A Diarylheptanoid from Lesser Galangal (Alpinia officinarum) Inhibits Proinflammatory Mediators via Inhibition of Mitogen-Activated Protein Kinase, p44/42, and Transcription Factor Nuclear Factor-κB

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ABSTRACT
The diarylheptanoid 7-(4’-hydroxy-3’-methoxyphenyl)-1-phenylhept-4-en-3-one (HMP) is a naturally occurring phytochemical found in lesser galangal (Alpinia officinarum). In the present study, we have demonstrated the anti-inflammatory properties of this compound on mouse macrophage cell line (RAW 264.7) and human peripheral blood mononuclear cells (PBMCs) in vitro. Treatment of RAW 264.7 cells with HMP (6.25–25 μM) significantly inhibited lipopolysaccharide (LPS)-stimulated nitric oxide (NO) production. This compound also inhibited the release of LPS-induced proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) from human PBMCs in vitro. In addition, Western blotting and reverse transcription-polymerase chain reaction analysis demonstrated that HMP decreased LPS-induced inducible nitric-oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein and mRNA expression in RAW 264.7 cells. Furthermore, HMP treatment also reduced nuclear factor-κB (NF-κB) DNA binding induced by LPS in RAW 264.7 cells. To elucidate the molecular mechanism for inhibition of proinflammatory mediators by HMP (25 μM), we have studied the effect of HMP on LPS-induced p38 and p44/42 mitogen-activated protein kinase (MAPK). We observed that the phosphorylation of p44/42 MAPK in LPS-stimulated RAW 264.7 cells was markedly inhibited by HMP, whereas activation of p38 MAPK was not affected. These results suggested that HMP from lesser galangal suppressed the LPS-induced production of NO, IL-1β, and TNF-α and expression of iNOS and COX-2 gene expression by inhibiting NF-κB activation and phosphorylation of p44/42 MAPK.

The use of herbal therapy or alternative medicine is becoming an increasingly attractive approach for the treatment of various inflammatory disorders. The majority of naturally occurring phytochemicals found in plants possess tremendous antioxidant and anti-inflammatory activities (Surh et al., 2001). Anti-inflammatory properties of various phytochemicals are mediated through the inhibition of nitric oxide (NO), prostaglandins, leukotrienes, and production of cytokines such as interleukin (IL)-1β, IL-6, IL-12, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). Antioxidants such as (--)-epigallocatechin-3-gallate (Lin and Lin, 1997), resveratrol (Tsai et al., 1999) and naturally occurring flavonoids, including apigenin and kaempferol (Liang et al., 1999) have been reported to suppress NO production through inhibition of nuclear factor-κB (NF-κB). Previous studies have shown that ginger (Zingiber officinale) and its constituents, which are used for the treatment of cancer, are potent inhibitors of immune cell activation and cytokine secretion (Ageel et al., 1989). Pharmacologically, ginger possess very complex mixture of compounds such as gingerols, capsaicin, caffeic acid, and curcumin. Various formulations of ginger have been shown to act as a dual inhibitor of both COX and lipoxygenase (Mustafa et al., 1993), to inhibit leukotriene synthesis (Kiuchi et al., 1992), and to reduce carrageenan-induced rat-paw edema (Mascolo et al., 1989; Jana et al., 1999).
al., 1999). Another closely related plant, commonly known as greater galanga (Alpinia galanga), has also traditionally been used for rheumatic diseases in South East Asian medicine. The German Commission E Monographs lists the use of lesser galanga, which is closely related to greater galanga, for dyspepsia and loss of appetite. The United States Food and Drug Administration lists ginger and lesser galanga as "generally regarded as safe" (21 CFR Section 182.10, 182.20).

Various preparations from lesser galanga have been used as traditional medicine in China due to its significant therapeutic properties for spleen and stomach. Most important compounds identified from lesser galanga are flavonoids and diarylheptanoids. The various gingeroids and diarylheptanoids, naturally occurring in lesser galanga, have been shown as potent inhibitor of prostaglandin synthase enzymatic activity (Kiuchi et al., 1992).

