jmb

A Novel Synthetic Compound, YH-1118, Inhibited LPS-Induced Inflammatory Response by Suppressing IκB Kinase/NF-κB Pathway in Raw 264.7 Cells

Chang Hyun Yun[1], Eun Jung Jang[1], Soon Cheon Kwon[1], Mee-Young Lee[2], Sangku Lee[2], Sei-Ryang Oh[2], Hyeong-Kyu Lee[2], Kyung-Seop Ahn[2], and Ho-Jae Lee[1*]

[1]Laboratory of Chemoprevention, Lee Gil Ya Cancer and Diabetes Institute, Gachon University, Incheon 406-840, Republic of Korea
[2]Natural Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology, Chungbuk 363-883, Republic of Korea

For the search of a potent first-in-class compound to inactivate macrophages responsible for inflammatory responses, in the present study, we investigated the anti-inflammatory effects of YH-1118, a novel synthetic compound, in a lipopolysaccharide (LPS)-stimulated mouse macrophage cell line, Raw 264.7. YH-1118 inhibited LPS-induced nitric oxide (NO) production and inducible NO synthase (iNOS) expression at both the protein and mRNA levels. The suppression of LPS-induced iNOS expression by YH-1118 was mediated *via* nuclear factor kappa B (NF-κB), but not activator protein-1 (AP-1) transcription factor. This was supported by the finding that YH-1118 attenuated the phosphorylation of inhibitor of κBα (IκBα) and nuclear translocation and DNA binding activity of NF-κB. Through the mechanisms that YH-1118 inhibited the activation of IκB kinases (IKKs), upstream activators of NF-κB, or p38 MAPK, YH-1118 significantly suppressed LPS-induced production of pro-inflammatory cytokines, tumor necrosis factor-α, interleukin-1β (IL-1β), and IL-6 ($p < 0.05$). In conclusion, our results suggest that YH-1118 inhibits LPS-induced inflammatory responses by blocking IKK and NF-κB activation in macrophages, and may be a therapeutic candidate for the treatment of various inflammatory diseases.

Keywords: YH-1118, iNOS, NF-κB, macrophages, inflammation

Introduction

Inflammation is the first response of the immune system to infection or injury, which is regulated by cytokines, growth factors, and inflammatory mediators produced by macrophages, neutrophils, and other inflammatory cells [10]. In the presence of stimuli such as lipopolysaccharide (LPS), activated macrophages produce pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, granulocyte/macrophage colony stimulating factor (GM-CSF), and inflammatory mediators, including nitric oxide (NO) and prostaglandin E_2 (PGE_2) [25, 29]. Therefore, LPS-activated macrophages have usually been used for evaluating the anti-inflammatory effects of drug candidates.

NO is a gaseous signaling molecule produced from L-arginine by nitric oxide synthase (NOS) and mediates a wide range of biological functions, including vasodilatation, neurotransmission, inflammation, and cytotoxicity [17]. Although NO has a beneficial role in the antimicrobial activity of macrophages against infectious organisms, excess production of NO leads to septic shock and many inflammatory diseases [32]. Inducible NOS (iNOS) is capable of producing a high output of NO during inflammation, whereas constitutively expressed NOS isoforms, endothelial NOS (eNOS) and neuronal NOS (nNOS), are implicated in diverse normal physiology [20]. Induction of inflammatory genes, including iNOS, is mediated *via* activation of inducible transcription factors [16, 30], among which nuclear factor-kappa B (NF-κB) is one of the pivotal transcription factors

that regulate gene expression involved in inflammatory responses and cellular proliferation. Therefore, aberrant regulation of NF-κB activity has been implicated in the pathogenesis of many diseases, which include immune deficiencies, autoimmune and inflammatory disease, and cancer [15], after which NF-κB has been investigated as a potential target for anti-inflammatory therapies.

In the canonical NF-κB pathway, NF-κB exists in a latent form, complexed to an inhibitor κB (IκB) protein in unstimulated cells. The activation of NF-κB occurs *via* the phosphorylation of IκBα at Ser32 and IκBβ at Ser36 [33]. Stimulation by cytokines such as TNF-α results in IκB kinase (IKK) complex activation, which in turn leads to the phosphorylation and proteasomal degradation of inhibitor of NF-κB (IκBα) [8]. The IKK complex contains two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ [34]. The activation of IKK is mediated by phosphorylation through NF-κB-inducing kinase (NIK), which targets preferentially IKKα and MEKK1 that phosphorylates IKKβ [11, 14]. Following activation, the NF-κB p65/p50 heterodimer is rapidly translocated to the nucleus, where it activates the transcription of target genes [12, 13]. In addition, several studies have reported that the activation of NF-κB is triggered by mitogen-activated protein kinases (MAPKs), such as p38 MAP kinase, extracellular signal-regulated kinase (ERK), and c-Jun NH₂-terminal kinase (JNK) [9, 22].

In an effort to find a chemical that can inhibit LPS-induced NO production in mouse macrophages, we screened a chemical library. As a result, we found that a novel synthetic compound, YH-1118, significantly inhibited NO production in Raw 264.7 cells. Since NO is an important mediator in several kinds of inflammation-related disease models, we speculated on the inhibitory effects of YH-1118 on NF-κB-mediated induction of iNOS and pro-inflammatory cytokines and its potential as a future drug candidate for inflammation-based diseases.

