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Inhibition of lipopolysaccharide-induced expression of inducible nitric oxide synthase by phenolic (3*E*)-4-(2-hydroxyphenyl)but-3-en-2-one in RAW 264.7 macrophages

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Abstract

The large amount of nitric oxide (NO) produced by inducible NO synthase (iNOS) contributes to cellular injury in inflammatory disease. In the present study, a novel synthetic compound (3*E*)-4-(2-hydroxyphenyl)but-3-en-2-one (HPB) was found to inhibit lipopolysaccharide (LPS)-induced NO generation, but not through the inhibition of iNOS activity, in RAW 264.7 macrophages. Administration of HPB into mice also inhibited the LPS-induced increase in serum nitrite/nitrate levels. To evaluate the underlying mechanisms of HPB inhibition of NO generation, the expression of the *iNOS* gene in RAW 264.7 macrophages was examined. HPB abolished the LPS-induced expression of iNOS protein, iNOS mRNA and iNOS promoter activity in a similar concentration-dependent manner. LPS-induced nuclear factor-κB (NF-κB) DNA binding and NF-κB-dependent reporter gene activity were both significantly inhibited by HPB. This effect was mediated through the inhibition of inhibitory factor-κBα (IκBα) phosphorylation and degradation, and of p65 nuclear translocation. HPB had no effect on the LPS-induced phosphorylation of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinases (MAPK), and c-Jun NH_2-terminal kinase (JNK). However, HPB suppressed the LPS-induced intracellular reactive oxygen species (ROS) production. These results indicate that HPB down-regulates *iNOS* gene expression probably through the inhibition of LPS-induced intracellular ROS production, which has been implicated in the activation of NF-κB.

Keywords: HPB; Nitric oxide; Nitric oxide synthase; Nuclear factor-κB; Antioxidant; RAW 264.7 macrophages

1. Introduction

Nitric oxide (NO) is a short-lived free radical that mediates many biological functions, including neurotransmission, vascular homeostasis, and host defense [1,2]. NO is synthesized from L-arginine by nitric oxide synthase (NOS). Three types of NOS have been identified: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [3]. In activated macrophages, the transcriptionally expressed iNOS is responsible for the prolonged and profound production of NO. The physiological generation of NO mediates the bactericidal and tumoricidal functions of macrophages. However, the excessive action of iNOS has been implicated in the pathogenesis of inflammatory tissue injury and several additional disease states [4–6]. Thus, the inhibition of NO production by blocking iNOS expression is an important target in the treatment of inflammatory diseases.

Expression of the *iNOS* gene in macrophages is regulated mainly at the transcriptional level, particularly by NF-κB [7]. In unstimulated cells, NF-κB is present

Abbreviations: DCFH-DA, 2′,7′-dichlorofluorescein diacetate; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; HPB, (3*E*)-4-(2-hydroxyphenyl)but-3-en-2-one; IκB, inhibitory factor-κB; IKK, IκB kinase; iNOS, inducible NO synthase; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor-κB; NO, nitric oxide; ROS, reactive oxygen species; and RT-PCR, reverse transcriptase-polymerase chain reaction

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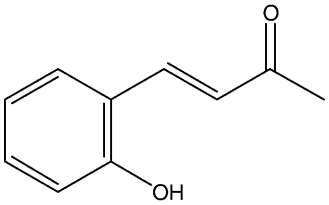

Fig. 1. Chemical structure of HPB.

constitutively in the cytosol as a homodimer or heterodimer and is linked to inhibitory IκB proteins (IκB). Activation of NF-κB results in phosphorylation, ubiquitination, and proteasome-mediated degradation of the IκB proteins, followed by the translocation of NF-κB to the nucleus and induction of gene transcription through binding to the *cis*-acting κB element [8]. Moreover, three well-defined mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun NH_2-terminal kinase (JNK), have been implicated in the transcriptional regulation of *iNOS* gene because the specific MAPKs inhibitors suppress the expression of the *iNOS* gene [9–13].