The critical role of NO in various pathological conditions has led to the discovery of new therapeutic agents from varied sources. NO is a short-lived free radical produced from l-arginine in a reaction catalyzed by NO synthase (NOS). It mediates diverse functions by acting on most cells of the body through the interaction with different molecular targets, which can either be activated or inhibited (Xie and Fidler, 1998). At least three types of NOS isoforms have been reported (Nathan and Xie, 1994a): endothelial NOS, neuronal NOS, and inducible NOS (iNOS). The endothelial NOS and neuronal NOS are constitutively expressed and are Ca²⁺/calmodulin-dependent, whereas expression of the high-output isoform iNOS is induced by LPS and various cytokines such as IFN-α, IFN-β, IFN-γ, IL-1α, IL-1β, and TNF-α (Nathan and Xie, 1994b). Low concentrations of NO produced by iNOS possess beneficial roles in antimicrobial activity of macrophages against pathogens (Cook and Cattell, 1996). At the same time, excessive production of NO and its derivatives, such as peroxynitrite and nitrogen dioxide, have been suggested to be mutagenic in vivo and to provoke the pathogenesis of septic shock and diverse autoimmune disorders (Kilbourn et al., 1990; Wink et al., 1991; Nguyen et al., 1992; Miller et al., 1993). Furthermore, NO and its oxidized forms have also been shown to be carcinogenic (Halliwell, 1994). Therefore, agents that can suppress high NO production by inhibiting iNOS expression or its activity can be used as potential therapeutic tools for management of NO-related disorders.

In the current study, we have evaluated a diarylheptanoid, HMP {[7-(4′-hydroxy-3′-methoxyphenyl)-1-phenyleth-4-en-3-one]} (chemical structure shown in Fig. 1), isolated from Alpinia officinarum (Kiuchi et al., 1992; Liu et al., 2003) for its anti-inflammatory properties, specifically by using in vitro model systems of inflammation. We demonstrate that HMP suppresses the LPS-induced proinflammatory cytokines (IL-1β and TNF-α) production from human PBMCs and NO production from mouse macrophage cells (RAW 264.7). HMP also inhibits LPS-induced iNOS and COX-2 mRNA and protein expression. Furthermore, we show that HMP reduces the activation of mitogen-activated protein kinase (MAPK) p44/42 and NF-κB DNA binding activity induced by LPS.

Materials and Methods

Reagents and Cells. Mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). DMEM, RPMI 1640 medium, LPS, Tri-reagent, Ficoll-hypaque, Griess reagent, monoclonal anti-β-actin antibody, and 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). IL-1β and TNF-α ELISA kits were purchased from R & D Systems (Minneapolis, MN). The relative RT-PCR kit for mouse iNOS and COX-2 were obtained from Ambion (Austin, TX), mouse monoclonal anti-iNOS and COX-2 were purchased from BD Biosciences Pharmingen (San Diego, CA), and anti-mouse and anti-rabbit IgG conjugated with horseradish peroxidase were purchased from DAKO (Carpinteria, CA). Monoclonal antibody against phospho-p44/p42 was obtained from Cell Signaling Technology, Inc. (Beverly, MA). Polyclonal antibodies against phospho-p38, total p38, and total p44/p42 were also obtained from Cell Signaling Technology Inc.

Isolation and Identification of HMP. We have isolated a diarylheptanoid from the rhizomes of lesser galanga by bioassay-directed fractionation. Normal phase column chromatography followed by semipreparative reversed-phase high-performance liquid chromatography was used to isolate this diarylheptanoid, which was identified to be HMP (Liu et al., 2003). This compound was confirmed to be 99% pure by high-performance liquid chromatography and NMR studies.

MTT Assay for Cell Viability. MTT is a pale yellow substrate that is reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not reduce significant amounts of MTT. Mouse macrophage cell line RAW 264.7 were cultured in 96-well flat-bottom plate at concentration of 0.25 million/ml and after 12 h of preconditioning, cells were treated with various concentrations of HMP for 48 h. Thereafter, culture medium was aspirated and 100 μl of MTT dye (1 mg/ml in PBS) was added to the cultures and further incubated for 4 h at 37°C. The formazan crystals made due to dye reduction by viable cells were dissolved using acidified isopropanol (0.1 N HCl). Index of cell viability was calculated by measuring the optical density of color produced by MTT dye reduction at 570 nm.