Materials and Methods

Chemicals and Reagents

Lipopolysaccharide (*Escherichia coli*; serotype 055:B5), JSH-23 (4-methyl-*N*-benzene-1,2-diamine), and SP600125 (anthra[1,9-cd]pyrazole-6 (2H)-one), were purchased from Sigma (St. Louis, MO). Griess reagent system was obtained from Promega (Madison, WI, USA). Anti-phospho-IKKα/β (Ser176/180), anti-phospho-IκBα (Ser32), anti-IκBα, anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-JNK (Thr183/Tyr185), and anti-JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-IKKα/β antibody was obtained from Santa Cruz Biotech (Santa Cruz, CA,

USA). The ELISA kit for TNF-α, IL-1β, and IL-6 were purchased from R&D Systems Inc. (Minneapolis, MN, USA).

Synthesis of YH-1118

YH-1118 (5-(2,2-dimethyl-2H-chromen-6-yl-methylene)-2-thioxo-imidazolidin-4-one) was synthesized by condensation of a benzopyranyl aldehyde with 2-thiohydantoin using a catalytic amount of piperidine in ethanol. Benzopyranyl aldehyde was prepared by an alkylation reaction of 4-hydroxybenzaldehyde with 3-chloro-3-methyl-1-butyne in the presence of potassium iodide and potassium carbonate, followed by cyclization of the alkylated adduct in *N,N*-diethylaniline, in a microwave oven. The structure was determined by NMR (proton and carbon) analysis. ¹H NMR (CD₃OD) 7.36–7.32 (m, 2H), 6.77 (d, *J*=7.8, 1H), 6.47 (s, 1H), 6.42 (d, *J*=9.6, 1H), 5.74 (d, *J*=9.9, 1H), 1.42 (s, 6H) ppm. ¹³C NMR (CD₃OD) 180.1, 167.9, 155.8, 132.6, 132.4, 128.8, 127.8, 126.8, 123.1, 122.7, 117.9, 113.9, 78.3, and 28.5 ppm.

Cell Culture

The Raw 264.7 cell line, derived from murine macrophages, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 0.1% sodium bicarbonate, 2 mM glutamine, penicillin G (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (Gibco), and maintained at 37°C in a humidified incubator containing 5% CO_2.

Cell Viability Assay

Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Amresco, OH, USA) to formazan. Briefly, 10 μl of 5 mg/ml MTT solution was added to the cell supernatant and incubated for 4 h at 37°C. The reagent was discarded and the formazan blue formed in cells was dissolved with dimethylsulfoxide. The optical density at 540 nm was measured in a microplate reader.

Nitric Oxide Assay

Nitric oxide levels in cell supernatants were determined by the measurement of total nitrite, a stable oxidation product of NO based on the Griess reaction. Nitrite in the culture supernatants was mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine hydrochloride and 1% sulfanilamide in 5% phosphoric acid) at room temperature for 10 min in the dark. The absorbance at 540 nm was measured, and the nitrite concentration was determined using sodium nitrite as a standard.

Plasmids, Transient Transfection, and Luciferase Activity Assay

The mouse iNOS promoter-luciferase reporter plasmid (pGL2-iNOS) was generously provided by Charles J. Lowenstein (Johns Hopkins University) *via* Addgene Inc. (Addgene plasmid 19296). pNF-κB-Luc and pAP-1-Luc were purchased from Stratagene (La Jolla, CA, USA). Raw 264.7 cells were transiently transfected with

pGL2-iNOS, pNF-κB-Luc, or pAP-1-Luc and the internal control pCMV-β-gal in 24-well plates using Fugene 6 reagent (Roche, Indianapolis, IN, USA). After 24 h transfection, cells were treated with 1 μg/ml of LPS for 16 h. Luciferase activities were quantified by using the Luciferase Assay System (Promega) according to the manufacturer's protocol. All of the values were normalized with the β-galactosidase activities. All experiments were performed in triplicate and repeated at least three times.

Reverse Transcription-PCR

Total RNA was isolated from Raw 264.7 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription was performed with 1 μg of pure RNA using M-MLV reverse transcriptase (Promega). The synthesized cDNA was amplified by PCR using specific primers. PCR products were visualized by electrophoresis on agarose gels with RedSafe (iNtRON) staining and analyzed using an Image Quant LAS 4000 image analyzer (GE Healthcare Life Sciences). The primer sequences of iNOS were forward primer 5'-CTG CAG CAC TTG GAT CAG GAA CCT G-3' and reverse primer 5'-GGG AGT AGC CTG TGT GCA CCT GGA A-3'. Primers specific for GAPDH were used as an internal control.

Western Immunoblot Analysis

Cells were lysed in lysis buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and protease inhibitor mixture (Complete; Roche Applied Science). Extracts were separated by SDS-PAGE followed by electro-transfer to polyvinylidene difluoride membranes and probed with polyclonal or monoclonal antisera, followed by horseradish peroxidase-conjugated anti-rabbit, anti-mouse IgG, and visualized by chemiluminescence, according to the manufacturer's instructions (Pierce).

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. For electrophoretic mobility shift assay (EMSA), 5 μg of the nuclear extract was mixed with ^{32}P-labeled double-stranded NF-κB consensus site oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and incubated in a binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 12.5% (v/v) glycerol, and 0.2 mM DTT) at room temperature for 30 min. Labeled samples were subsequently electrophoresed on a 5% non-denaturing polyacrylamide gel with 0.5× TAE buffer at 4ºC. Gels were dried and exposed to Kodak X-Omat film (Kodak, Rochester, NY, USA) for visualization of NF-κB bands. The specificity of binding was also examined by competition with the unlabeled oligonucleotides.

