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Benzoxathiole Derivative Blocks Lipopolysaccharide-Induced Nuclear Factor-κB Activation and Nuclear Factor-κB-Regulated Gene Transcription through Inactivating Inhibitory κB Kinase β

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ABSTRACT

Benzoxathiole derivatives have been used in the treatment of acne and have shown cytostatic, antipsoriatic, and antibacterial properties. However, little is known about the molecular basis for these pharmacological properties, although nuclear factor (NF)-κB activation is closely linked to inflammation and cell proliferation. Here, we demonstrate that the novel small-molecule benzoxathiole 6,6-dimethyl-2-(phenylimino)-6,7-dihydro-5*H*-benzo-[1,3]oxathiol-4-one (BOT-64) inhibits NF-κB activation with an IC_{50} value of 1 μM by blocking inhibitory κB (IκB) kinase β (IKKβ), and suppresses NF-κB-regulated expression of inflammatory genes in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. BOT-64 inhibits IKKβ-mediated IκBα phosphorylation in LPS-activated macrophages, resulting in sequential prevention of downstream events, including proteolytic degradation of IκBα, DNA binding ability, and transcriptional activity of NF-κB. BOT-64 inhibits LPS-inducible IKKβ activity in the cells and catalytic activity of highly purified IKKβ. Moreover, the effect of BOT-64 on cell-free IKKβ was abolished by substitution of Ser-177 and Ser-181 residues in the activation loop of IKKβ to glutamic acid residues, indicating a direct interaction site of benzoxathiole. BOT-64 attenuates NF-κB-regulated expression of inflammatory genes such as inducible nitric-oxide synthase, cyclooxygenase-2, tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 in LPS-activated or expression vector IKKβ-transfected macrophages. Furthermore, BOT-64 dose-dependently increases the survival rates of endotoxin LPS-shocked mice.

Nuclear factor (NF)-κB is a family of eukaryotic transcription factors that play an important role in the regulation of immune and inflammatory processes, cell proliferation and survival, and cellular stress responses (O'Sullivan et al., 2007; Olivier et al., 2006). Mammalian NF-κB consists of homo- or heterodimers of the Rel protein family. In normal cells, NF-κB is sequestered in the cytoplasm as an inactive complex bound to inhibitory κB (IκB) proteins such as IκBα, IκBβ, and IκBε (Baeuerle and Baltimore, 1988). In response to lipopolysaccharide (LPS), an endotoxin recognized by toll-like receptor-4 and its accessory protein MD-2 on immune cells (Nagai et al., 2002), cellular IκB kinase (IKK) complex is activated and phosphorylates cytoplasmic IκBs (Zandi et al., 1998). In the case of IκBα, the most studied member of the class, Ser-32 and Ser-36 residues are phosphorylated by the IKK complex (DiDonato et al., 1996). Analogous serine residues have been identified in both IκBβ and IκBε. This phosphorylation is essential for subsequent ubiquitination fol-

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ABBREVIATIONS: NF, nuclear factor; BOT-64, 6,6-dimethyl-2-(phenylimino)-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one; PTN, parthenolide; IκB, inhibitory κB; IKK, IκB kinase; LPS, lipopolysaccharide; iNOS, inducible nitric-oxide synthase; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α; IL, interleukin; ERK-1/2, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SEAP, secretory alkaline phosphatase; NPT, neomycin phosphotransferase; PGE_2, prostaglandin E_2; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; WB, Western blot; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; RFU, relative fluorescence unit; Luc, luciferase; TNF, tumor necrosis factor; AT buffer, HEPES, Triton X-100, glycerol, EDTA, EGTA, NaF, $Na_4P_2O_7$, dithiothreitol, Na_3VO_4, phenylmethylsulfonyl fluoride, leupeptin, and pepstatin; PS-1145, XXX; IRFI 042, mono[2-[2-(acetylthio)ethyl]-2,3-dihydro-4,6,7-trimethyl-5-benzofuranyl] ester; YS 51, 1-(β-naphtylmethyl)-6,7-dihydroxy-1,2,3,4,-tetrahydroisoquinoline; BMS-345541, (4(2′-aminoethyl)amino-1,8-dimethylimididazo(1,2-*a*)quinoxaline)-4,5-dihydro-1,8-dimethylimidazo(1,2-*a*)quinoxalin-4-one-2-carboxylic acid; PS-1145, *N*-(6-chloro-9*H*-β-carbolin-8-yl)-nicotinamide.

lowed by proteasome-mediated degradation of IκBs, after which NF-κB moves into the nucleus (Karin and Ben-Neriah, 2000). There NF-κB binds to the κB sequences for transcriptional regulation of immune and inflammatory genes, including inducible nitric-oxide synthase (iNOS), cyclooxygenase (COX)-2, and cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 (Tian and Brasier, 2003).

IKK complex-catalyzed IκB phosphorylation is a prerequisite for the activation and nuclear translocation of NF-κB (Karin and Ben-Neriah, 2000). The IKK complex consists of catalytic subunits of IKKα and IKKβ and a regulatory subunit of NEMO/IKKγ. Although inducible serine/threonine kinase activity of IKK complex is regulated by NEMO/IKKγ, it is known that phosphorylation of Ser-177 and Ser-181 residues in the activation loop of IKKβ is crucial (Delhase et al., 1999). Genetic study of IKK subunit knockout mice has provided evidence that almost all inflammatory stimuli, including LPS, require the IKKβ subunit for NF-κB activation (Li et al., 1999b). Dominant-negative IKKβ but not dominant-negative IKKα blocks NF-κB-regulated gene transcription in inflammatory states (Aupperle et al., 1999). IKKα does not seem to play a major role in the classic pathway of NF-κB activation but instead is important for developmental processes (Li et al., 1999a).

Benzoxathiole derivatives have been used in the treatment of acne and are reported to have cytostatic, antipsoriatic, and antibacterial properties (Goeth and Wildfeuer, 1969; Lius and Sennerfeldt, 1979). However, the molecular basis of these pharmacological properties remains to be defined. We postulated that benzoxathiole derivatives could mediate some of their pharmacological effects by modulating NF-κB activation, which is closely linked to inflammation and cell proliferation. In this study, we demonstrate that a novel benzoxathiole derivative (BOT-64) of 6,6-dimethyl-2-(phenylimino)-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (Fig. 1A) is an efficient inhibitor of IKKβ in NF-κB activation, and this mechanism of action down-regulates NF-κB-regulated expression of inflammatory genes in macrophages and increases the survival rate of endotoxin LPS-shocked mice.

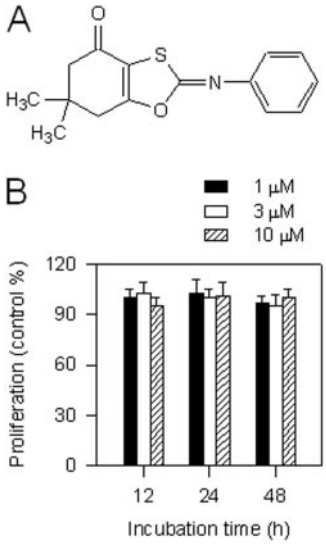

Fig. 1. Effect of BOT-64 on cell proliferation. A, chemical structure of BOT-64. B, RAW 264.7 cells were incubated with various concentrations (1–10 μM) of BOT-64 for the indicated times. Proliferation of the cells was analyzed by WST-1 method. Values are represented as percentage of the control, media alone-treated group. Data are means ± S.E. from three separate experiments.