Nitric Oxide Measurement. The RAW 264.7 cells were cultured in DMEM supplemented with 15% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cell suspension of 0.5 million cells/well was cultured for 12 h. Cells were then treated with either LPS (0.5 μg/ml) alone or LPS with various concentrations of HMP (6.25–25 μM) for 24 h. The cell supernatants were collected at the end of culture for nitrite assay, which were used as a measure of NO production (Eigler et al., 1995). Equal volume of Griess reagent (Sigma-Aldrich) was mixed with each group of cell supernatant (100 μl), and the absorbance was measured at 570 nm. The concentration of nitrite (micromolar) was calculated from standard curve drawn with known concentration of sodium nitrite dissolved in DMEM. The results are presented as mean ± S.D. of four replicates of one representative experiment and this experiment was repeated five times with similar results.

TNF-α and IL-1β ELISA. The PBMCs were separated from peripheral blood of normal healthy human volunteers. Cell suspension of 0.5 × 10⁶ cells/ml in RPMI 1640 medium supplemented with 10%...
FBS was prepared and 200 µl/well of this cell suspension was cultured in 96-well flat-bottom plate. PBMC suspension was treated with LPS (10 ng/ml) either alone or in combination of different concentrations of HMP (6.25–25 µM). Cell suspensions from each group were harvested after 18 h and stored at –70 °C until tested. The quantity of IL-1β and TNF-α present in supernatants was estimated by ELISA (R & D Systems) following manufacturer’s instructions. The concentrations of IL-1β and TNF-α in samples were calculated from standard curve drawn with known concentration of recombinant IL-β and TNF-α. The results are presented as mean (picograms per milliliter) ± S.D. of three replicates of one representative experiment and this experiment was repeated three times with similar results.

Preparation of Total Protein Lysate for Western. The RAW 264.7 cells were cultured with LPS alone or with various concentrations of HMP (12.5 and 25 µM) for indicated time points. At the end of incubation, cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium orthovanadate, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM sodium pyrophosphate, and 20% glycerol. After incubation for 30 min on ice, lysates were centrifuged (12,500 rpm, 15 min.) and supernatants were collected and protein concentration in samples was estimated by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) following manufacturer’s instructions.

Western Blotting. Equal amount of protein (40 µg) from each sample was resolved on SDS-polyacrylamide electrophoresis gel (8 and 10% separating gels for iNOS and p42/44MAPK, respectively). After electrophoresis the proteins were transferred to Hybond membranes.

Determination of Relative Change in iNOS and COX-2 mRNA Expression. The RAW 264.7 cells were cultured (10^4/well) in six-well plate for 24 h followed by treatment with LPS either alone or with different concentrations of HMP for 12 h. Total RNA was isolated using Tri-reagent (Sigma-Aldrich), and 5 µg of this total RNA was reverse transcribed to make cDNA using random hexamer and superscript reverse transcriptase (Invitrogen, Carlsbad, CA), following manufacturer’s instructions. Linear range of amplification of iNOS and COX-2 cDNA was determined using gene-specific primers from Ambion, following manufacturer’s instructions. Briefly, the optimum amount of 18S primer and competitor for iNOS and COX-2 gene was determined. The PCR for iNOS (2 µl of cDNA, 30 cycles) and COX-2 (1 µl of cDNA, 25 cycles) was performed in a final volume of 50 µl containing dNTPs (each at 2.5 mM), 1× PCR buffer, 5 units of TaqDNA polymerase, 0.4 µM gene specific primer, and optimum ratio of 18S primer and competitor (3:7). Finally, PCR products from each sample (10 µl) were resolved in 2% agarose gel (Fisher Scientific Co., Fair Lawn, NJ), stained with ethidium bromide, and image of gel was captured at appropriate exposure time. Densitometric analysis was performed using image analysis software (Scion).

Electrophoretic Mobility Shift Assay (EMSA). RAW 264.7 cells were treated with either LPS (0.5 µg/ml) alone or with various concentration of HMP for 2 h. Thereafter, nuclear extracts were prepared using a modified method (Lahti et al., 2000). Briefly, cells were washed once with PBS (pH 7.2) and were suspended in hypotonic buffer A [10 mM HEPES (pH 7.6), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF] for 10 min on ice, and vortexed for 10 s. Nuclei were pelleted by centrifugation at 12,000g for 5 min. Then the pellets were suspended in buffer B [20 mM HEPES (pH 7.6), 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF] for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 12,000g for 20 min and stored at –80 °C. For electrophoretic mobility shift assays, 6 µg of each nuclear extract was mixed with the 32P-labeled double-stranded NF-kB binding consensus oligonucleotides (5′-AGTTGAGGGACCTTTCCAGGC-3′) (Promega, Madison, WI) and incubated at room temperature for 20 min. The incubation mixture contains 1 µg of poly(dI-dC)-in a binding buffer [25 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl]. The DNA/protein complex was electrophoresed on 5% nondenaturing polyacrylamide gels in Tris/acetate/EDTA buffer. The specificity of binding was also examined by competition with the unlabeled oligonucleotides. Mobility shift of DNA due to binding of NF-kB complex was detected by PhosphorImager-445 SI (Amersham Biosciences, Inc.).