Immunofluorescence

Cells seeded on chamber slides were fixed with 4% paraformaldehyde

solution for 15 min. Cells were blocked with 5% bovine serum albumin for 1 h and incubated with anti-p65 antibody overnight at 4ºC. Antibody-bound cells were detected by Alexa Fluor 546-conjugated secondary antibody (Invitrogen). Immunofluorescence images were obtained using a LSM-710 confocal microscope (Zeiss).

Cytokine Assay

Levels of TNF-α, IL-1β, and IL-6 in culture medium were quantified by ELISA kits according to the manufacturer's instructions.

Statistical Analysis

Data are expressed as means ± SEMs and are representative of three independent experiments. Statistical analysis was performed using the Student's *t*-test, and *p* values of less than 0.05 were considered statistically significant.

Results

Effect of YH-1118 on LPS-Induced NO Production in Raw 264.7 Cells

The chemical structure of YH-1118, 5-(2,2-dimethyl-2H-chromen-6-ylmethylene)-2-thioxo-imidazolidin-4-one, is shown in Fig. 1A. To examine the effect of YH-1118 on LPS-induced NO production in Raw 264.7 cells, cells were treated with different concentrations of YH-1118, followed by stimulation with LPS for 16 h. The concentration of nitrite, as an index of NO production, was measured by the Griess method. As shown in Fig. 1B, stimulation of cells with LPS resulted in a significant increase of the nitrite concentration in conditioned medium, compared with that of non-stimulated cells. YH-1118 reduced LPS-induced nitrite production in a dose-dependent manner. YH-1118 at below 10 μM did not show significant cytotoxic effects to the Raw 264.7 cells (Fig. 1C), indicating that the inhibitory effect of YH-1118 on LPS-induced NO production was not attributable to its nonspecific cell toxicity.

Effect of YH-1118 on LPS-Induced iNOS Protein, mRNA Expression, and Transcriptional Activity in Raw 264.7 Cells

To examine whether the inhibitory effect of YH-1118 on LPS-induced NO production was attributable to its influence on iNOS protein synthesis, western immunoblot analysis was carried out. As shown in Fig. 2A, iNOS protein was hardly detectable in resting Raw 264.7 cells, but a pronounced amount of iNOS protein was induced by LPS stimulation. Treatment of YH-1118 decreased LPS-induced iNOS protein levels in a dose-dependent manner. To investigate the effect of YH-1118 in iNOS gene expression, the steady-state level of
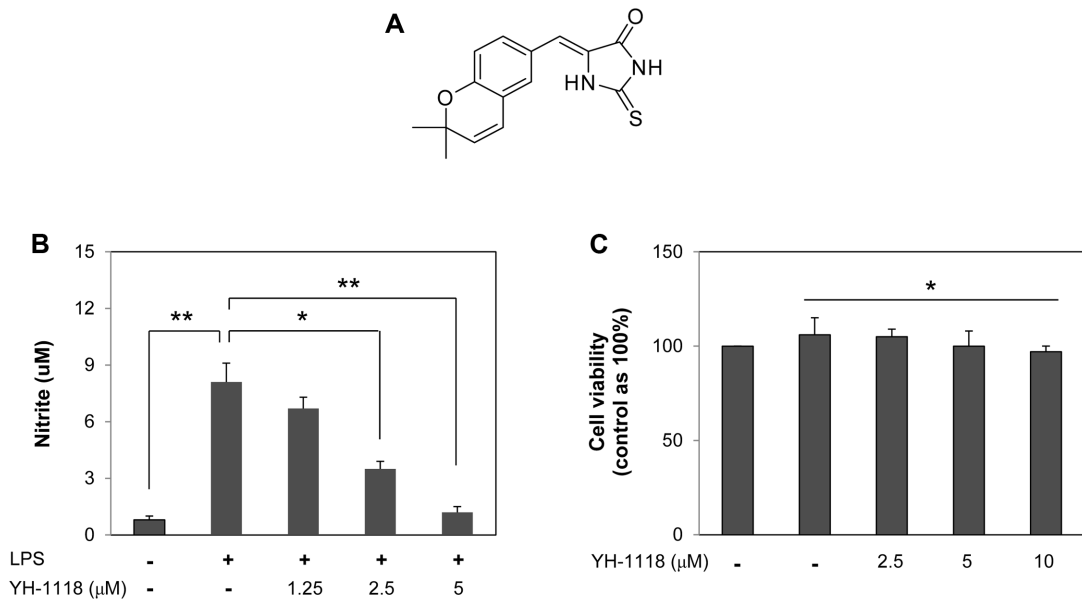
Fig. 1. Effect of YH-1118 on LPS-induced NO production in Raw 264.7 cells.

(**A**) Chemical structure of YH-1118. (**B**) Raw 264.7 cells were treated with indicated concentrations of YH-1118 for 4 h, and then stimulated with 1 μg/ml of LPS for 16 h. Nitrite levels were measured using the Griess reagent. (**C**) Raw 264.7 cells were treated with indicated concentrations of YH-1118 for 24 h, and cell viability was determined using the MTT assay. Data are the mean ± SD in triplicate assays. *$p < 0.05$, **$p < 0.01$.

mRNA was determined by RT-PCR. Treatment of YH-1118 decreased the iNOS mRNA level in a dose-dependent manner (Fig. 2B). To assess the transcriptional activity of YH-1118 on the regulation of iNOS, Raw 264.7 cells were transfected with murine iNOS-promoter fused to the luciferase gene as a reporter. As shown in Fig. 2C, LPS stimulation significantly increased the luciferase activity, and YH-1118 suppressed the LPS-induced luciferase activity in a dose-dependent manner.