Materials and Methods

Reagents, Antibodies, and Plasmids. LPS (*Escherichia coli* 055:B5), parthenolide (PTN), and anti-FLAG M2 affinity gel freezer-safe beads were purchased from Sigma-Aldrich (St. Louis, MO), and Lipofectamine was from Invitrogen (Carlsbad, CA). Antibodies against IκBα, IKKβ, iNOS, COX-2, extracellular signal-regulated kinase (ERK)-1/2, or c-Jun N-terminal kinase (JNK) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and those against p-IκBα, p-ERK-1/2, or p-JNK were from Cell Signaling Technology (Danvers, MA). pSV-β-galactosidase control vector was purchased from Promega (Madison, WI). A reporter plasmid of pNF-κB-secretory alkaline phosphatase (SEAP)-neomycin phosphotransferase (NPT) expression vectors encoding IKKα, IKKβ, IKKβ (C/A), IKKβ (SS/EE), NF-κB p65 or p50, and luciferase (Luc) reporter plasmids of piNOS (−1592/+183)-Luc, pTNF-α (−1260/+60)-Luc, pIL-1β (−1856/+1)-Luc, or pIL-6 (−250/+1)-Luc have been described previously (Hiscott et al., 1993; Lowenstein et al., 1993; Zhang et al., 1994; Yao et al., 1997; Moon et al., 2001; Kim et al., 2007).

Novel Benzoxathiole BOT-64. 5,5-Dimethylcyclohexa-1,3-dione (1 mM) in 10 ml of methylene chloride was added drop-wise to a solution of iodosylbenzene (1 mM) in 15 ml of methylene chloride and stirred for 1 h at room temperature. After the methylene chloride was evaporated under vacuum, the reaction mixture was dissolved in 15 ml of benzene and then added with a solution of rhodium (II) acetate dimer (20 mg) and phenylisothiocyanate (1 mM) in 15 ml of benzene. The resulting mixture was refluxed for 5 h, concentrated under vacuum, and then subjected to column chromatography (hexane/ethyl acetate = 8:1) to separate BOT-64 with >95% purity. BOT-64: yield, 35%; yellow solid; melting point, 91 to 93°C; IR (KBr) 2950, 2920, 2870, 1660, 1620 cm^{-1}; NMR ($CDCl_3$): δ 1.19 (s, 6H), 2.42 (s, 2H), 2.67 (s, 2H), 7.04 (d, J = 7.6 Hz, 2H), 7.17 (t, J = 7.2 Hz, 1H), 7.38 (t, J = 7.6 Hz, 2H).

Cell Culture. RAW 264.7 and THP-1 cells are murine and human macrophages, respectively (American Type Culture Collection, Manassas, VA). They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, benzylpenicillin potassium (143 U/ml), and streptomycin sulfate (100 μg/ml) at 37°C in a 5% CO_2 atmosphere. RAW 267.4 cells harboring pNF-κB-SEAP-NPT reporter construct were cultured in the same media supplemented with Geneticin (500 μg/ml).

Cell Proliferation Assay. RAW 264.7 cells were seeded at 1×10^4 cells/well in 96-well culture plates and incubated overnight. After cells were treated with BOT-64 for various periods, they were exposed to 10 μl of WST-1 reagent (Dojindo Laboratories, Kumamoto, Japan) for 3 h, and the absorbance values were measured at a wavelength of 450 nm.

SEAP Assay. RAW 264.7 cells harboring pNF-κB-SEAP-NPT reporter construct or THP-1 cells transfected with pNF-κB-SEAP reporter construct were treated with BOT-64 for 2 h and then stimulated with LPS (1 μg/ml) for 16 h. SEAP activity was measured as described previously (Moon et al., 2001). In brief, aliquots of the culture media were heated at 65°C for 5 min and reacted with 4-methylumbellifery phosphate (500 μM) in the dark for 1 h. SEAP activity was measured as relative fluorescence units (RFUs) with emission at 449 nm and excitation at 360 nm. In another experiment, Lipofectamine was used to transiently transfect RAW 264.7 cells harboring the pNF-κB-SEAP-NPT construct with pSV-β-galactosidase control vector in combination with expression vectors encoding IKKα, IKKβ, NF-κB p65, or p50. The transfected cells were treated with BOT-64 for 16 h and then subjected to the SEAP assay.

Electrophoretic Mobility Shift Assay. RAW 264.7 cells were treated with BOT-64 for 2 h and then stimulated with LPS (1 μg/ml)

for 1 h. The cells were disrupted in a lysis buffer (10 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 10 mM KCl, 0.5% Nonidet P-40, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF) and incubated on ice for 10 min. After centrifugation, cell pellets were resuspended in a lysis buffer (20 mM HEPES, pH 7.9, 50 mM $MgCl_2$, 420 mM KCl, 20% glycerol, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF) and incubated on ice for 30 min. After centrifugation, supernatants were used as the sources of nuclear extracts. A double-stranded oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′, in which the NF-κB binding site is underlined) was ^{32}P-end labeled using [γ-^{32}P]ATP and polynucleotide kinase and then reacted with nuclear extracts in a binding buffer [10 mM Tris, pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 0.05 μg/μl poly(dI-dC), and 4% glycerol] on ice for 30 min. The DNA-protein complex was resolved on native 6% acrylamide gel by electrophoresis, and radioactive bands from the dried gels were visualized by exposure to X-ray film. Supershift assays were performed by incubating the DNA-binding reactions with 4 μg each of anti-NF-κB p65 antibody or anti-NF-κB p50 antibody for an additional 20 min on ice before electrophoresis.

Western Blot Analysis. RAW 264.7 cells were treated with BOT-64 for 2 h and then stimulated with LPS (1 μg/ml) for various times. Cell extracts were prepared in an AT buffer (20 mM HEPES, pH 7.9, 1% Triton X-100, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM Na_3VO_4, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Equal amounts of protein were resolved on SDS-acrylamide gels by electrophoresis and transferred to PVDF membrane. Either 5% nonfat milk in phosphate-buffered saline with Tween 20 or 5% bovine serum albumin in Tris-buffered saline with Tween 20 was used as the blocking buffer. The blots were incubated at 4°C overnight with primary antisera. The antisera and dilutions are as follows: anti-IκBα (1:300), anti-p-IκBα (1:1000), anti-IKKβ (1:200), anti-p-ERK-1/2 (1:1000), anti-p-JNK (1:500), anti-iNOS (1:1500), and anti-COX-2 (1:200). The blots were then incubated at room temperature for 2 h with appropriate horseradish peroxidase-labeled secondary antisera. Immune complexes on the blots were visualized by exposure to X-ray film after reacting with an enhanced chemiluminescence reagent (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Enzyme Assay of IKKβ. The catalytic activity of IKKβ was measured as described previously (Kim et al., 2007). In brief, either highly purified IKKβ (Millipore Corporation, Billerica, MA) or immunoprecipitated enzyme was incubated with GST-IκB (2 μg) as the substrate and [γ-^{32}P]ATP (5 μCi) in an assay buffer (20 mM HEPES, pH 7.7, 2 mM $MgCl_2$, 50 μM ATP, 10 mM β-glycerophosphate, 10 mM NaF, 300 μg/ml Na_3VO_4, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT) at 30°C for 1 h. The reaction mixtures were resolved on SDS-10% acrylamide gel by electrophoresis, and radioactive bands from the dried gels were visualized by exposure to X-ray film. For preparation of IKK immunoprecipitates, RAW 264.7 cells were treated with BOT-64 for 2 h and then stimulated with LPS (1 μg/ml) for 10 min. Cell extracts were incubated with anti-IKKβ antibody (2 μg) and protein A beads (GE Healthcare). To obtain IKKβ immunoprecipitates, RAW 264.7 cells were transfected with FLAG-tagged expression vectors encoding IKKβ (C/A) or IKKβ (SS/EE). Cell extracts were incubated with anti-FLAG affinity gel freezer-safe beads on ice for 2 h. Beads were washed three times with the AT buffer, twice with 20 mM HEPES, pH 7.7, and then subjected to the kinase assay.

