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Magnolol Inhibits iNOS, p38 Kinase, and NF-κB/Rel in Murine Macrophages

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ABSTRACT. We demonstrate that magnolol, a hydroxylated biphenyl compound isolated from *Magnolia officinalis*, inhibits LPS-induced expression of iNOS gene in RAW 264.7 cells (murine macrophage cell line). Treatment of RAW 264.7 cells with magnolol inhibited LPS-stimulated nitric oxide production in a dose-related manner. RT-PCR analysis showed that the decrease of NO was due to the inhibition of iNOS gene expression. Western immunoblot analysis of phosphorylate p38 kinase showed magnolol significantly inhibited the phosphorylation of p38 kinase which is important in the regulation of iNOS gene expression. The specific p38 inhibitor SB203580 abrogated the LPS-induced NO generation and iNOS expression, whereas the selective MEK-1 inhibitor PD98059 did not affect the NO induction. Immunostaining of p65 and reporter gene assay showed that magnolol inhibited NF-κ/Rel nuclear translocation and transcriptional activation, respectively. Collectively, this series of experiments indicates that magnolol inhibits iNOS gene expression by blocking NF-κ/Rel and p38 kinase signaling. Due to the critical role that NO release plays in mediating inflammatory responses, the inhibitory effects of magnolol on iNOS suggest that magnolol may represent a useful anti-inflammatory agent.

Keywords: Magnolol, Macrophages, p38 kinase, iNOS, NF-κB/Rel.

INTRODUCTION

Magnolol, a compound purified from *Magnolia officinalis* (Magnoliaceae) which has long been used for the treatment of fever, headache, anxiety, diarrhea, asthma, and stroke, has strong anti-inflammatory effects (Wang *et al.*,1992). It has been reported that magnolol relaxes rat vascular smooth muscle (Teng *et al.*, 1990), scavenges hydroxyl radicals (Fujita and Taira, 1994), inhibits meutrophil aggregation and superoxide anion generation (Wang *et al.*, 1998, 1999), suppresses the expression of vascular cell adhesion molecule-1 in endothelial cells (Chen *et al.*, 2002), inhibits nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages (Matsuda *et al.*, 2001). Recently, it has been reported that anti-inflammatory effects of magnolol are mediated through inhibition of the downstream pathway of MEKK-1 in NF-κB activation signaling (Lee *et al.*, 2005).

Bacterial 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 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 kB 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 kB site is necessary to confer inducibility by LPS (Xie *et al.*, 1994).

A p38 kinase is an important mediator of stress-induced gene expression (Raingeaud *et al.*, 1995). In

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particular, the p38 kinase is known to play a key role in LPS-induced signal transduction pathways leading to cytokine synthesis (Lee and Young, 1996). It was deomonstrated that p38 kinase activation is involved in iNOS expression in tumor necrosis factor-α (TNF-α) 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).

In the present studies, we investigated the effect of magnolol on the production of NO, an important indicator of inflammation. To further investigate the mechanism by which magnolol inhibits the expression of iNOS gene, we assessed the effects of magnolol on the activation of NF-κB/Rel and p38 kinase. The present studies demonstrate that magnolol inhibits iNOS gene expression through the inhibition of NF-κB/Rel and p38 kinase pathways.

MATERIALS AND METHODS

Materials

Magnolol was isolated from *Magnolia officinalis* (Magnoliaceae). LPS from *Salmonella thyposa* was purchased from Sigma (St. Louis, MO, USA). Reagents used for cell culture were purchased from HyClone (Logan, UT, USA). Anti-iNOS and anti-p65 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Anti-phosphorylated p38 and p38 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

RAW 264.7 cells (murine macrophage cell line) were purchased from American Type Culture Collection (Bethesda, MD, USA). Cells were grown in DMEM/High glucose supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were then cultured in the presence of 5% CO_2 at 37°C. Peritoneal cells were harvested by sterile peritoneal larvage with Hanks' balanced salt solution, washed, resuspended in culture medium, and plated at 5×10^5 cells/ml. Nonadherent cells were removed by repeated 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 pretreated with magnolol for 1 h before incubation with LPS (200 ng/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. Using $NaNO_2$ to generate a standard curve, nitrite production was measured by an O.D. reading at 540 nm.