In the evaluation of anti-inflammatory activities of chalcone derivatives [14–17], which have been shown to exert a wide range of activities including the inhibition of NO and TNF-α production from macrophages, release of mediators from mast cells and neutrophils, and the blockade of superoxide anion generation from neutrophils, an intermediate phenolic (3*E*)-4-(2-hydroxyphenyl)but-3-en-2-one (HPB) (Fig. 1) was found to be a potent inhibitor of NO production in LPS-stimulated RAW 264.7 macrophages. Phenolic compounds have been reported to exert anti-inflammatory activity mediated by inhibition of NF-κB or iNOS in various cell types [18]. In this study, we undertook experiments to assess the inhibitory effects of HPB on NO production and to evaluate the underlying mechanisms.

2. Materials and methods

2.1. Materials

HPB (purity > 99%) was synthesized (S.-C. Kuo, unpublished observation) and dissolved in DMSO. The RAW 264.7 mouse macrophage-like cell line was obtained from the American Type Culture Collection. Dulbecco's modified Eagle's medium, penicillin, streptomycin, fetal bovine serum and lipofectAMINE were purchased from Invitrogen. Polyvinylidene difluoride membrane was obtained from Millipore. ECL Western blotting reagent and Hybond-N^+ nylon membranes were purchased from Amersham Bioscience. REzol^{TM} C&T reagent was purchased from Protech Technology (Taiwan). Express Hyb hybridization solution, pNF-κB-LUC and antibody against iNOS were obtained from BD Biosciences. Random primer fluorescein labeling kit was obtained from PerkinElmer. Nitrate reductase and DIG gel shift kit were purchased from Roche Applied Science. pRL-TK and a Dual-luciferase reporter assay system were obtained from Promega. Antibodies against p65, IκBα, p38 MAPK, phospho-JNK, JNK and ERK2 were obtained from Santa Cruz Biotechnology. Antibodies against phospho-ERK1/2, phospho-p38 MAPK and phospho-IκBα were purchased from New England Biolabs. Antibody against actin was obtained from Chemicon. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes. All other reagents and chemicals were purchased from the Sigma-Aldrich.

2.2. Cell culture

RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin, and were maintained in a humidified incubator with 5% CO_2. Stock cells were passaged 2–3 times/week with 1:10 split ratio and used within 10 passages.

2.3. Nitrite determination

Cells were seeded onto 96-well plates with 2×10^5 cells/well and allowed to adhere overnight. Cells were then treated with vehicle or test drug at 37 °C for 1 h before stimulation with 1 μg/ml of LPS (*Escherichia coli*, serotype 0111:B4) for 24 h in a final volume of 0.2 ml. The cell-free culture medium was collected and stored at −70 °C until NO determination. The production of NO was determined based on the Griess reaction [19]. Briefly, 40 μl of 5 mM sulfanilamide, 10 μl of 2 M HCl and 20 μl of 40 mM naphthylethylenediamine were added to 150 μl of culture medium. After a 10-min incubation period at room temperature, absorbance was measured with a microplate reader at 550 nm. A standard nitrite curve was generated in the same fashion using $NaNO_2$.

2.4. iNOS activity

The iNOS activity in the cell lysate was measured as L-arginine- and NADPH-dependent generation of nitrite/nitrate [20]. Briefly, cells were stimulated or not stimulated with 1 μg/ml of LPS for 16 h, then resuspended in lysis buffer (20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin and leupeptin, and 5 μg/ml of pepstain A) and sonicated. Protein concentration of samples was determined by Bradford assay. The lysates (200 μg protein) were incubated with or without HPB for 3 h at room temperature in a 200 μl reaction mixture containing 20 mM Tris–HCl, pH 8.0, 2 mM NADPH, 2 mM L-arginine and 10 μM FAD. Nitrate formation in the reaction mixture was reduced to nitrite by incubation at 37 °C for 15 min with 0.1 U/ml of nitrate reductase, 0.1 mM NADPH and 5 μM FAD. The reaction was stopped by the addition of 10 U/ml of lactic

dehydrogenase (LDH) and 10 mM pyruvate. Nitrite value of the control test (without NADPH/L-arginine) was subtracted from the experimental values. The iNOS activity in cytosol was expressed as nmol/mg of protein.