Nitrite Quantification. RAW 264.7 cells were treated with BOT-64 for 2 h and then stimulated with LPS (1 μg/ml) for 24 h. Amounts of nitrite were measured as described previously (Archer, 1993). In brief, aliquots (100 μl) of the culture media were reacted with 1:1 mixture (100 μl) of 1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine, and the absorbance values were measured at 540 nm.

Enzyme-Linked Immunosorbent Assay. RAW 264.7 cells were treated with BOT-64 for 2 h and then stimulated with LPS (1 μg/ml) for 24 h. Amounts of prostaglandin E_2 (PGE_2), TNF-α, IL-1β, or IL-6 in the culture media were measured using enzyme-linked immunosorbent assay (ELISA) kits (GE Healthcare).

Reverse Transcription-Polymerase Chain Reaction. RAW 264.7 cells were treated with BOT-64 for 2 h and then stimulated with LPS (1 μg/ml) for 4 to 6 h. Total RNA of the cells was subjected to semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) using an RNA PCR kit (Bioneer Co., Daejeon, Korea). In brief, total RNA was reverse-transcribed at 42°C and then subjected to 25 to 30 cycles of PCR consisting of 30-s denaturation at 94°C, 30-s annealing at 50 to 60°C, and 90-s extension at 72°C. The sequences of primers for RT-PCR and the sizes of PCR products are as follows: iNOS: sense, 5′-GTCAACTGCAAGAGAACGGAGAC-3′, and antisense, 5′-GAGCTCCTCCAGAGGGTAGGCTTG-3′, 457 bp; COX-2: sense, 5′-ACTCACTCAGTTTGTTGAGTCATTC-3′, and antisense, 5′-TTTGATTAGTACTGTAGGGTTAATG-3′, 583 bp; TNF-α: sense, 5′-AGGTTCTGTCCCTTTCACTCACTGG-3′, and antisense, 5′-AGAGAACCTGGGAGTAGACAAGGT-3′, 487 bp; IL-1β: sense 5′-CCTGTCCTGTGTAATGAAAGACGGC-3′, and antisense, 5′-GTGCTGCCTAATGTCCCCTTGAATC-3′, 525 bp; IL-6: sense, 5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′, and antisense, 5′-CCTTCTGTGACTCCAGCTTATCTGT-3′, 549 bp; and β-actin: sense, 5′-CACCACACCTTCTACAATGAGCTGC-3′, and antisense, 5′-GCTCAGGAGGAGCAATGATCTTGAT-3′, 745 bp. RT-PCR products were resolved on agarose gels by electrophoresis and stained with ethidium bromide.

Luciferase Assay. RAW 264.7 cells were transiently transfected with pSV-β-galactosidase control vector in combination with each Luc reporter construct of p-iNOS (−1592/+183)-Luc, p-TNF-α (−1260/+60)-Luc, p-IL-1β (−1856/+1)-Luc, or pIL-6 (−250/+1)-Luc, using Lipofectamine. The transfected cells were treated with BOT-64 for 2 h and then stimulated with LPS (1 μg/ml) for 16 h. Cell extracts were subjected to luciferase assay and β-galactosidase assay using the appropriate kits (Promega). In another experiment, the cells were transiently transfected with pSV-β-galactosidase control vector and expression vector encoding IKKβ in combination with each of the Luc reporter constructs. The transfected cells were treated with BOT-64 for 16 h and subjected to the luciferase assay.

Septic Shock in Mice. Male ICR mice (20 ± 2 g body weight, 10 mice per group) were purchased from Samtako Animal Center (Kyeongki, Korea) and acclimated under semi-specific pathogen-free conditions with free access to standard diet and water ad libitum. The mice were administered BOT-64 (3–30 mg/kg, i.p.), and septic shock was induced 2 h after by injection of LPS (1.5 mg/mouse, i.p.). Survival rates of the mice were monitored over the next 48 h. Animal studies have been carried out in a veterinary facility of Chungbuk National University, Korea, in accordance with all institutional and federal ethical regulations for experimental animal care.

Statistical Analysis. Data are expressed as means ± S.E., and were analyzed by analysis of variance followed by Dunnett's test apart from survival rates in septic shock of mice, which were analyzed by the logrank test. Different values of $P < 0.01$ were considered significant.

Results

In the present study, we investigated the effects of novel benzoxathiole BOT-64 (Fig. 1A) on the NF-κB activation pathway and NF-κB-regulated expression of inflammatory genes in macrophages and on septic shock in mice. The concentrations of BOT-64 and the duration of exposure used in this study had minimal effects on the proliferation of RAW 264.7 macrophages (Fig. 1B). We focused on LPS-induced NF-κB activation since the activating pathway triggered by LPS has been relatively well characterized.

Nontoxic Doses of BOT-64 Efficiently Blocked LPS-Induced NF-κB Activation in Macrophages. To investigate whether BOT-64 could affect cellular NF-κB activation,

we determined LPS-induced NF-κB transcriptional activity using RAW 264.7 macrophages harboring the pNF-κB-SEAP-NPT construct that contains four copies of the κB sequence fused to SEAP gene as a reporter (Moon et al., 2001). The cells were pretreated with BOT-64 and then exposed to LPS for NF-κB activation. As shown in Fig. 2A, basal levels of SEAP were quite low in the cells. Upon exposure to LPS alone, however, we observed a profound increase in SEAP expression over the basal levels, indicating that cellular NF-κB is functional. Although BOT-64 had no effect on the basal levels of SEAP expression, it dose-dependently blocked LPS-induced SEAP expression in RAW 264.7 cells, corresponding to 19% inhibition at 0.3 μM, 52% at 1 μM, 70% at 3 μM, and 97% at 10 μM, with an IC_{50} value of 1 μM. A positive control, PTN inhibited the LPS-induced SEAP expression in RAW 264.7 cells with an IC_{50} value of 4 μM. Furthermore, BOT-64 blocked LPS-induced NF-κB transcriptional activity in another cell line, THP-1 macrophages transfected with the pNF-κB-SEAP reporter construct, causing 27% inhibition at 1 μM, 73% at 3 μM, and 90% at 10 μM (Fig. 2B).

We next determined whether BOT-64 could affect the DNA binding ability of NF-κB complex in the cells. Electrophoretic mobility shift assay with NF-κB-specific oligonucleotide showed that DNA binding ability of the NF-κB complex, p65/p50, and p50 homodimer was markedly increased upon exposed RAW 264.7 cells with LPS alone (Fig. 2C). BOT-64 dose-dependently blocked LPS-increased DNA binding ability of the NF-κB complex in the cells (Fig. 2C). Therefore, BOT-64 is an efficient inhibitor of LPS-induced NF-κB activation in macrophages.

BOT-64 Inhibited Signal-Induced Degradation and Phosphorylation of IκBα in LPS-Activated Macrophages. The nuclear phenomena of DNA binding ability and transcriptional activity of the NF-κB complex are preceded by phosphorylation and proteolytic degradation of cytoplasmic IκB in the LPS-induced signal cascade for NF-κB activation (Karin and Ben-Neriah, 2000; Guha and Mackman, 2001). To determine whether the prevention of LPS-induced NF-κB activation by BOT-64 was due to the inhibition of IκBα degradation, RAW 264.7 cells were pretreated with BOT-64 and exposed to LPS for various times. We then examined LPS-induced IκBα degradation by Western blot analysis. Upon exposure to LPS alone, cellular IκBα was dramatically degraded within 30 to 45 min, and its levels were returned to normal by 75 to 100 min (data not shown). BOT-64 dose-dependently reduced the degradation of cellular IκBα when examined 30 min after LPS stimulation (Fig. 2D).