Effect of YH-1118 on LPS-Induced NF-κB and AP-1 Transcriptional Activities in Raw 264.7 Cells

To investigate the effect of YH-1118 on the transcriptional activities of NF-κB and AP-1, reporter gene assays were performed. Raw 264.7 cells were transfected with a pNF-κB-Luc or pAP-1-Luc reporter construct and stimulated with LPS for 16 h. As shown in Fig. 3A, YH-1118 suppressed the LPS-induced NF-κB transcriptional activity in a dose-dependent manner. Furthermore, treatment with JSH-23, a
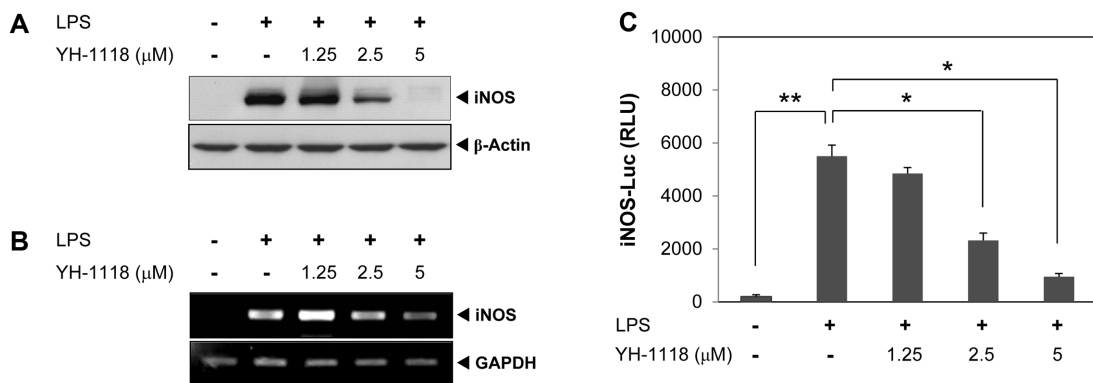

Fig. 2. Effect of YH-1118 on LPS-induced iNOS protein, mRNA expression, and transcriptional activity in Raw264.7 cells.

(**A**) Raw 264.7 cells were treated with indicated concentrations of YH-1118 for 4 h, and then stimulated with 1 μg/ml of LPS for 16 h. Protein lysates were immunoblotted with anti-iNOS or β-actin antibodies. (**B**) Total RNAs were isolated and the levels of iNOS mRNA were analyzed by RT-PCR. (**C**) Raw 264.7 cells transfected with the iNOS-Luc reporter construct were treated with the indicated concentrations of YH-1118 for 4 h. Luciferase activity was measured after 16 h of LPS stimulation. Data shown are the mean ± SD of three separate experiments. *$p < 0.05$, **$p < 0.01$.
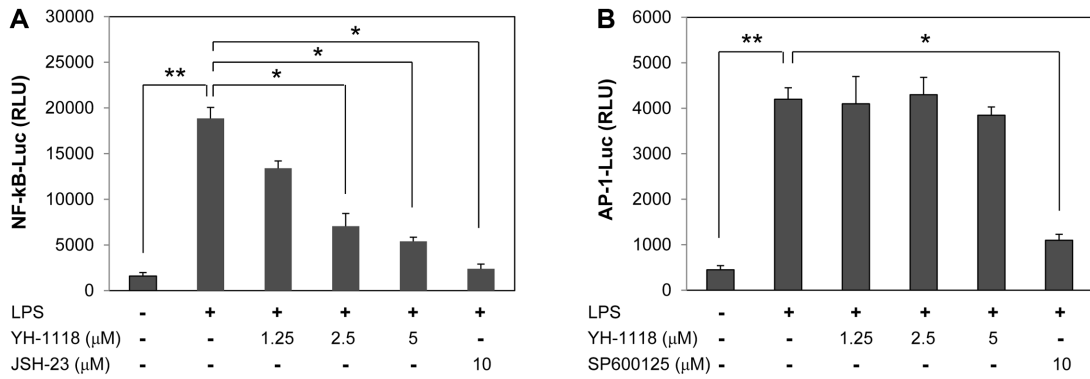
Fig. 3. Effect of YH-1118 on LPS-induced NF-κB and AP-1 transcriptional activities in Raw 264.7 cells.
Raw 264.7 cells transfected with pNF-κB-Luc (**A**) or pAP-1-Luc (**B**) were treated with the indicated concentrations of compounds for 4 h. Luciferase activity was measured after 16 h of LPS stimulation. Data shown are the mean ± SD of three separate experiments. *$p < 0.05$, **$p < 0.01$.

specific NF-κB activation inhibitor [26], substantially attenuated the LPS-induced NF-κB transcriptional activity. However, YH-1118 did not suppress the LPS-induced AP-1 transcriptional activity, whereas SP600125, a specific JNK inhibitor, significantly inhibited the LPS-induced AP-1 transcriptional activity (Fig. 3B).