Statistical Analysis. Data are expressed as mean ± standard deviation of indicated experiments. Statistical significance between two groups was determined by Student’s t test. The significance level was set at p < 0.05.

Results

Effect of HMP on RAW 264.7 Cell Viability. To find out whether HMP is cytotoxic, RAW 264.7 cells were treated with various concentrations (6.25–25 µM) of HMP for 48 h, and cell viability assay was performed using MTT dye, as described under Materials and Methods. We observed that HMP shows almost no cytotoxic effect on RAW 264.7 cells. There was no significant difference in viability of cells treated with HMP (6.25–25 µM) in comparison with control (data not shown).

Inhibition of LPS-Induced Nitric Oxide Production from Mouse Macrophage RAW 264.7 Cells by HMP. The role of NO in pathogenesis of various inflammatory diseases is well known. The endotoxins such as LPS have been shown to stimulate NO release from macrophages, which play an important role in inflammation. Because half-life of NO is very short, we measured nitrite as an indicator of NO inhibition. The HMP also inhibited LPS-induced NO production (Table 1). However, HMP alone has no effect on NO production. More importantly, even the lowest dose of HMP was also able to inhibit the nitric oxide production (p < 0.05).

Suppression of LPS-Induced Secretion of Proinflammatory Cytokines (IL-1β and TNF-α) by HMP. The production of proinflammatory cytokines from LPS-induced human PBMCs in vitro have been shown previously (Burkart et al., 2002). In addition to suppressive effect of HMP on NO release from RAW 264.7 cells, effect of HMP on LPS-induced secretion of proinflammatory cytokines IL-1β and TNF-α from human PBMCs was also measured. The amount of IL-1β and TNF-α in culture supernatant of human PBMCs after 18 h of treatment with LPS in presence or absence of various doses of HMP (6.25–25 µM) was tested by ELISA. In concordance to NO inhibition, the HMP also inhibited LPS-induced secretion of IL-1β significantly in dose-dependent manner (Table 1). However, the inhibition of TNF-α by HMP...
was only at 25 μM concentration (Table 1). The production of TNF-α from human PBMCs without any treatment (control) or with HMP alone was found below the detection limit (15.62 pg/ml) of assay.

**Inhibition of LPS-Induced iNOS and COX-2 Protein Expression by HMP.** To confirm that whether the inhibition of NO production is due to less enzymatic activity or decreased protein expression of iNOS, we further studied the effect of HMP on iNOS protein expression by Western blotting. In addition to iNOS, we have also studied the effect of HMP on the expression of COX-2 protein, known to be activated in LPS-stimulated macrophages (Liang et al., 1999). Equal amount of protein (40 μg) was resolved to detect the expression of iNOS and COX-2 by Western blot. We found that HMP treatment for 18 h has markedly inhibited iNOS and COX-2 protein expression in RAW 264.7 cells (Fig. 2). The inhibitory concentration of HMP for iNOS protein expression was similar to that for reduction of NO production. The detection of β-actin was also performed in the same blot as an internal control. These experiments have been repeated four times with similar observations.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Nitrite (µM)</th>
<th>IL-1β (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.77 ± 0.82</td>
<td>21.8 ± 0.395</td>
<td>&lt;15.62</td>
</tr>
<tr>
<td>LPS</td>
<td>20.04 ± 3.7</td>
<td>512.01 ± 26.68</td>
<td>290.97 ± 2.41</td>
</tr>
<tr>
<td>HMP (6.25 µM)</td>
<td>3.66 ± 1.08</td>
<td>22.12 ± 3.33</td>
<td>&lt;15.62</td>
</tr>
<tr>
<td>HMP (12.5 µM)</td>
<td>14.54 ± 2.5</td>
<td>339.21 ± 10.53</td>
<td>342.24 ± 11.50</td>
</tr>
<tr>
<td>HMP (12.5 µM) + LPS</td>
<td>3.88 ± 1.14</td>
<td>24.45 ± 1.03</td>
<td>&lt;15.62</td>
</tr>
<tr>
<td>HMP (25 µM)</td>
<td>13.75 ± 2.65*</td>
<td>113.43 ± 3.81*</td>
<td>298.49 ± 5.47</td>
</tr>
<tr>
<td>HMP (25 µM) + LPS</td>
<td>3.86 ± 0.97</td>
<td>23.28 ± 1.41</td>
<td>&lt;15.62</td>
</tr>
<tr>
<td>HMP (25 µM) + LPS</td>
<td>9.95 ± 1.41**</td>
<td>77.27 ± 4.6**</td>
<td>165.38 ± 8.48**</td>
</tr>
</tbody>
</table>