To determine whether IκBα stabilization by BOT-64 was indeed due to its effect on LPS-induced IκBα phosphorylation, another Western blot analysis was carried out. Upon exposure to LPS alone for 10 min, significant IκBα phosphorylation had already taken place in the cells, whereas IκBα degradation had not yet occurred (Fig. 2E). BOT-64 dose-

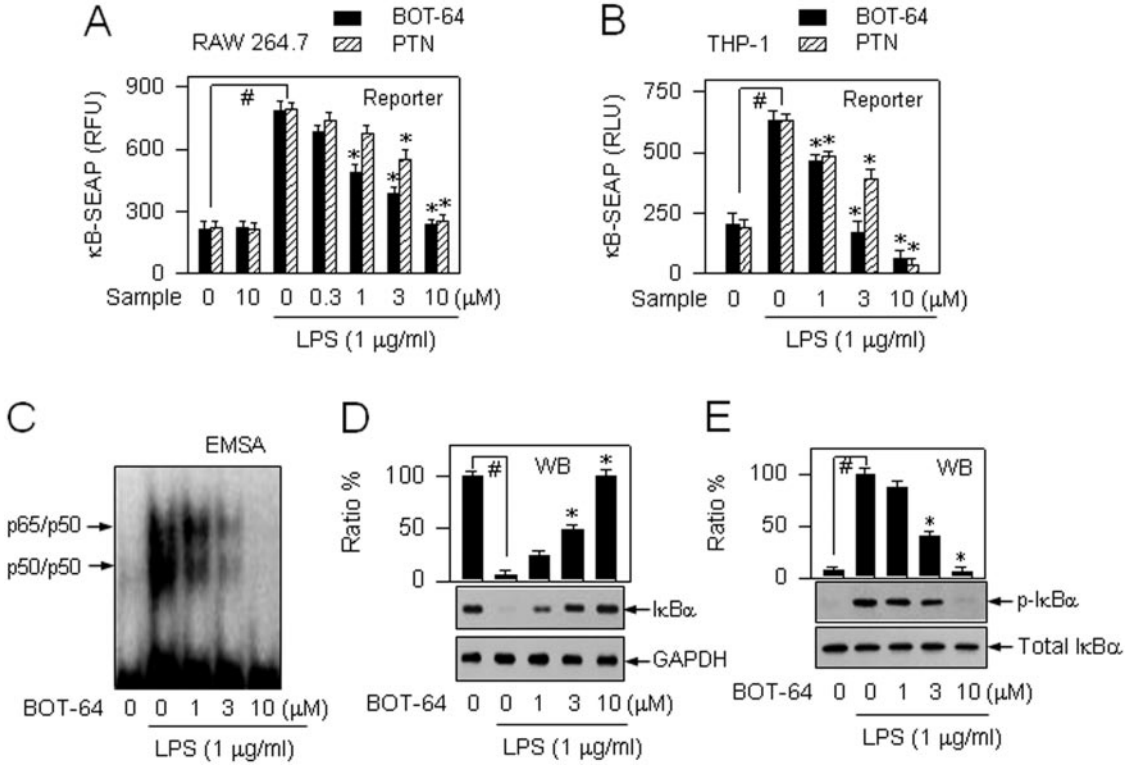

Fig. 2. Effect of BOT-64 on LPS-induced NF-κB activation. RAW 264.7 cells harboring pNF-κB-SEAP-NPT reporter construct (A) or THP-1 cells transfected with pNF-κB-SEAP reporter construct (B) were pretreated with BOT-64 or PTN for 2 h and stimulated with LPS for 16 h. SEAP activity in the culture media was measured as RFUs. RAW 264.7 cells were pretreated with BOT-64 for 2 h and stimulated with LPS for 1 h (C), 30 min (D), and 10 min (E). C, nuclear extracts were subjected to electrophoretic mobility shift assay with a ^{32}P-labeled oligonucleotide containing the κB sequence. D, cytoplasmic extracts were subjected to Western blot (WB) analysis with anti-IκBα antibody. One of similar results is represented, and the relative ratio percentage is also indicated, where IκBα signal was normalized to glyceraldehyde 3-phosphate dehydrogenase signal. E, cytoplasmic extracts were subjected to WB analysis with anti-p-IκBα (Ser-32/36) antibody or anti-IκBα antibody. One of similar results is represented, and the relative ratio percentage is also indicated, where p-IκBα signal was normalized to total IκBα signal. Data are means ± S.E. from three separate experiments. #, $P <$ 0.01 versus media alone-treated group. ∗, $P < 0.01$ versus LPS alone-treated group.

dependently inhibited LPS-induced IκBα phosphorylation in the cells (Fig. 2E).

BOT-64 Targeted IKKβ-Mediated IκBα Phosphorylation in NF-κB Activating Pathway. As described above, BOT-64 inhibited the phosphorylation and degradation of cytoplasmic IκBα and the DNA binding and transcriptional activity of nuclear NF-κB (Fig. 2) in LPS-activated RAW 264.7 cells, which are sequential events in the NF-κB activating pathway. To elucidate a primary target of BOT-64, we transfected RAW 264.7 cells harboring the pNF-κB-SEAP-NPT reporter construct with expression vectors encoding either IKKβ, IKKα, or NF-κB p65 or p50 and then examined the effect of BOT-64 directly on the resultant NF-κB activation. Transfection with IKKβ vector resulted in a significantly increased the expression of SEAP reporter in the cells, which was dose-dependently down-regulated by treatment of BOT-64 (Fig. 3A). Likewise, transfection with IKKα vector elicited a profound expression of the SEAP reporter, but this was only weakly inhibited at a high concentration of BOT-64 (Fig. 3B). In contrast, BOT-64 did not significantly affect NF-κB p65 or p50 vector-elicited SEAP expressions in the cells (Fig. 3, C and D). These results indicate that a target event of BOT-64 could be upstream IκB degradation in the NF-κB activating pathway, presumably IKKβ-mediated IκBα phosphorylation.

BOT-64 Inhibited LPS-Inducible Kinase Activity of Cellular IKK Complex. In response to inflammatory stimuli, including LPS, the kinase activity of cellular IKK complex is inducible through phosphorylation of Ser-177 and Ser-181 residues in the activation loop of IKKβ and then catalyzes IκBα phosphorylation, resulting in NF-κB activation (Delhase et al., 1999; Karin and Ben-Neriah, 2000). We tested the kinase activity of the IKK complex using whole extracts from RAW 264.7 cells stimulated with LPS in the absence or presence of BOT-64. Catalytic activity of GST-IκB phosphorylation by the IKK complex was hardly detectable in the normal cells but was markedly increased upon exposure to LPS alone for 10 min (Fig. 4A). BOT-64 dose-dependently inhibited LPS-induced IKK activity in the cells, and as expected, neither LPS nor BOT-64 had any effect on the expression of IKKβ protein (Fig. 4A). In contrast, BOT-64 had no apparent effect on the activation of ERK-1/2 and JNK in LPS-activated RAW 264.7 cells (Fig. 4B). These are involved in the alternative LPS signaling through toll-like receptor-4 (Guha and Mackman, 2001). This result indicates that BOT-64 inhibits LPS-induced cellular IKK activity, and its mode of action is kinase-specific.