2.5. Measurement of cell viability

Cell viability was assessed by 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded and cultured as described above. After treatment with vehicle or test drug at 37 °C for 1 h, cells were stimulated with 1 μg/ml of LPS for 24 h, and MTT (0.5 mg/ml) was added at the last 3 h. After the removal of the medium and the addition of 200 μl of DMSO to the well, the absorbance at 570 nm was measured using a microplate reader.

2.6. Animal treatment and the preparation of blood sample

BALB/c mice (20–25 g), obtained from the National Laboratory Animal Center (Taiwan), were used in the in vivo test. The experimental design consisted of four experimental groups, in which DMSO (1 ml/kg) or test drug was intraperitoneally given 0.5 h before a single intravenous injection of saline (1 ml/kg) or LPS (7.5 mg/kg). In the first group ($n = 5$), animals received DMSO followed by a saline injection. In the second group ($n = 8$), animals received DMSO before LPS administration. In the third group ($n = 8$), HPB (45 μmol/kg) was injected before LPS challenge. In the fourth group ($n = 8$), after a bolus dose of HPB (135 μmol/kg), LPS was given. Six hours later, mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and blood was collected by cardiac puncture. Serum was removed and stored at −70 °C for the determination of nitrite/nitrate concentration. All experiments in the present study were performed under the guidelines of the Institutional Experimental Laboratory Animal Committee and were in strict accordance with the principles and guidelines contained in the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health.

2.7. Nuclear extracts preparation and electrophoretic mobility shift assay (EMSA)

For the preparation of cell lysates, cells were washed twice with phosphate buffer saline and harvested in Laemmli SDS sample buffer. Nuclear extracts were prepared as described previously [21]. Briefly, cells were washed twice with ice-cold phosphate buffer saline and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM NaF, and 1 mM Na_3VO_4) and incubated on ice for 15 min. Then the cells were lysed by the addition of 0.5% Nonidet P-40 and vigorous vortexing for 10 s. The nuclei were pelleted by centrifugation at 12,000 × g for 30 s at 4 °C and resuspended in extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, and 1 mM Na_3VO_4). After 15 min on ice, lysates were centrifuged at 12,000 × g for 10 min at 4 °C. Supernatants were obtained and stored at −70 °C.

For EMSA, the oligonucleotide sequence 5′-tcgaCCAACTGGGGACTCTCCCTTTGGGAACA-3′, corresponding to the consensus B site (−92 to −65) of the iNOS promoter [22] was synthesized. Nuclear extract (5 μg) was used for EMSA with a DIG gel shift kit according to the protocol of the manufacturer.

2.8. Western blot analysis

Samples of cell lysate (40 μg) or nuclear extracts (5 μg) were separated by 7.5% SDS-PAGE (for iNOS and β-tubulin) or 10% SDS-PAGE (for MAPKs, IκB, p65, and actin) and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were incubated with 5% nonfat milk in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h and blotted with antibodies against iNOS (1:1000), β-tubulin (1:5000), MAPKs (1:1000), phospho-MAPKs (1:1000), IκB (1:2000), phopho-IκB (1:1000), p65 (1:500) or actin (1:5000). After washing with TBST buffer, a 1:10,000 dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 h. The blots were developed using enhanced chemiluminescence Western blotting reagents. The band intensity was detected and quantitated by Luminescent Image Analyzer (Fujifilm LAS-3000) using MultiGauge software.