Western immunoblot analysis

Whole cell lysates (20 μg) were separated by 10% SDS-PAGE, then electro-transferred to nitrocellulose membranes (Amersham International, Bucking-hamshire, UK). The membranes were incubated in blocking buffer [0.1% Tween-20 and 5% skim milk in Tris-buffered saline (TBS, pH 7.6)] for 1 h at room temperature. The nitrocellulose membranes were incubated with iNOS, phosphorylated p38, or p38-specific antibodies (1,000 : 1 dilution, rabbit polyclonal) overnight at 4°C. Immunoreactive bands were then detected by incubation with conjugates of anti-rabbit IgG with WEST-ZOLTM PLUS (Intron Bio, Korea).

RT-PCR

Total RNA was isolated using TRIzol Reagent (Carlsbad, CA, USA) as described previously (Chomczynski *et al.*, 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)12-18 primer. 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, 57°C for 1 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 1% Seakem LE agarose gels (BMA, 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.*, 1993), 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 h. The transfectants were treated with LPS and magnolol. Eighteen hours later the cells were lysed with lysis buffer (250 ml). 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.

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 magnolol on nitrite production in macrophages

To investigate the effects of magnolol on NO production, we measured the accumulation of nitrite, the stable end product of NO, in the culture media using Griess reagent. RAW 264.7 cells were pretreated with magnolol for 1 h before incubation with LPS (200 ng/ml) for 24 h. Potent macrophage activator LPS alone increased the production of nitrite ≥ 9-fold over basal levels in RAW 264.7 cells (Fig. 1A). This induction in nitrite generation by LPS was inhibited by magnolol in a dose-dependent manner. Treatment of peritoneal macrophages with magnolol also inhibited the production of nitrite in a dose-related manner (Fig. 1B).

Effect of magnolol on the gene expression of iNOS

After RAW 264.7 cells were exposed to magnolol in the presence of LPS, the expression level of iNOS gene was monitored by Western immunoblot analysis and RT-PCR. As shown in Fig. 2A, iNOS protein pro-

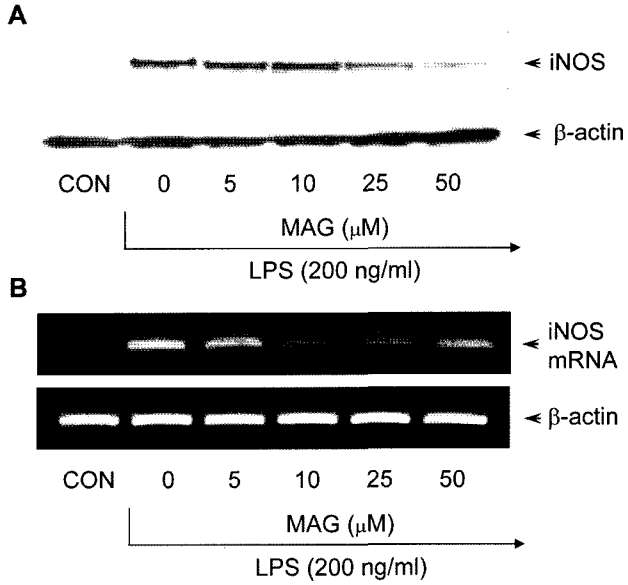

Fig. 2. Inhibition of iNOS gene expression by magnolol in LPS-stimulated RAW 264.7 cells. (A) Cells (5×10^5 cells/ml) were treated with magnolol (5, 10, 25, and 50 μM) before treatment with LPS (200 ng/ml) for 16 h. Cell lysates were then prepared and subjected to Western immunoblotting. (B) Cells (5×10^5 cells/ml) were preincubated with magnolol (5, 10, 25, and 50 μM) before treatment with LPS (200 ng/ml) for 8 h. Total RNA was isolated and analyzed for the magnitude of mRNA expression of iNOS using RT-PCR. One of two representative experiments is shown.