BOT-64 Directly Inhibited Catalytic Activity of Purified IKKβ. To determine whether BOT-64 could directly affect the catalytic activity of IKKβ, highly purified IKKβ was incubated with various concentrations of BOT-64. The kinase activity of purified IKKβ was inhibited only when the enzyme source was preincubated with BOT-64 before reacting with the substrate GST-IκB and cofactor ATP (Fig. 5A). The IKKβ protein was also identified on SDS-acrylamide gel

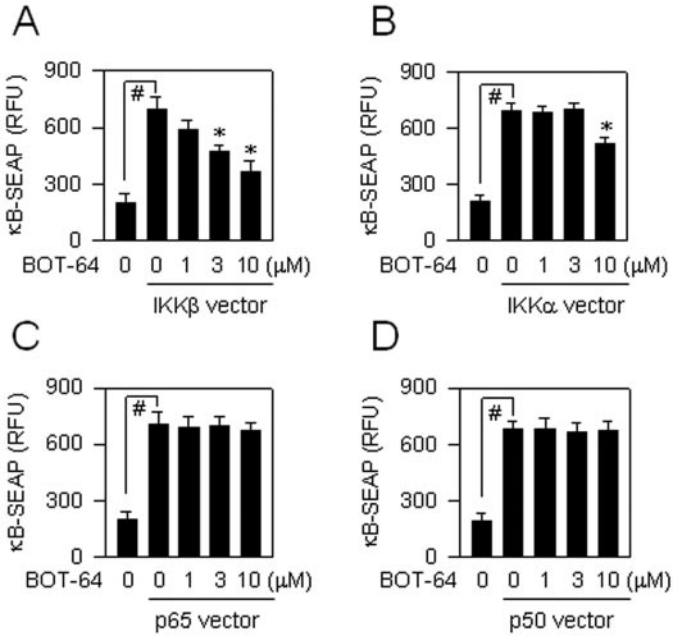

Fig. 3. Effect of BOT-64 on expression vector-elicited NF-κB activation. RAW 264.7 cells harboring pNF-κB-SEAP-NPT construct were transfected with pSV-β-galactosidase control vector, in combination with each expression vector encoding IKKβ (A), IKKα (B), NF-κB p65 (C), or p50 (D). The transfected cells were treated with BOT-64 for 16 h. SEAP activity in the culture media was measured as RFUs and then normalized to β-galactosidase activity. Data are mean ± S.E. from three separate experiments. #, $P < 0.01$ versus pSV-β-galactosidase control vector alone-transfected group. ∗, $P < 0.01$ versus each expression vector plus pSV-β-galactosidase control vector alone-transfected group.

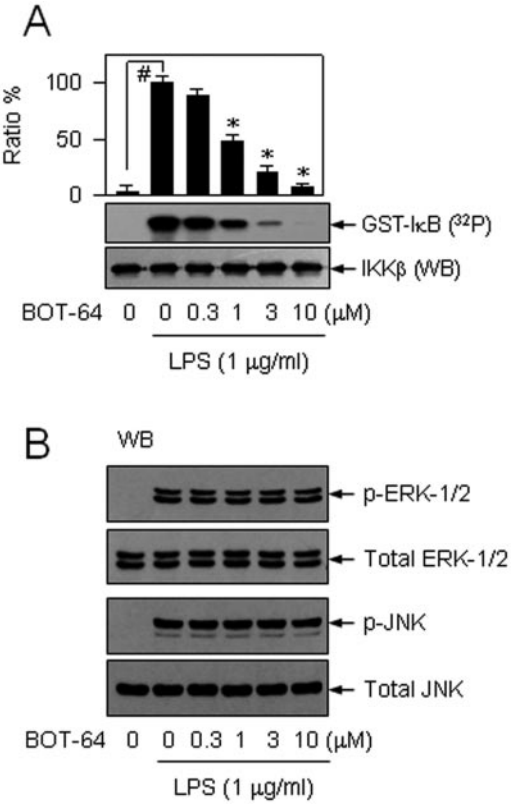

Fig. 4. Effect of BOT-64 on LPS-induced activation of IKK, ERK-1/2, and JNK. RAW 264.7 cells were pretreated with BOT-64 for 2 h and stimulated with LPS for 10 (A) or 25 min (B). A, cell extracts were used for the kinase assay of GST-IκB phosphorylation (^{32}P) and were also subjected to WB analysis with anti-IKKβ antibody. Data are means ± S.E. from three separate experiments. One of similar results is represented, and relative ratio percentage is also indicated, where GST-IκB signal was normalized to IKKβ signal. #, $P < 0.01$ versus media alone-treated group. ∗, $P < 0.01$ versus LPS alone-treated group. B, cell extracts were subjected to WB analysis with antibodies against p-ERK-1/2, ERK-1/2, p-JNK, or JNK.

by electrophoresis, and as expected, its size corresponds to 88 kDa (Fig. 5B).

The positive control, PTN, inhibited LPS-induced NF-κB activation in this study (Fig. 2, A and B). The α,β-unsaturated carbonyl structures of PTN and other compounds, including artemisolide, directly react with Cys-179 residue in the activation loop of IKKβ, in a Michael addition, inhibiting the catalytic activity of IKKβ for NF-κB activation (Hehner et al., 1999; Kim et al., 2007). As shown in Fig. 1A, BOT-64 also contains an α,β-unsaturated carbonyl structure.

We determined next whether the mechanism by which BOT-64 inhibits the catalytic activity of IKKβ could be attributable to its direct interaction with the activation loop of IKKβ. RAW 264.7 cells were transfected with FLAG-tagged expression vectors encoding IKKβ (C/A) with alanine residue substituted for Cys-179 residue that is altered by thiol-reactive IKKβ inhibitors or those encoding IKKβ (SS/EE) with glutamic acid residues substituted for two Ser-177 and Ser-181 residues that are phosphorylated for the activation of IKKβ. The point-mutated IKKβ proteins were purified as anti-FLAG immunoprecipitates from the cells and still exhibited the kinase activity of GST-IκB phosphorylation (Fig. 5, C and D). Preincubation of BOT-64 with IKKβ (C/A) immunoprecipitates, before the kinase reaction, inhibited GST-IκB phosphorylation in a dose-dependent manner (Fig. 5C). In contrast, BOT-64 had no apparent effect on the kinase activity of IKKβ (SS/EE) immunoprecipitates (Fig. 5D). These findings proved that BOT-64 inhibited the catalytic activity of IKKβ, and this mechanism of action is probably mediated by direct interaction with Ser-177 and/or Ser-181 residues in the activation loop of IKKβ.

BOT-64 Suppressed LPS-Induced Production of Inflammatory Mediators. NF-κB activation has been evidenced to play a pivotal role in the LPS-induced production of inflammatory mediators such as nitric oxide (NO), PGs, and cytokines (Guha and Mackman, 2001; Tian and Brasier, 2003). We quantified the inflammatory mediators in LPS-activated RAW 264.7 cells. Amounts of nitrite, a stable metabolite of NO, were quite low in normal cells (7.7 ± 3.2 μM) but markedly increased to 48.8 ± 4.5 μM upon exposure to LPS alone (Fig. 6A). Pretreatment of the cells with BOT-64 inhibited LPS-induced nitrite production in a dose-dependent manner, with an IC_{50} value of 0.7 μM (Fig. 6A), which correlates well with its inhibitory potency on NF-κB activation (Fig. 2A). LPS alone increased PGE_2 levels to 1464 ± 58 pg/ml from basal levels of 267 ± 43 pg/ml. This increase was also inhibited by BOT-64 (Fig. 6B). To understand whether the inhibitory effects of BOT-64 on nitrite and PGE_2 production were mainly due to the LPS-inducible amounts of iNOS or COX-2 protein, we performed Western blot analysis using whole extracts from RAW 264.7 cells exposed to LPS in the absence or presence of BOT-64. As shown in Fig. 6C, iNOS and COX-2 signals were hardly detectable in normal cells but markedly increased upon exposure to LPS alone. BOT-64 dose-dependently suppressed LPS-induced production of both iNOS and COX-2 proteins. However, neither LPS nor BOT-64 had any effects on the cellular levels of housekeeping glyceraldehyde 3-phosphate dehydrogenase.