2.9. RNA extraction, reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis

Total cellular RNA was isolated from RAW 264.7 macrophages using REzol™ C&T reagent according to the instructions of the manufacturer. Total RNA (20 μg) was subjected to electrophoresis on 1% agarose gels, transferred onto Hybond-N^+ nylon membranes by electroblotting. After UV cross-linking, the membranes were prehybridized, and then hybridized with a cDNA probe [23]. The iNOS and GAPDH cDNA probes were made by reverse transcription by using 2.5 μg of total RNA and reverse-transcribed into cDNA using oligo(dT) primer, then amplified using two oligonucleotide primers derived from published iNOS and GAPDH sequence, including 5′-TCATTGTACTCTGAGGGCTGACACA-3′ and 5′-GCCTTCAACACCAAGGTTGTCTGCA-3′ (iNOS), 5′-TATGACAACTCCCTCAAGAT-3′ and 5′-AGATCCACAACGGATACATT-3′ (GAPDH) [24]. PCR amplification was performed by 25 cycles of 1 min denaturation at 95 °C, annealing for 1 min at 58 °C, with an extension for 1.5 min at 72 °C. The PCR products were labeled with a random primer fluorescein labeling kit. After hybridization, the

membranes were washed and subsequently probed with an antifluorescein-horseradish peroxidase conjugated antibody, before visualization with the nucleic acid chemiluminescence reagent. To ensure equal loading of RNA, a GAPDH probe was used as an internal control to normalize iNOS mRNA expression. The densities of the bands were detected and quantitated by Luminescent Image Analyzer (Fujifilm LAS-3000) using MultiGauge software.

2.10. Plasmids, transient transfection, and reporter assay

Firefly *luciferase* gene under the control of the iNOS promoter, piNOS-LUC [25], and firefly *luciferase* gene under the control of four tandem copies of the consensus NF-κB site, pNF-κB-LUC, were used to quantify iNOS promoter activity and NF-κB transcriptional activity, respectively. A *Renilla* luciferase reporter under the control of the herpes simplex virus thymidine kinase promoter, pRL-TK, was used as an internal control to normalize the reporter gene activity. RAW 264.7 cells were transiently transfected by lipofectAMINE liposomal transfection reagent according to the instructions of the manufacturer. Twenty-four hours later, the culture medium was replaced, and cells were treated with vehicle or HPB for 1 h followed by stimulation with LPS. After 6 or 24 h, the cells were lysed, and the luciferase activity was determined by a luminometer using a Dual-luciferase reporter assay according to the instructions of the manufacturer.

2.11. Determination of reactive oxygen species (ROS)

The production of ROS was determined by labeling with cell-permeable DCFH-DA [26]. Briefly, cells were incubated with vehicle or test drug at 37 °C for 1 h before stimulation with LPS for another 3 h, and then 10 μM of DCFH-DA was added to cells for 15 min at 37 °C. The cells were trypsinized and the fluorescent intensity was analyzed by a FACSCalibur flow cytometry system (BD Biosciences) [27].

2.12. Statistical analysis

Statistical analyses were performed using the Bonferroni *t* test method after ANOVA for multigroup comparison; $P < 0.05$ was considered statistically significant. Analysis of the regression line test was used to calculate IC_{50} values. Values are expressed as means ± S.D.

3. Results

3.1. Effects of HPB on LPS-induced production of NO in vitro and in vivo

RAW 264.7 cells were treated with various concentrations of HPB for 1 h before stimulation with LPS for another 24 h. The culture media were collected, and the nitrite content within the media was determined. The