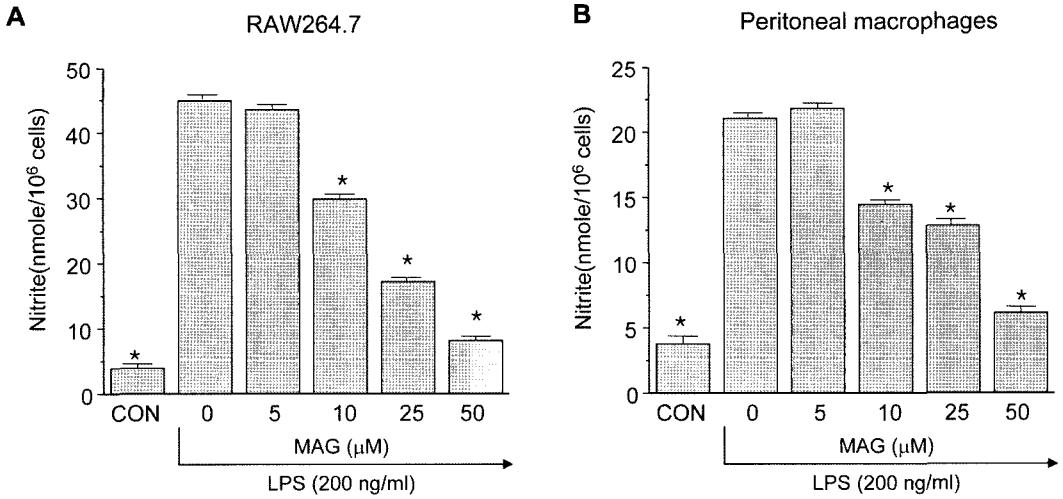

Fig. 1. Inhibition of nitrite production by magnolol in LPS-stimulated RAW 264.7 cells and peritoneal macrophages. Cells (5×10^5 cells/ml) were pretreated with magnolol (5, 10, 25, and 50 μM) for 1 h before treatment with LPS (200 ng/ml) for 24 h. The supernatants were subsequently isolated and analyzed for nitrite. Each value shows the mean ± S.D. of triplicate determinations.

duction was inhibited by magnolol treatment in a dose-dependent manner. Consistent with this finding the transcription of iNOS mRNA was dose-dependently inhibited by magnolol (Fig. 2B). The result reflected that the decreased production of NO in macrophage was mediated by the inhibition of iNOS gene expression. Control β-actin was constitutively expressed and was not affected by the treatment with magnolol. These results indicate that magnolol decreases the gene expression of iNOS, which is involved in inflammation (Hibbs *et al.*, 1987).

Inhibition of p38 kinase by magnolol in LPS-stimulated macrophages

We investigated the effect of magnolol on the activation of p38 in LPS-stimulated RAW 264.7 cells. Activation of p38 kinase requires phosphorylation at threonine and tyrosine residues. Immunoblot analysis with antiphospho-specific 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 1 h treatment (data not shown). When cells were pretreated with magnolol (5, 10, 25, or 50 μM) for 1 h before incubation with LPS (200 ng/ml) for 20 min, LPS-induced activation of p38 was attenuated in a dose-dependent manner (Fig. 3).

We further investigated whether p38 kinase pathway is involved in LPS-induced nitrite generation. We specifically blocked p38 kinase pathway and monitored the nitrite production when RAW 264.7 cells were challenged with LPS. SB203580, a bicyclic imidazole compound, is a specific inhibitor of p38 (Cuenda *et al.*, 1995). PD98059 is a specific inhibitor of MEK-1, mitogen activated protein kinase/extracellular signal-regulated kinase 1, which is responsible for ERK1/2 activation (Dudley *et al.*, 1995). SB203580 inhibited LPS-induced nitrite generation, while PD98059 did not inhibit the nitrite production (Fig. 4A). The LPS-induced iNOS expression was also specifically inhibited by SB203580 but not by PD98059 (Fig. 4B). These results suggest that p38 kinase pathway is important in the regulation of iNOS expression by LPS.

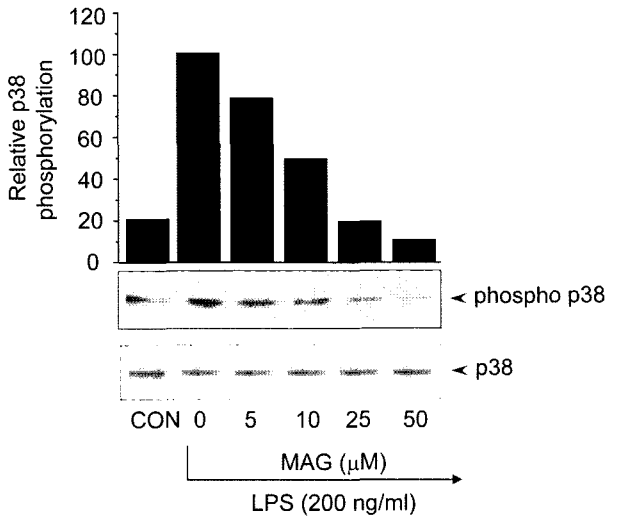

Fig. 3. Inhibition of p38 kinase phosphorylation by magnolol in LPS-stimulated RAW 264.7 cells. Cells were pretreated with magnolol (5, 10, 25, and 50 μM) for 30 min before incubation with LPS (200 ng/ml) for 20 min. Cell extracts were then prepared and subjected to Western immunoblotting with antibodies specific for phosphorylated form of p38 or for p38. One of two representative experiments is shown.