* Represents the statistical significance of difference between HMP + LPS and LPS alone (*p < 0.05; **p < 0.01).

**Inhibition of LPS-Induced Activation of MAPKs.** Because p44/42 and p38 MAPKs have been shown to be involved in iNOS expression induced by LPS in mouse macrophages (Chen and Wang, 1999; Lahti et al., 2000), we investigated the effect of HMP on the activation of p38 and p44/42 MAPK in LPS-stimulated RAW 264.7 macrophages. The phosphorylations of threonine and tyrosine residues are required for the activation of MAPK (Raiingeaud et al., 1995). We have demonstrated that activation of p38 and p44/42 by LPS is highest at 30 min of LPS treatment followed by lower level of activity. When the cells were cotreated with HMP (25 µM) and LPS (0.5 µg/ml), the LPS-induced phosphorylation of p44/42 MAPK was markedly inhibited by HMP at 30-min time point (Fig. 4). However, no effect of HMP was observed on LPS induced phosphorylation of p38 (Fig. 4).

**Inhibition of LPS-Induced NF-κB Activation by HMP.** The involvement of transcription factor NF-κB in the expression of iNOS stimulated by proinflammatory cytokines and LPS is well known. Therefore, to investigate whether inhibition of iNOS expression by HMP involves modulation of NF-κB, EMSA was performed. For this, NF-κB DNA binding activity was studied in nuclear lysates of RAW 264.7 cells.
after 2 h of treatment with LPS alone or with HMP. As shown in Fig. 5, the induction of specific NF-κB DNA binding activity by LPS was inhibited by HMP. The relative levels of NF-κB DNA binding activity with the treatment of 12.5 and 25 μM HMP were less in comparison with LPS alone and marked inhibition was found at 25 μM concentration of HMP. The specificity of binding was examined by competition with the addition of unlabeled/cold oligonucleotides, in excess (data not shown).

Discussion

Herbal remedies and nutriceuticals or botanicals are widely used by both the healthy and the diseased. A number of traditional herb-derived medicines have been developed as an anti-inflammatory drugs and only few of them have been studied for molecular mechanism of action (Surh et al., 2001). To address this issue, we first evaluated the anti-inflammatory properties of HMP isolated from lesser galangal and an attempt was made to dissect out the molecular mechanism of action of this compound. In this study, we demonstrated that HMP is not cytotoxic and inhibits the LPS-induced NO production from RAW 264.7 cells. It also inhibits proinflammatory cytokine IL-1β production from LPS-stimulated human PBMCs, in a dose-dependent manner. However, inhibition of LPS stimulated TNF-α was observed only at 25 μM concentration of HMP (Table 1). We demonstrated that inhibition of NO production is due to inhibition of iNOS expression at mRNA as well as protein level as shown by RT-PCR and Western blot. In addition, we also studied another important mediator of inflammation, COX-2, which acts on arachidonic acid and releases prostaglandins that further orchestrates the process of inflammation (Willoughby et al., 2000). It has been shown previously that LPS stimulates COX-2 expression in macrophages (Zhou et al., 2002). Similar to iNOS inhibition, HMP also inhibits LPS induced COX-2 protein and mRNA expression in a dose-dependent manner as observed by Western blot and RT-PCR (Figs. 2 and 3).