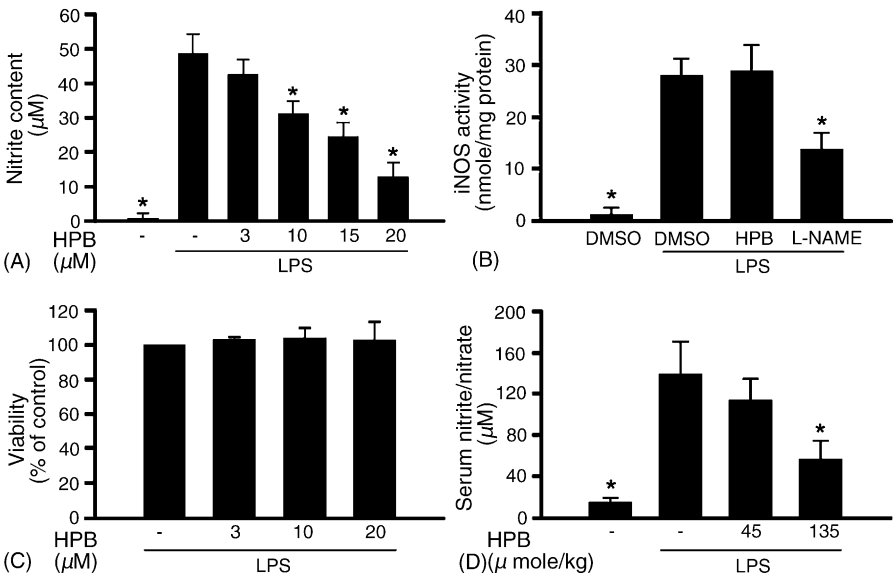

Fig. 2. Effects of HPB on nitrite production, iNOS activity and cell viability in macrophages stimulated with LPS and on serum nitrite/nitrate content in mice challenged with LPS. (A) RAW 264.7 macrophages were pretreated with DMSO or the indicated concentrations of HPB for 1 h before stimulation with LPS (1 μg/ml) for another 24 h. The nitrite content of culture media was analyzed. Values are means ± S.D. of nine independent experiments. $^{*}P < 0.01$, compared with the control value (2nd column). (B) Cell lysates were prepared after treatment of cells with or without LPS for 16 h, and then incubated with 20 μM HPB or 1 mM L-NAME for 5 min prior to the iNOS activity assay. Values are means ± S.D. of three independent experiments. $^{*}P < 0.01$, compared with the control value (2nd column). (C) Cells were treated with DMSO or the indicated concentrations of HPB for 1 h before stimulation with LPS for another 24 h. The cytotoxicity of HPB was measured using MTT assay. Values are means ± S.D. of four independent experiments. (D) DMSO or the indicated concentrations of HPB were injected intraperitoneally 0.5 h prior to LPS (7.5 mg/kg, i.v.) or an equal volume of saline challenge in BALB/c mice. Six hours later, the serum nitrite/nitrate level was determined. Values are means ± S.D. of five to eight mice per group. $^{*}P < 0.01$, compared with the control value (2nd column).

production of nitrite was significantly increased by the LPS treatment. HPB suppressed the LPS-induced nitrite production in a concentration-dependent manner with an IC_{50} value of 14.7 ± 3.2 μM (about 74.9% inhibition at 20 μM HPB) (Fig. 2A). The NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME, 1 mM), inhibited iNOS activity to about 50% of the LPS alone-treated value, whereas HPB (20 μM) failed to change the induced iNOS activity (Fig. 2B). MTT (Fig. 2C) and LDH (data not shown) assays revealed that the inhibition of LPS-induced nitrite production by HPB was not due to cytotoxicity (viability >95% at 20 μM HPB). In the in vivo experiments, LPS (7.5 mg/kg, i.v.) treatment for 6 h resulted in a 9.4-fold increase in the serum nitrite/nitrate levels in BALB/c mice. Administration of HPB (135 μmol/kg or 21.8 mg/kg, i.p.) prior to LPS challenge significantly attenuated the serum nitrite/nitrate concentration (about 65.8% inhibition) (Fig. 2D). The pharmacokinetic analyses of plasma concentration–time profiles of HPB await further study.

3.2. Effects of HPB on LPS-induced iNOS gene expression

To assess the effect of HPB on LPS-induced *iNOS* gene expression, Western and Northern blot analyses were performed. The expression of the iNOS protein and mRNA were barely detectable in unstimulated cells, but markedly increased after treatment with LPS for 24 h. Treatment with HPB showed a concentration-dependent inhibition of iNOS protein and mRNA expression in LPS-stimulated RAW 264.7 cells with IC_{50} values of 15.1 ± 3.2 and 12.4 ± 2.7 μM, respectively (about 68.3% inhibition of iNOS protein expression at 20 μM HPB and 74.4% inhibition of mRNA expression at 15 μM HPB) (Fig. 3A and B). In addition, HPB decreased LPS-induced iNOS promoter activity with an IC_{50} value of 11.0 ± 3.6 μM (Fig. 3C). The similar inhibition of iNOS promoter activity and iNOS mRNA expression by HPB implies that blockade of the transcriptional process has a critical role.