Amounts of the cytokines were measured, by use of ELISA, in the culture media of LPS-activated RAW 264.7 cells. Upon exposure to LPS alone, the cells released pronounced amounts of TNF-α (14.0 ± 1.1 ng/ml) compared with the basal level (0.9 ± 0.5 ng/ml) (Fig. 6D). BOT-64 inhibited LPS-induced TNF-α production in a dose-dependent manner (Fig. 6D). In a parallel experiment, LPS-induced productions of IL-1β and IL-6 were also decreased by treatment with BOT-64 (Fig. 6, E and F).

BOT-64 Attenuated NF-κB-Regulated Expression of Inflammatory Genes in LPS-Activated Macrophages. To understand whether the inhibitory action of BOT-64 on LPS-induced expression of iNOS, COX-2, or cytokines had taken place at the transcriptional level, semiquantitative RT-PCR was carried out. As shown in Fig. 7A, the transcripts encoding iNOS, COX-2, TNF-α, IL-1β, or IL-6 were barely

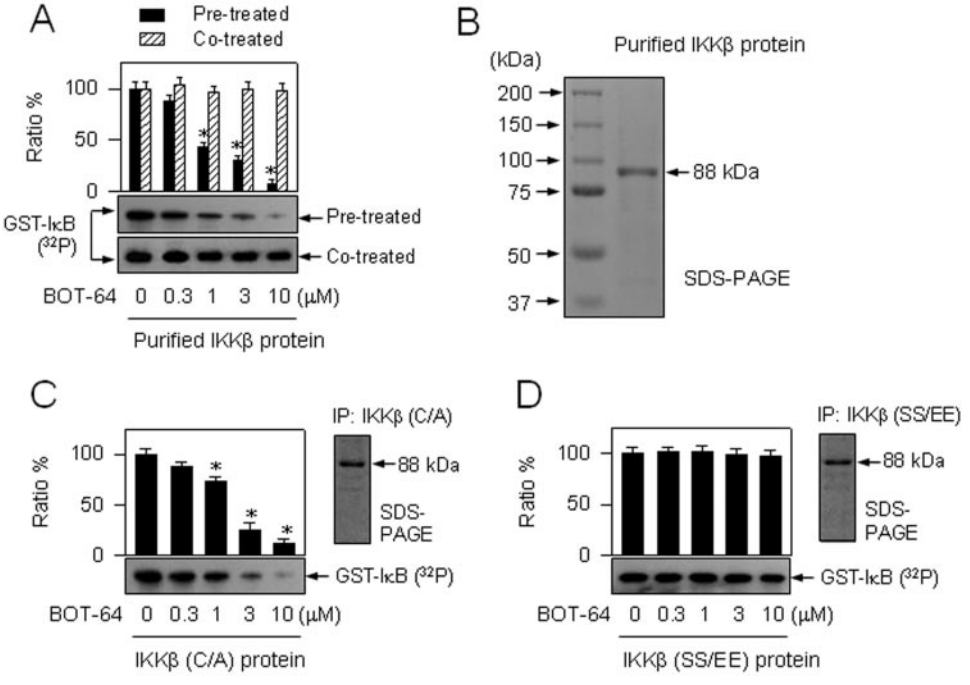

Fig. 5. Effect of BOT-64 on catalytic activity of IKKβ. A, highly purified IKKβ was preincubated with BOT-64 for 30 min before reacting with substrate GST-IκB and cofactor ATP (pretreated, ■) or incubated with BOT-64 in the presence of GST-IκB and ATP (cotreated, ▨). Catalytic activity of the enzyme was measured by GST-IκB phosphorylation (^{32}P). B, purified IKKβ corresponding to 88 kDa in size is identified on SDS 10% acrylamide gel by electrophoresis [SDS-polyacrylamide gel electrophoresis (PAGE)]. RAW 264.7 cells were transfected with FLAG-tagged expression vector encoding IKKβ (C/A) (C) or IKKβ (SS/EE) (D). Cell extracts were subjected to immunoprecipitation (IP) with anti-FLAG M2 affinity gel freezer-safe beads followed by in vitro kinase assay of GST-IκB phosphorylation (^{32}P), in which immunoprecipitates were preincubated with BOT-64 before the kinase reaction. The enzyme sources are also identified by SDS-PAGE. Data are means ± S.E. from three separate experiments. *, $P < 0.01$ versus enzyme alone-containing group.

detectable in normal cells but markedly increased upon exposure to LPS alone. BOT-64 differentially attenuated LPS-induced synthesis of the inflammatory transcripts in the cells.

Transcriptional regulation of iNOS and cytokines by BOT-64 was further delineated using the promoter activity assay with Luc reporter. RAW 264.7 cells were transfected with p-iNOS (−1592/+183)-Luc, p-TNF-α (−1260/+60)-Luc, p-IL-1β (−1856/+1)-Luc, or p-IL-6 (−250/+1)-Luc construct (Hiscott et al., 1993; Lowenstein et al., 1993; Zhang et al.,

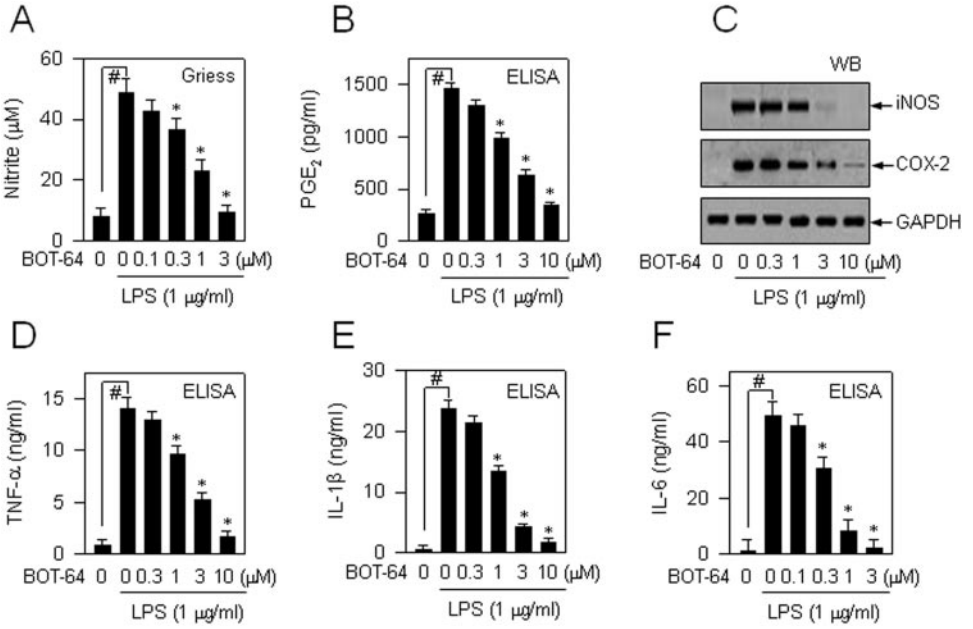

Fig. 6. Effect of BOT-64 on LPS-induced production of inflammatory mediators. RAW 264.7 cells were pretreated with BOT-64 for 2 h and stimulated with LPS for 24 h. The culture media were subjected to Griess reaction for nitrite quantification (A) or ELISAs for PGE_2 (B), TNF-α (D), IL-1β (E), and IL-6 (F). Data are means ± S.E. from three separate experiments. #, $P < 0.01$ versus media alone-treated group. *, $P < 0.01$ versus LPS alone-treated group. C, cell extracts were subjected to WB analysis with anti-iNOS antibody or anti-COX-2 antibody.