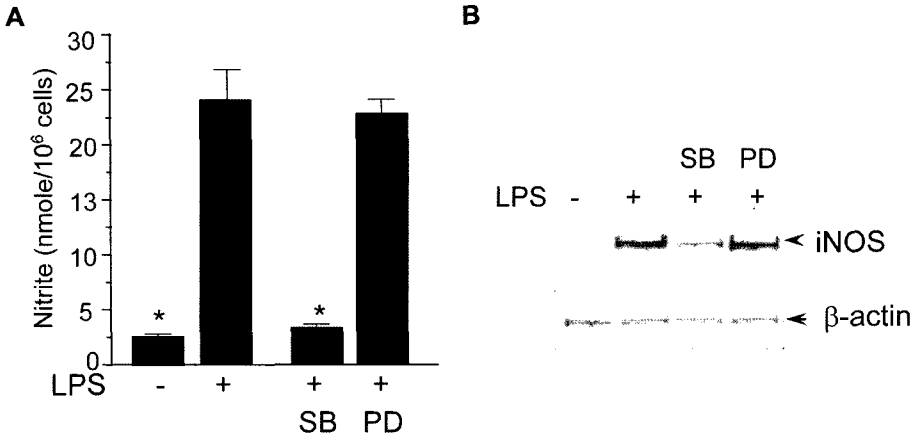

Fig. 4. Effects of SB203580 and PD98059 on nitrite production and iNOS expression in LPS-stimulated RAW 264.7 cells. Cells (5×10^5 cells/ml) were pretreated with SB203580 (30 μM) and PD98059 (50 μM) for 30 min before incubation with LPS (200 ng/ml) for 24 h. (A) Nitrite generation was determined from the culture supernatant. (B) Cell extracts were then prepared and subjected to Western immunoblotting with antibodies specific for iNOS.

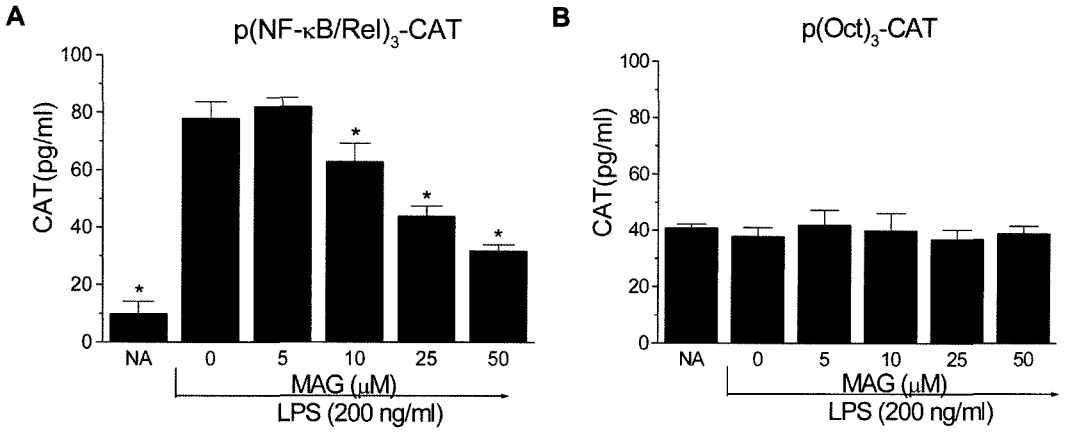

Fig. 5. Inhibition of NF-κB/Rel transcriptional activation by magnolol in LPS-stimulated RAW 264.7 cells. Cells (5×10^5 cells/ml) were transfected with $p(NF\text{-}\kappa B/Rel)_3$-CAT (A) or $p(Oct)_3$-CAT (B) by DEAE dextran method. Twenty-four hours after transfection, cells were treated with the indicated concentrations of magnolol in the presence of LPS (200 ng/ml) for 18 h. Cell extracts were then prepared and analyzed for the expression of CAT using CAT ELISA kit. One of two representative experiments is shown.