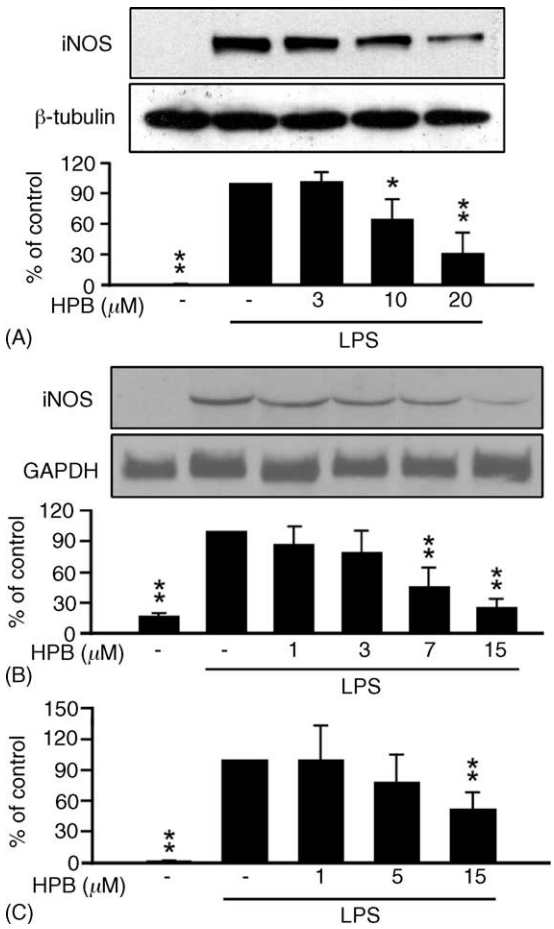

Fig. 3. Effect of HPB on LPS-induced *iNOS* gene expression in RAW 264.7 macrophages. Cells were treated with DMSO or the indicated concentrations of HPB for 1 h followed by stimulation with LPS (1 μg/ml) for another 24 h. (A) The protein levels of iNOS and β-tubulin were determined by Western blot analysis. The ratio of immunointensity between the iNOS and the β-tubulin was calculated. Values are means ± S.D. of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control value (2nd column). (B) The iNOS mRNA was determined by Northern blot analysis. The density ratios of iNOS to GAPDH were calculated. Values are means ± S.D. of five independent experiments. $^{**}P < 0.01$, compared with the control value (2nd column). (C) The piNOS-LUC and the pRL-TK-co-transfected cells were treated with DMSO or the indicated concentrations of HPB for 1 h before stimulation with LPS for another 24 h. The firefly and *Renilla* luciferase activities in the cell lysates were detected by the Dual-luciferase reporter assay system. The former activity was normalized to the respective latter activity. Values are means ± S.D. of five independent experiments. $^{**}P < 0.01$, compared with the control value (2nd column).

3.3. Effects of HPB on LPS-stimulated NF-κB DNA binding and transcriptional activity

Activation of NF-κB is necessary for induction of the *iNOS* gene [7], we therefore examined the effect of HPB on the activation of NF-κB. LPS treatment caused a significant increase in the DNA binding activity of NF-κB as determined by EMSA (Fig. 4A). The NF-κB DNA binding complexes, p65/50 and p50/50, were identified with supershift analysis as in our previous study [23]. In the presence of HPB, LPS-induced NF-κB DNA binding decreased in a concentration-dependent manner (about 42% inhibition at 20 μM HPB). Since p65 was the major component of NF-κB induced by LPS in macrophages, we next determined the levels of p65 in the nuclear extract by immunoblot analysis. The LPS-induced nuclear translocation of p65 (about 52.1% inhibition at 20 μM HPB) (Fig. 4B) and the NF-κB DNA binding activity were both inhibited by HPB in a similar concentration-dependent manner. The expression of reporter genes in cells co-transfected with pNF-κB-LUC and pRL-TK was also analyzed. Consistent with the EMSA assay, the expression of NF-κB luciferase activity was concentration-dependently inhibited by HPB (about 41.4% inhibition at 20 μM HPB) (Fig. 4C).