Effect of YH-1118 on LPS-Induced DNA Binding Activity of the NF-κB Complex and Nuclear Translocation of NF-κB p65 in Raw 264.7 Cells

To determine whether YH-1118 affects the DNA binding activity of NF-κB in LPS-stimulated Raw 264.7 cells, EMSA was performed using NF-κB-specific ^{32}P-labeled oligonucleotide.
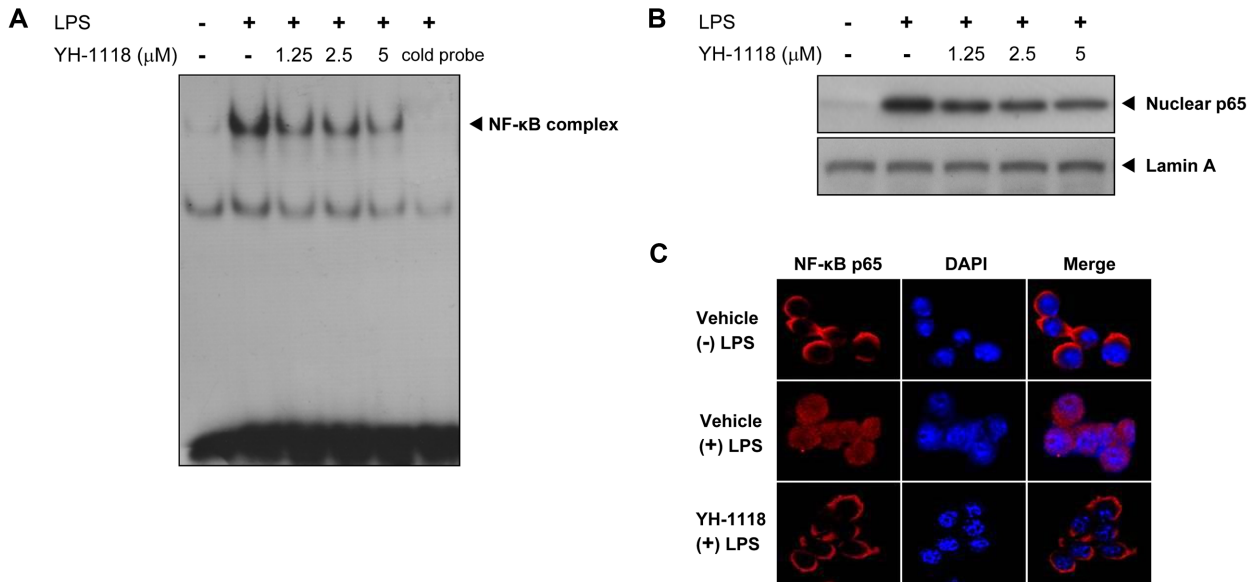

Fig. 4. Effect of YH-1118 on the LPS-induced DNA binding activity of the NF-κB complex and nuclear translocation of NF-κB p65 in Raw 264.7 cells.
(**A**) Raw 264.7 cells were treated with the indicated concentrations of YH-1118 for 4 h and then stimulated with 1 μg/ml of LPS for 30 min. Nuclear extracts were reacted with NF-κB-specific ^{32}P-labeled oligonucleotides and then resolved on a non-denaturing polyacrylamide gel by electrophoresis. Competitive EMSA using an unlabeled NF-κB oligonucleotide probe confirmed the specificity of NF-κB binding. Data shown are representative of two independent experiments. (**B**) Nuclear extracts were prepared and immunoblotted with anti-NF-κB p65 and lamin A antibodies. (**C**) Raw 264.7 cells were treated with 10 μM of YH-1118 and then stimulated with 1 μg/ml of LPS for 1 h. Cells were fixed in 4% PFA, and then stained with anti-NF-κB (p65) antibody, followed by Alexa Fluor goat anti-rabbit IgG. The data are representative of at least three experiments.

Raw 264.7 cells significantly increased the DNA binding activity of the NF-κB complex upon stimulation with LPS for 30 min (Fig. 4A). YH-1118 decreased the LPS-stimulated DNA binding activity of the NF-κB complex in a dose-dependent manner. To further investigate whether YH-1118 inhibits nuclear translocation of NF-κB in response to LPS, Raw 264.7 cells were treated with the indicated concentrations of YH-1118 for 4 h and then stimulated with 1 μg/ml of LPS for 30 min. Nuclear extracts were analyzed by western immunoblotting. As shown in Fig. 4B, the amount of NF-κB complex in the nucleus was significantly increased upon stimulation with LPS, and YH-1118 inhibited the LPS-stimulated nuclear translocation of NF-κB p65 in a dose-dependent manner. To confirm the cellular localization of NF-κB p65, confocal microscopy was performed in Raw 264.7 cells. NF-κB p65 was found in the cytoplasm in the absence of LPS treatment. Upon stimulation with LPS, NF-κB p65 was predominantly translocated to the nucleus, but treatment of 10 μM of YH-1118 significantly blocked the LPS-stimulated nuclear translocation of NF-κB p65 in Raw 264.7 cells (Fig. 4C).

Effect of YH-1118 on LPS-Induced Activation of IκB Kinase and MAP Kinases in Raw 264.7 Cells

To examine the effect of YH-1118 on the LPS-induced phosphorylation and degradation of IκBα, Raw 264.7 cells were treated with YH-1118 followed by stimulation with LPS, and total cell lysates were analyzed by western immunoblotting. As shown in Fig. 5A, stimulation with LPS significantly increased the levels of phosphorylated IκBα, and YH-1118 reduced the LPS-induced IκBα phosphorylation in a dose-dependent manner. In order to investigate the effect of YH-1118 on the inhibition of IKK activation, the phosphorylation levels of IKKα/β were determined by immunoblot analysis. YH-1118 inhibited the LPS-induced phosphorylation of IKK in a dose-dependent manner. The LPS-induced activation of MAP kinases, including p38, ERK, and JNK, were assessed in Raw 264.7 cells. LPS treatment significantly increased the levels of phosphorylated p38, ERK1/2, and JNK (Fig. 5B). YH-1118 inhibited the LPS-induced phosphorylation of p38 in a dose-dependent manner, and moderately inhibited the phosphorylation of ERK1/2 and JNK.