LPS is known to transduce its signal via activating various signaling proteins such as protein tyrosine kinase, MAPK, and protein kinase C. The p38 MAPK is an important mediator of stress-induced gene expression (Raingeaud et al., 1995). In particular, the p38 MAPK is known to play a key role in the LPS-induced signal transduction pathway (Lee et al., 1994; Lee and Young, 1996). Badger et al. (1996) reported that infusion of the p38 inhibitor SB203580 reduces mortality in LPS-treated mice. However, the involvement of p38 kinase and iNOS expression is controversial. Paul et al. (1999) described no effect of the specific p38 kinase inhibitor SB203580 on iNOS expression in LPS-induced RAW 264.7 macrophages. Also, Chan et al. (1999) found no effect of SB203580 on IFN-γ/TNF-α-induced iNOS expression in mouse macrophages. In contrast, it has been 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 (Chen and Wang, 1999).
lates the induction of iNOS by cytokines and LPS. Among the 22 elements homologous to consensus sequence binding sites (Lowenstein et al., 1993; Xie et al., 1994), the binding of this complex in a dose-dependent manner. These data together suggest that HMP regulates the expression of iNOS by suppressing p44/42 and inhibiting NF-κB. To best of our knowledge, this is the first report to show the anti-inflammatory properties of a diarylheptanoid, HMP, from lesser galangal. To establish the specific mechanism for the action of HMP, studies are in progress.

Fig. 5. Inhibition of LPS induced NF-κB activation by HMP. Nuclear protein lysates of RAW 264.7 cells were prepared after 2 h of treatment with LPS (0.5 μg/ml) either alone or with different concentrations of HMP. Six micrograms of nuclear protein was used to DNA binding assay, and total binding reaction was resolved in 5% nondenaturing polyacrylamide gel. The retarded bands were indicated with arrow. Data represent one of three experiments performed separately with similar results. Lane 1, control; 2, LPS (0.5 μg/ml); 3, LPS + HMP (25 μM); and 4, HMP (12.5 μM). NS, nonspecific.

References

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Jeon et al. (2000) have also shown that the p38 MAPK pathway is specifically involved in LPS-induced iNOS expression and a specific inhibitor of p44/42 MAPK, PD98059, did not show any effect on iNOS expression. In contrast, Lahti et al. (2000) reported that PD98059 suppresses LPS-induced iNOS expression. In agreement, in this study, we report that LPS-induced activation of p44/42-MAPK and iNOS is suppressed/inhibited by HMP. However, HMP did not show any effect on LPS-induced p38 activation. It might be possible that HMP inhibits iNOS expression via p44/42 MAPK pathway. NF-κB is clearly one of the most important regulators of proinflammatory gene expression such as TNF-α, IL-1β, IL-6, IL-8, iNOS, and COX-2 (Jeon et al., 2000; Yamamoto and Gaynor, 2001; Zhou et al., 2002). The promoter region of the murine gene encoding iNOS contains two NF-κB binding sites (Lowenstein et al., 1993; Xie et al., 1994). The binding of NF-κB to the κB sites in promoter region is important for iNOS induction by LPS. The promoter of the murine iNOS contains at least 22 elements homologous to consensus sequences for the binding of transcription factors, which regulates the induction of iNOS by cytokines and LPS. Among those transcription factors, NF-κB is necessary to confer inducibility by LPS in mouse macrophages (Xie et al., 1994). Similarly, Kleiner et al. (1996) also suggested that in 3T3 cells, there are three different signal transduction pathways that could stimulate iNOS mRNA expression: the receptor tyrosine kinase pathway (by IFN-γ, TNF-α, and LPS), the protein kinase A pathway (by forskolin, 8-bromo-cAMP, 3-isobutyl-1-methylxanthine), and the protein kinase C pathway (by 12-O-tetradecanoylphorbol-13-acetate). All these pathways seem to converge with the activation of NF-κB, although a marked intercell variability exists (Feuillard et al., 1991; Vincenti et al., 1992; Muroi and Suzuki, 1993). NF-κB is composed mainly of two proteins: p65 and p50. Normally, in an unstimulated cell, the NF-κB is present in cytosol bound with inhibitory IκB (IκB). After stimulating the cells with various agents, IκB is phosphorylated and subsequently degraded by ubiquitination. Releasing IκB from NF-κB leads to activation and nuclear translocation of NF-κB subunits (Wang et al., 2002). In the present study using EMSA, we have clearly shown that HMP inhibits LPS-induced binding of NF-κB complex in a dose-dependent manner.


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*Anti-Inflammatory Compound from Alpinia officinarum* 931