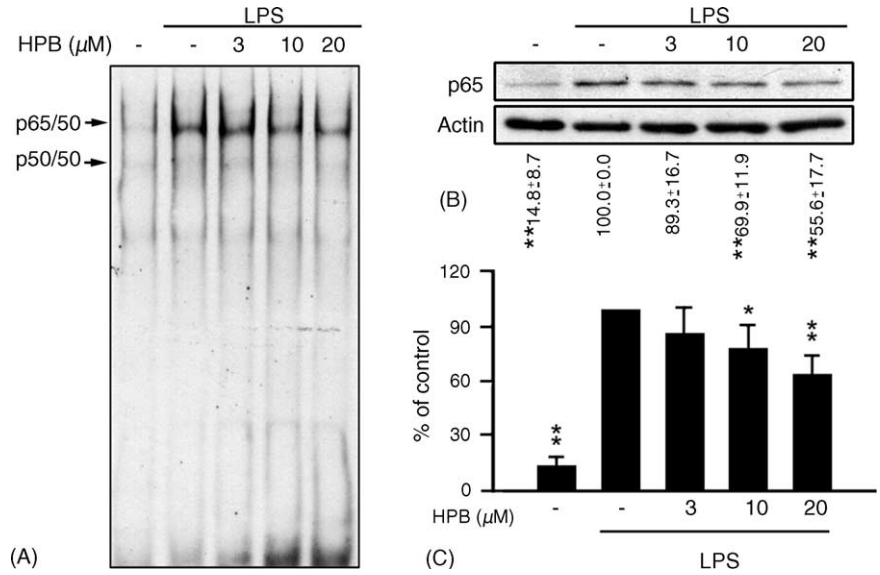

Fig. 4. Effect of HPB on LPS-induced NF-κB activation in RAW 264.7 macrophages. Cells were treated with DMSO or the indicated concentrations of HPB for 1 h before stimulation with LPS for another 1 h. (A) Nuclear extracts were prepared and analyzed by EMSA for NF-κB. The result shown is representative of four independent experiments. (B) The p65 subunit of NF-κB and actin in nuclear extracts was determined by Western blot analysis. The ratio of immunointensity between the p65 and actin is shown. Values are means ± S.D. of six independent experiments. $^{**}P < 0.01$, compared with the control value (lane 2). (C) The pNF-κB-LUC and pRL-TK-co-transfected cells were treated with DMSO or the indicated concentrations of HPB for 1 h before stimulation with LPS for another 6 h. The firefly and *Renilla* luciferase activities in the cell lysates were detected by the Dual-luciferase reporter assay system. The former activity was normalized to the respective later activity. Values are means ± S.D. of five independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control value (2nd column).

3.4. Effects of HPB on phosphorylation and degradation of IκB

The nuclear translocation and DNA binding of NF-κB are preceded by the phosphorylation of IκBα at serine residues and the subsequent proteasome-mediated degradation of IκBα [8]. Pretreatment of RAW 264.7 cells with HPB blocked LPS-induced phosphorylation of IκBα (about 92.5% inhibition at 20 μM HPB) (Fig. 5A). Moreover, IκBα degradation was reduced to 42.8% of the LPS alone-treated value when cells were pretreated with 20 μM HPB (Fig. 5B).