Effect of YH-1118 on LPS-Induced Production of Pro-Inflammatory Cytokines in Raw 264.7 Cells

The release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 plays an important role in the inflammatory response and contributes to the development of inflammatory diseases. Therefore, we examined the effects of YH-1118 on the LPS-induced production of TNF-α, IL-1β, and IL-6, using ELISA kits. To reach detectable ranges of secreted cytokines in the culture media, LPS stimulation was extended up to 24 h. The stimulation of Raw 264.7 cells with LPS significantly increased the levels of TNF-α (Fig. 6A), IL-1β (Fig. 6B), and IL-6 (Fig. 6C) compared with
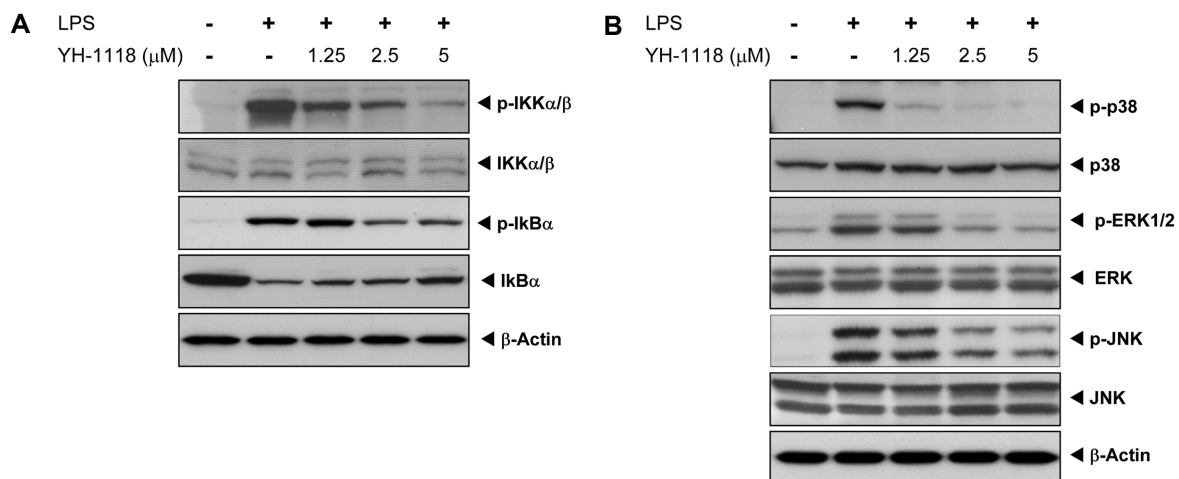

Fig. 5. Effect of YH-1118 on LPS-induced IKK and MAPK activation in Raw 264.7 cells.
(**A** and **B**) Raw 264.7 cells were treated with the indicated concentrations of YH-1118 for 4 h and then stimulated with 1 μg/ml of LPS for 30 min. Total cell lysates were analyzed by immunoblotting with anti-phospho-IKKα/β, anti-phospho-IκBα, anti-phospho-p38, anti-phospho-ERK, or anti-phospho-JNK antibodies.
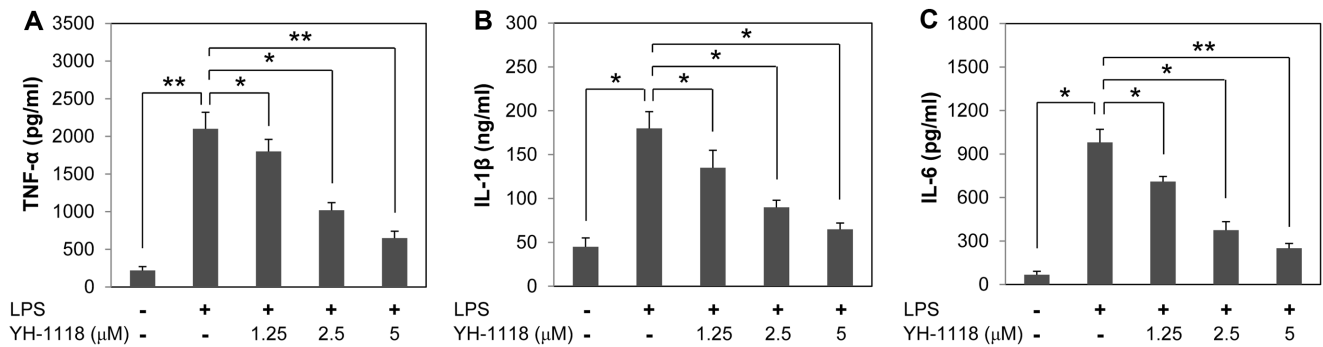
Fig. 6. Effect of YH-1118 on LPS-induced production of pro-inflammatory cytokines in Raw 264.7 cells.
Raw 264.7 cells were treated with the indicated concentrations of YH-1118 for 4 h and then stimulated with 1 μg/ml of LPS for 24 h. Levels of TNF-α (**A**), IL-1β (**B**), and IL-6 (**C**) in the culture media were quantified using mouse ELISA kits. Data shown are the mean ± SD of three separate experiments. $*p < 0.05$, $**p < 0.01$.

those of the control group. However, treatment with YH-1118 significantly inhibited the LPS-induced production of TNF-α, IL-1β, and IL-6.