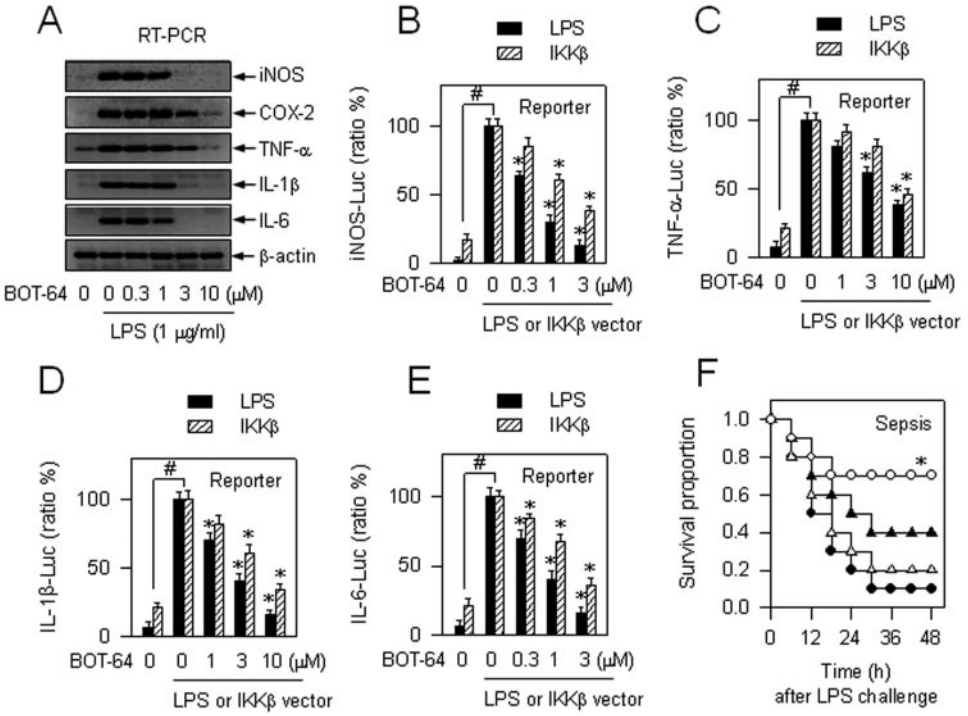

Fig. 7. Effect of BOT-64 on NF-κB-regulated gene transcription and LPS-induced septic shock. A, RAW 264.7 cells were pretreated with BOT-64 for 2 h and stimulated with LPS for 4 to 6 h. Total RNA was subjected to RT-PCR with housekeeping β-actin as an internal control. The cells were transfected with pSV-β-galactosidase control vector in combination with each Luc reporter construct for iNOS (B), TNF-α (C), IL-1β (D), or IL-6 promoter (E) (■). The transfected cells were pretreated with BOT-64 for 2 h and stimulated with LPS (1 μg/ml) for 16 h. In another experiment, the cells were transfected with pSV-β-galactosidase control vector and expression vector of IKKβ, in combination with each Luc reporter construct for iNOS (B), TNF-α (C), IL-1β (D), or IL-6 promoter (E) (▨). The transfected cells were treated with BOT-64 for 16 h. Luciferase activity was measured in the cell extracts and is represented as relative ratio percentage, in which luciferase activity was normalized to β-galactosidase activity. Data are means ± S.E. from three separate experiments. #, $P < 0.01$ versus media alone-treated group or each Luc reporter construct alone-transfected group. *, $P < 0.01$ versus LPS alone-treated group or each Luc reporter construct plus IKKβ vector alone-transfected group. F, 10 mice per group were treated with vehicle only (●) or BOT-64 at doses of 3 mg/kg (△), 10 mg/kg (▲), and 30 mg/kg (○) and after 2 h were injected with LPS (1.5 mg/mouse, i.p.). Survival rates of these mice were observed over the next 48 h. *, $P < 0.01$ versus LPS alone-challenged group in the logrank test.

1994; Yao et al., 1997). Upon exposure to LPS alone, the cells harboring the piNOS (−1592/+183)-Luc construct had approximately 40-fold increased luciferase expression over the basal levels (Fig. 7B). BOT-64 inhibited LPS-induced luciferase expression, a reporter of iNOS promoter activity, in a dose-dependent manner (Fig. 7B). Likewise, LPS-induced promoter activities, from p-TNF-α (−1260/+60)-Luc (Fig. 7C), p-IL-1β (−1856/+1)-Luc (Fig. 7D), or p-IL-6 (−250/+1)-Luc construct (Fig. 7E), were also inhibited by treatment with BOT-64. Therefore, BOT-64 down-regulates LPS-induced expression of inflammatory genes encoding iNOS, COX-2, TNF-α, IL-1β, and IL-6 at the transcriptional level.

Because BOT-64 inhibits NF-κB activation and also transcriptionally down-regulates inflammatory gene expression in LPS-activated macrophages, we decided to determine whether BOT-64 could affect NF-κB-regulated expression of the inflammatory genes. RAW 264.7 cells were transfected with an expression vector encoding IKKβ in combination with each Luc reporter construct for promoter activity. Upon transfecting the cells with IKKβ vector, luciferase expression from p-iNOS (−1592/+183)-Luc construct increased almost 20-fold over the basal levels (Fig. 7B). BOT-64 dose-dependently inhibited IKKβ vector-elicited iNOS promoter activity in the cells (Fig. 7B). In a parallel experiment, IKKβ vector-elicited promoter activities, from p-TNF-α (−1260/+60)-Luc (Fig. 7C), p-IL-1β (−1856/+1)-Luc (Fig. 7D), or p-IL-6 (−250/+1)-Luc construct (Fig. 7E) were also inhibited by treatment of BOT-64.

BOT-64 Prevented LPS-Induced Septic Death in Mice. NF-κB-regulated expression of the gene network, which includes iNOS, COX-2, and cytokines, has been shown to play a central role in the pathophysiology of septic shock (Liu and Malik, 2006). To confirm whether the suppressive action of BOT-64 on NF-κB-regulated gene transcription in macrophages could result in anti-inflammatory efficacy in an animal model, we investigated LPS-induced septic death in mice. As shown in Fig. 7F, most of the mice were sacrificed within 30 h after injection with LPS alone, and 10 to 60% of the LPS-shocked mice were rescued by treatment with BOT-64 at doses of 3 to 30 mg/kg.

Discussion

The molecular basis of multiple pharmacological properties assigned to benzoxathiole derivatives has not been defined, even though NF-κB activation is closely linked to inflammatory and proliferative disorders. A goal of this study is to demonstrate the effects of benzoxathiole BOT-64 (Fig. 1A) on LPS-induced NF-κB activation and NF-κB-regulated gene transcription at the molecular level.

In the present study, we demonstrated that BOT-64 could inhibit NF-κB activation, the transcriptional activity, and DNA binding ability of nuclear NF-κB (Fig. 2, A–C) and the degradation and phosphorylation of cytoplasmic IκBα in LPS-activated RAW 264.7 cells (Fig. 2, D and E). To clarify the primary target of BOT-64 on the LPS-induced NF-κB activating pathway, we performed cellular NF-κB activation directly due to the transfection of expression vector encoding IKKβ, IKKα, or NF-κB p65 or p50. BOT-64 inhibited IKKβ vector-elicited NF-κB activation in a dose-dependent manner (Fig. 3A), but its effect on IKKα vector-elicited NF-κB activation was very weak (Fig. 3B). On the other hand, BOT-64 did not inhibit NF-κB p65 or p50 vector-elicited NF-κB activation (Fig. 3, C and D). Therefore, a target event of BOT-64 is the IKKβ-mediated IκBα phosphorylation in the cytoplasm, resulting in sequential prevention of downstream events for NF-κB activation.