Inhibition of NF-κB/Rel in response to magnolol in LPS-stimulated macrophages

To further investigate the molecular mechanism of magnolol-mediated inhibition of macrophage, we focused on the activation of transcription factors whose binding sites are in the promoter of iNOS gene. Since it has been reported that protein binding at the kB binding site is necessary to confer inducibility by LPS of iNOS (Xie *et al.*, 1994). We assessed the effect of magnolol on NF-κB/Rel using a transient transfection assay. When RAW 264.7 cells were transiently transfected with $p(NF\text{-}\kappa B/Rel)_3$-CAT, the CAT gene expressions were found to be inhibited by magnolol in the presence of LPS (Fig. 5A). Basal levels of CAT expression in unstimulated RAW 264.7 cells were < 10 pg/ml ± 5.5 (mean ± standard deviation, two experiment). On LPS-stimulation, CAT expression in RAW 264.7 cells was increased about 8.3-fold. Magnolol 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 magnolol (Fig. 5B).

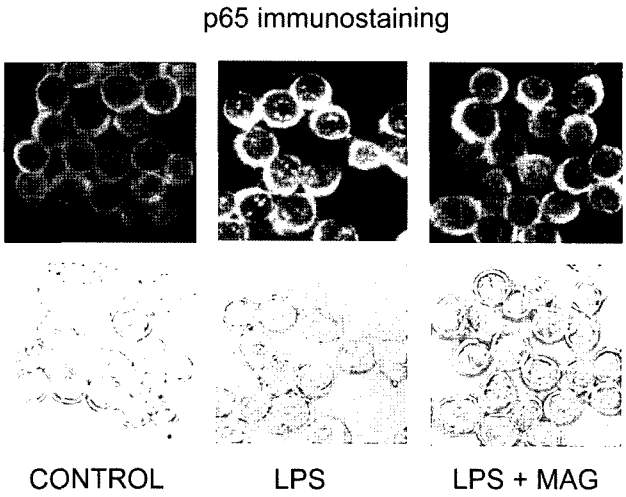

Fig. 6. Inhibition of p65 nuclear translocation by magnolol in LPS-stimulated RAW 264.7 cells. Cells (1×10^5 cells/ml) were pre-incubated with magnolol (50 μM) for 1 h before treatment with LPS (200 ng/ml) for 2 h on cover slide in 12 well plates. Cells were subjected to immunohistochemical staining using an antibody specific for murine p65. One of two representative experiments is shown.

Inhibition of NF-κB/Rel nuclear translocation by magnolol in LPS-stimulated macrophages

To further investigate whether magnolol 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. Magnolol treatment significantly inhibited LPS-induced nuclear translocation of p65 (Fig. 6). These results indicate that magnolol decreases the nuclear translocation of NF-κB/Rel, which is important in the regulation of iNOS gene expression.

DISCUSSION

We demonstrate that magnolol treatment significantly attenuates LPS-induced NO production and iNOS transcription through the blocking of NF-κB/Rel and nega-

tive regulation of p38 kinase pathway in the macrophage cell line RAW 264.7. The major finding of the present study is that magnolol significantly inhibits the iNOS expression in the RAW 264.7. Since magnolol 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 magnolol promotes mRNA instability.

We also showed that magnolol significantly inhibits the p38 kinase pathway in LPS-stimulated RAW 264.7 cells. The p38 kinase is an important mediator of stress-induced gene expression (Raingeaud *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 and Young, 1996). 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 previous study (Jeon *et al.*, 2000) 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 MEK1 had no effect on iNOS expression. Thus, magnolol 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 (Foey *et al.*, 1998, Beyaert *et al.*, 1996). 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 study showed that NF-κB/Rel is positively regulated by LPS for iNOS gene expression, and magnolol 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 kB motifs in the regulatory region of a variety of genes. NF-κB/Rel reporter gene assay showed strong induction of reporter gene expression by LPS. Magnolol inhibited LPS-induced expression of the reporter gene (Fig. 5). The inhibition of nuclear translocation of NF-κB/Rel by magnolol was further confirmed by the immunostaining of p65 (Fig. 6).

In summary, these experiments demonstrate that magnolol, a hydroxylated biphenyl compound isolated from *Magnolia officinalis*, 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-κ/Rel through negative regulation of p38 kinase pathway. At least two significant points are brought out by these studies. First, these experiments further confirm the critical role of the p38 kinase pathway and 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 magnolol on iNOS suggest that magnolol may represent a useful anti-inflammatory agent.

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