Discussion

In the current study, we demonstrated that YH-1118 has significant anti-inflammatory activity, with a potent ability to regulate the production of NO, TNF-α, IL-1β, and IL-6 through suppressing the activation of NF-κB and its upstream regulator IKK in LPS-induced Raw 264.7 cells. Considering the effects of YH-1118 on the LPS-induced production of NO and pro-inflammatory cytokines, our findings provide a molecular basis for the anti-inflammatory properties of YH-1118 and its therapeutic potential for the treatment of various inflammatory diseases.

LPS, a component of the outer membranes of gram-negative bacteria, provides a key signal leading to activation of the immune system, in particular *via* the stimulation of macrophages [2]. LPS is recognized by Toll-like receptor 4 (TLR4) on cell membranes such as macrophages and triggers an acute and early release of inflammatory mediators through TLR4-NF-κB signaling pathways that mediate host damage [21, 28]. Therefore, inhibitors of the production of these inflammatory mediators and cytokines have been considered as candidates for anti-inflammatory agents. Previous reports have shown that transcriptional induction of iNOS is largely dependent on cooperative activities of NF-κB and activator protein 1 (AP-1) transcription factors, which act on cognate *cis*-acting elements in the iNOS promoter [18, 31]. The present study clearly demonstrated that YH-1118 suppresses NF-κB transcriptional activity and

resulted in decreased expression of iNOS in LPS-stimulated macrophages. As shown in Fig. 3B, YH-1118 does not inhibit AP-1 transcriptional activity. These results indicate that the inhibitory effect of YH-1118 on LPS-induced iNOS expression is mediated *via* NF-κB, but not the AP-1 transcription factor.

The NF-κB transcription factor is a critical regulator of immunologically mediated immediate transcriptional responses [24]. The activation of NF-κB is linked to a sequential cascade that includes IKK-dependent IκBα phosphorylation, ubiquitination, and proteolytic degradation, as well as translocation of cytosolic NF-κB to the nucleus [12, 13]. The phosphorylation of IκBα at serine residues is essential for the activation and release of NF-κB [3]. The current study showed that the phosphorylation of IκBα and nuclear localization of NF-κB p65 by LPS were significantly abolished in LPS-stimulated Raw 264.7 cells with YH-1118 treatment. The cellular levels of IκBα were also determined for whether YH-1118 inhibited their degradation. As seen in Fig. 5A, the recovery of IκBα levels in LPS-stimulated Raw 264.7 cells provided strong evidence that YH-1118 inhibits NF-κB activation as a consequence of the inhibition of IκBα phosphorylation. Members of the protein tyrosine kinase family play important roles in macrophage activation to LPS [7]. Phosphorylation of IκBα bound to NF-κB in LPS-stimulated Raw 264.7 cells is considered to be mediated by the NF-κB-inducing kinase (NIK) and the subsequent I-κB kinase (IKK) complexed with other proteins in the plasma membrane [27]. This is supported by evidence that the inhibition of NIK and subsequently IKK reduced the expression of iNOS in LPS-stimulated macrophages [19]. The pathways of NIK and

MEKK1 regulate the phosphorylation of IκBα *via* IKKs [11, 14]. The present study demonstrated that the activation of IKKα and IKKβ by LPS was significantly inhibited in LPS-stimulated Raw 264.7 cells with YH-1118 treatment.

MAP kinases play important roles in the regulation of inflammatory responses and in coordinating the induction of many gene-encoding inflammatory mediators [35]. MAPKs play important roles in the LPS-stimulated activation of macrophages by triggering a cascade reaction and ultimately resulting in expression of pro-pro-inflammatory cytokines. Upon stimulation with LPS, activation of MAPKs in the phosphorylated forms mediates the signaling cascades, leading to activation of NF-κB and AP-1 in activated macrophages [9]. Recent study showed that MAPKs, including p38, ERK, and JNK, play important roles in LPS-induced iNOS expression in mouse macrophages [5, 6]. Activation of p38 by LPS resulted in the stimulation of NF-κB-specific DNA-protein binding activity and subsequent expression of iNOS and NO release in Raw 264.7 cells [6]. Activation of p38 MAPK has been demonstrated in the mediation of NF-κB translocation through the phosphorylation of IκBα [4]. Although the exact mechanisms of each type of these MAPKs are unclear, there might be a signal cross-talk and signal convergence among these MAPKs. All of these results suggested that the inhibition of IκBα phosphorylation and nuclear translocation of NF-κB by YH-1118 might be mediated by the suppression of IKK and MAPK activation.

Like NO produced by iNOS, pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are induced by the regulation of NF-κB and play pivotal roles in the immune responses to various inflammatory stimuli [23]. It has been well studied that excess production of these inflammatory mediators is seen in many acute and chronic human diseases, including septic shock, multiple sclerosis, rheumatoid arthritis, and atherosclerosis [1]. Our results demonstrated that YH-1118 inhibits the production of these pro-inflammatory cytokines in LPS-stimulated macrophages. These results suggest that YH-1118 is a potential inhibitor for the initial phase of the inflammatory cascade under LPS stimulation. Conclusively, in this study, we elucidated the effects and molecular mechanisms of YH-1118, a synthetic compound, on the production of inflammatory mediators in mouse macrophages, in that YH-1118 significantly suppresses the production of NO and iNOS in LPS-induced Raw 264.7 cells. Further documentation in *in vivo* models is now under way to elucidate the potential application of YH-1118 in inflammation-based models such as bronchial asthma and inflammatory bowel disease.

