al., 1995) rather than by controlling transcription of the genes, whereas the expression of iNOS by p38 kinase is mainly regulated at the transcriptional level (Da Silva et al., 1997; Chen and Wang, 1999). Thus, the control of cytokine synthesis by p38 kinase can function at different levels, even within the same cell. We cannot exclude the possibility that factors other than p38 kinase can play a role in the sensitivity differences in mRNA expressions. For example, NF-κB/Rel transcription factors are critical in the transcription of iNOS, whereas the expression of TNF-α and IL-1β genes requires other transcription factors such as activating protein-1, NF-IL6, and cAMP response element-binding protein/ATF as well as NF-κB/Rel (Novotny et al., 1998; Zagariya et al., 1998; Baldassare et al., 1999).

As p38 kinase is involved in the induction of TNF-α and IL-1β, and these cytokines induce iNOS gene expression, the effects of radicicol may be due to the down-regulation of TNF-α and IL-1β. However, we observed that low concentrations of radicicol (0.03 and 0.1 μg/ml) inhibited the p38 kinase activity (Fig. 4) and NF-κB/Rel DNA-binding activity (Fig. 7) but not the expression of TNF-α and IL-1β (Fig. 2).

Thus, we suggest that radicicol directly inhibits the gene expression of iNOS through blocking p38 kinase pathways and NF-κB/Rel pathways.

Our study showed that NF-κB/Rel is positively regulated by LPS for iNOS gene expression, and radicicol treatment of RAW 264.7 cells 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 60 min. 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 and p50/c-Rel heterodimers, whereas the lower band appears to consist of p50 homodimers. It has been shown that p50 proteins have DNA-binding activity and p65 (Schmitz and Baueuerle, 1991) and c-Rel (Bull et al., 1990) proteins have transactivation domains in their C termini and thus are able to activate transcription of target genes. This finding suggests that radicicol may inhibit the formation of either p50/c-Rel or p50/p65 heterodimers based on the gel supershift studies (Fig. 7B).

In summary, these experiments demonstrate that radicicol 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 NF-κB/Rel through negative regulation of p38 kinase pathway. Inhibition of p38 kinase pathway attenuates the activation of NF-κB/Rel-binding proteins, which are necessary for the activation of the iNOS gene. At least two significant points are brought out by these studies. First, these experiments further confirm the critical role of the p38 kinase pathway in the regulation of iNOS via NF-κB/Rel. Second, due to the critical role that NO release plays in mediating inflammatory responses, the inhibitory effects of radicicol on iNOS suggest that this family of anti-fungal compounds may represent a useful anti-inflammatory agent. This is further supported by recent findings that radicicol compounds inhibit the production of proinflammatory cytokines and cyclooxygenase 2, another critical mediator of macrophage-mediated inflammation (Hwang et al., 1996).

References


Novotny V, Prieschl EE, Csonga R, Fabjani G and Baumruker T (1998) Nrf1 in a
Mirrington RN, Ritchie E, Shoppee CW, Tayler WC and Sternhell S (1964) The
McCapra F, Scott A, Delmotte P, Delmotte-Plaquee J and Bhacca NS (1964) The
Kwon HJ, Yoshida M, Abe K, Horinouchi S and Beppu T (1992a) Radicicol, an agent
Lee JC and Young PR (1996) Role of CSBP/p38/RK stress response kinase in LPS
Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russel SW and
Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D,
Kinoshita Y, Ohtake K, Fujisawa Y and Watanabe T (1991) Specific and signal-

Send reprint requests to: Hwan M. Kim, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea.
E-mail: hwanmok@kribb.re.kr