To elucidate an inhibitory mechanism of BOT-64 on IKKβ-mediated IκBα phosphorylation, we demonstrated that BOT-64 could inhibit LPS-inducible IKK activity in RAW 264.7 cells without affecting IKKβ expression (Fig. 4A). However, BOT-64 could not affect the activation of ERK-1/2 or JNK in the same cells (Fig. 4B). Therefore, the inhibitory action of BOT-64 on serine/threonine kinases is specific to the IKK complex. Furthermore, BOT-64 inhibited the catalytic activity of highly purified IKKβ in a dose-dependent manner only when the compound was preincubated with IKKβ before the kinase reaction (Fig. 5A). To understand whether this inhibition was attributable to the direct interaction of BOT-64 with the activation loop of IKKβ, FLAG-tagged IKKβ (C/A) and IKKβ (SS/EE) proteins were obtained as immunoprecipitates. The point-mutated IKKβ proteins still exhibited the catalytic activity of GST-IκB phosphorylation (Fig. 5, C and D). Pretreatment of BOT-64 inhibited in vitro kinase activity of IKKβ (C/A) protein in a dose-dependent manner (Fig. 5C) but not that of IKKβ (SS/EE) protein (Fig. 5D). These findings indicate that a molecular target of BOT-64 is Ser-177 and/or Ser-181 residues in the activation loop of IKKβ, resulting in inhibition of LPS-induced IKK activity in the cells and of the catalytic activity of purified IKKβ, which are prerequisites for LPS-induced NF-κB activation.

Because IKKβ-mediated phosphorylation of its cytoplasmic IκB partner represents a key convergent point for most pathogenic stimuli leading to NF-κB activation, IKKβ is widely considered to be a drug target for inflammatory and proliferative disorders (O'Sullivan et al., 2007; Olivier et al., 2006). Arsenic trioxide, at present under clinical trials for treatment of leukemia and solid tumors, and the gold compound auranofin are thiol-reactive drugs that directly interact with Cys-179 residue in the activation loop of IKKβ, resulting in inhibition of NF-κB activation (Jeon et al., 2003; Mathas et al., 2003). Aspirin, sulindac and some COX-2 inhibitors have been reported recently to inhibit LPS- or TNF-α-induced NF-κB activation, because they inhibit the catalytic activity of IKKβ by a competitive mechanism on ATP binding to the enzyme (Yin et al., 1998; Yamamoto et al., 1999). Sulfasalazine is routinely used in the treatment of inflammatory bowel disease and rheumatoid arthritis and has been shown to inhibit NF-κB activation through inhibition of ATP binding to IKKα and IKKβ (Weber et al., 2000). Epoxyquinoid derivatives such as manumycin A and jesterone dimer are another class of IKKβ inhibitors that induce a covalent dimerization of IKKβ, preventing the association of NEMO/IKKγ with IKKα and IKKβ (Liang et al., 2003; Bernier et al., 2006). In this study, BOT-64 exhibited approximately 3- to 4-fold stronger inhibitory potency, as assessed by the IC_{50} values, on NF-κB activation than PTN (Fig. 2, A and B). Furthermore, the potencies of IKKβ-inhibiting drugs on the regulation of NF-κB activation could be in order of BOT-64 > auranofin = manumycin A > sulfasalazine > aspirin.

Selective inhibitors of the IKK complex, which target IKKβ preferentially over IKKα, have recently undergone preclinical studies by the pharmaceutical industry. The imidazoqui-

noxaline derivative BMS-345541 is an allosteric inhibitor of IKKβ, preventing collagen-induced arthritis and inducing apoptosis of melanoma cells (McIntyre et al., 2003). The β-carboline derivative PS-1145 is a selective inhibitor of the IKK complex, preventing proliferation of multiple myeloma cells (Castro et al., 2003). Another IKKβ inhibitor, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl-nicotinonitrile, is an anti-inflammatory agent that has shown antiproliferative potentials in multiple myeloma and leukemia cells (Murata et al., 2004).

To investigate the influence of BOT-64 on NF-κB-regulated gene transcription, we have shown that BOT-64 inhibited LPS-induced production of inflammatory mediators such as nitrite, PGE_2, TNF-α, IL-1β, and IL-6 in RAW 264.7 cells (Fig. 6). The effects of BOT-64 on nitrite or PGE_2 production were mainly attributable to a dose-dependent decrease in the LPS-inducible amounts of iNOS or COX-2 protein in the cells (Fig. 6C). Furthermore, BOT-64 differentially attenuated LPS-induced synthesis of iNOS, COX-2, TNF-α, IL-1β, or IL-6 transcript in the cells (Fig. 7A) and inhibited LPS-induced promoter activities of iNOS or the cytokines (Fig. 7, B–E), indicating that BOT-64 down-regulates LPS-induced expression of inflammatory genes at the transcription level.

Even though multiple regulatory elements are involved, NF-κB has been evidenced to play a major role in the LPS-induced expression of inflammatory genes in macrophages (Guha and Mackman, 2001; Tian and Brasier, 2003). LPS-responsive κB sequences are identified in the inflammatory genes: iNOS with two sites at −8287/−8270 and −119/−102 regions relative to the transcription start; COX-2 with one site at −223/−214; TNF-α with three sites at −594/−577, −217/−200, and −103/−86; IL-1β with two sites at −2800/−2720 and −296/−286; and IL-6 with one site at −72/−63 (Hiscott et al., 1993; Lowenstein et al., 1993; Zhang et al., 1994; Yao et al., 1997). In this study, we have demonstrated that BOT-64 differentially inhibited cellular promoter activity of iNOS, TNF-α, IL-1β, or IL-6 gene elicited by overexpression of IKKβ (Fig. 7, B–E). This result indicates that BOT-64 could suppress NF-κB-regulated expression of inflammatory genes in the cells.

Endotoxin-induced transcriptional activation of multiple inflammatory genes is the hallmark of septic pathophysiology, in which NF-κB activation is a central pathogenic mechanism (Chauhan et al., 2003; Liu and Malik, 2006). iNOS knockout mice ameliorate the impaired vasodilator response and improve the survival rates in LPS model of septic shock (Chauhan et al., 2003). Mice deficient in p55 TNF receptor or p80 IL-1 receptor are resistant to LPS-induced septic shock and inflammation (Acton et al., 1996). Moreover, NF-κB inhibitors with diverse chemical properties and mechanism of action, such as PTN, pyrrolidine dithiocarbamate, benzofuran derivative IRFI 042, and isoquinoline alkaloid YS 51, have been demonstrated to rescue LPS-shocked animals with differential efficacy (Altavilla et al., 2002; Sheehan et al., 2002). In this study, BOT-64 suppressed NF-κB-regulated expression of inflammatory genes in LPS-activated RAW 264.7 cells (Fig. 7, B–E) and increased the survival rates of LPS-shocked mice in a dose-dependent manner (Fig. 7F).

Taken together, benzoxathiole BOT-64 is a small-molecule inhibitor of IKKβ, directly targeting Ser-177 and/or Ser-181 residues, that prevents LPS-induced NF-κB activation in the cells. This mechanism of action contributes to suppressive effect of BOT-64 on NF-κB-regulated transcription of inflammatory genes in macrophages and to pharmacological potential in LPS-induced septic shock of mice.

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