References

1. Bertolini A, Ottani A, Sandrini M. 2001. Dual acting anti-inflammatory drugs: a reappraisal. *Pharmacol. Res.* **44:** 437-450.
2. Broad A, Jones DE, Kirby JA. 2006. Toll-like receptor (TLR) response tolerance: a key physiological "damage limitation" effect and an important potential opportunity for therapy. *Curr. Med. Chem.* **13:** 2487-2502.
3. Brockman JA, Scherer DC, McKinsey TA, Hall SM, Qi X, Lee WY, Ballard DW. 1995. Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation. *Mol. Cell. Biol.* **15:** 2809-2818.
4. Carter AB, Knudtson KL, Monick MM, Hunninghake GW. 1999. The p38 mitogen-activated protein kinase is required for NF-kappaB-dependent gene expression. The role of TATA-binding protein (TBP). *J. Biol. Chem.* **274:** 30858-30863.
5. Chan ED, Riches DW. 2001. IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. *Am. J. Physiol. Cell Physiol.* **280:** C441-C450.
6. Chen CC, Wang JK. 1999. p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. *Mol. Pharmacol.* **55:** 481-488.
7. Geng Y, Zhang B, Lotz M. 1993. Protein tyrosine kinase activation is required for lipopolysaccharide induction of cytokines in human blood monocytes. *J. Immunol.* **151:** 6692-6700.
8. Ghosh S, Karin M. 2002. Missing pieces in the NF-kappaB puzzle. *Cell* **109(Suppl):** S81-S96.
9. Guha M, Mackman N. 2001. LPS induction of gene expression in human monocytes. *Cell. Signal.* **13:** 85-94.
10. Kanterman J, Sade-Feldman M, Baniyash M. 2012. New insights into chronic inflammation-induced immunosuppression. *Seminars Cancer Biol.* **22:** 307-318.
11. Karin M. 1999. The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. *J. Biol. Chem.* **274:** 27339-27342.
12. Klemm S, Ruland J. 2006. Inflammatory signal transduction from the Fc epsilon RI to NF-kappa B. *Immunobiology* **211:** 815-820.
13. Lawrence T. 2009. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor Perspect. Biol.* **1:** a001651.
14. Lee FS, Peters RT, Dang LC, Maniatis T. 1998. MEKK1 activates both IkappaB kinase alpha and IkappaB kinase

beta. *Proc. Natl. Acad. Sci. USA* **95:** 9319-9324.

15. Liu SF, Malik AB. 2006. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **290:** L622-L645.

16. Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW, Murphy WJ. 1993. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* **90:** 9730-9734.

17. MacMicking J, Xie QW, Nathan C. 1997. Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15:** 323-350.

18. Marks-Konczalik J, Chu SC, Moss J. 1998. Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor kappaB-binding sites. *J. Biol. Chem.* **273:** 22201-22208.

19. Matsushima A, Kaisho T, Rennert PD, Nakano H, Kurosawa K, Uchida D, *et al.* 2001. Essential role of nuclear factor (NF)-kappaB-inducing kinase and inhibitor of kappaB (IkappaB) kinase alpha in NF-kappaB activation through lymphotoxin beta receptor, but not through tumor necrosis factor receptor I. *J. Exp. Med.* **193:** 631-636.

20. Michel T, Feron O. 1997. Nitric oxide synthases: which, where, how, and why? *J. Clin. Invest.* **100:** 2146-2152.

21. Miller SI, Ernst RK, Bader MW. 2005. LPS, TLR4 and infectious disease diversity. *Nature Rev. Microbiol.* **3:** 36-46.

22. Nakano H, Shindo M, Sakon S, Nishinaka S, Mihara M, Yagita H, Okumura K. 1998. Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proc. Natl. Acad. Sci. USA* **95:** 3537-3542.

23. Netea MG, van der Meer JW, van Deuren M, Kullberg BJ. 2003. Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? *Trends Immunol.* **24:** 254-258.

24. Perkins ND. 2000. The Rel/NF-kappa B family: friend and foe. *Biochem. Sci.* **25:** 434-440.

25. Raetz CR, Whitfield C. 2002. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71:** 635-700.

26. Shin HM, Kim MH, Kim BH, Jung SH, Kim YS, Park HJ, *et al.* 2004. Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-kappaB without affecting IkappaB degradation. *FEBS Lett.* **571:** 50-54.

27. Stancovski I, Baltimore D. 1997. NF-kappaB activation: the I kappaB kinase revealed? *Cell* **91:** 299-302.

28. Takeda K, Akira S. 2004. TLR signaling pathways. *Seminars Immunol.* **16:** 3-9.

29. Wu JT, Kral JG. 2005. The NF-kappaB/IkappaB signaling system: a molecular target in breast cancer therapy. *J. Surg. Res.* **123:** 158-169.

30. Xie QW, Kashiwabara Y, Nathan C. 1994. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269:** 4705-4708.

31. Xie QW, Whisnant R, Nathan C. 1993. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J. Exp. Med.* **177:** 1779-1784.

32. Zamora R, Vodovotz Y, Billiar TR. 2000. Inducible nitric oxide synthase and inflammatory diseases. *Mol. Med.* **6:** 347-373.

33. Zandi E, Chen Y, Karin M. 1998. Direct phosphorylation of IkappaB by IKKα and IKKβ: discrimination between free and NF-kappaB-bound substrate. *Science* **281:** 1360-1363.

34. Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. 1997. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* **91:** 243-252.

35. Zhang W, Liu HT. 2002. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res.* **12:** 9-18.