3.5. Effects of HPB on activation of ERK, JNK, and p38 MAPK

To investigate whether ERK, JNK, and p38 MAPK signaling pathways are involved in the suppression of iNOS expression by HPB, the activation of these three MAPKs was assessed by immunodetecting their dually phosphorylated forms. Treatment of cells with LPS for 30 min resulted in a significant increase in the phosphorylation of p38 MAPK, JNK, and ERK (Fig. 6). HPB had no prominent inhibitory effect on the LPS-induced phosphorylation of p38 MAPK, JNK, and ERK. To standardize protein loading in each lane, blots were stripped and reprobed with the corresponding antibodies against p38 MAPK, JNK, and ERK2.

3.6. Effects of HPB on reactive oxygen species production in RAW 264.7 cells

ROS has been implicated in the transduction pathway leading to NF-κB activation [28,29]. Stimulation of cells with LPS caused an intracellular ROS elevation as assessed by cell labeling with permeant DCFH-DA (Fig. 7), which was oxidized by intracellular peroxides to highly fluorescent 2′,7′-dichlorofluorescein. HPB treatment resulted in concentration-dependent inhibition of LPS-induced ROS production (about 61.5% inhibition at 20 μM HPB). Treatment of cells with a cell permeant thiol antioxidant *N*-acetyl-L-cysteine (NAC, 5 mM), which has been reported to inhibit the production of NO in macrophages through the inhibition of NF-κB activation [30,31], also decreased LPS-induced ROS production (about 66.4% inhibition) in RAW 264.7 cells (Fig. 7).

4. Discussion

Because the overproduction of NO can be harmful and result in various inflammatory and autoimmune diseases [4–6], pharmacological interference with the NO production cascade presents promising strategies for therapeutic intervention in inflammatory disorders. In the present study, we demonstrated that a novel synthetic compound HPB inhibits LPS-induced NO production in in vitro and in vivo experiments. To explore the mechanism of inhibition

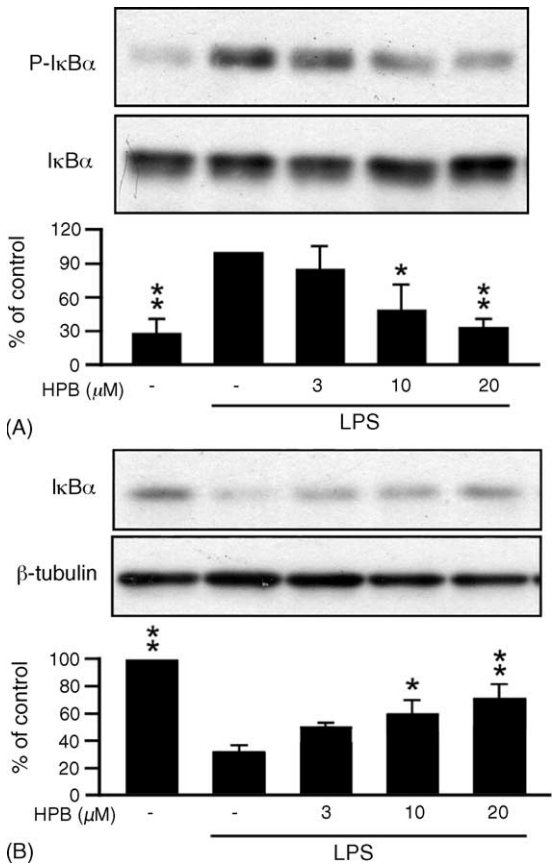

Fig. 5. Effects of HPB on LPS-induced IκBα phosphorylation and degradation in RAW 264.7 cells. (A) Cells were treated with DMSO or the indicated concentrations of HPB for 1 h before stimulation with LPS for 5 min. IκBα phosphorylation was detected by immunoblot analysis using anti-phospho-IκBα. The blots above were then stripped and reprobed with anti-IκBα. The ratio of immunointensity between the phospho-IκBα and the IκBα was calculated. Values are means ± S.D. of six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control value (2nd column). (B) Cells were incubated with DMSO or the indicated concentrations of HPB for 1 h before stimulation with LPS for 10 min. The protein levels of IκBα and β-tubulin were determined by Western blot analysis. The ratio of immunointensity between IκBα and β-tubulin was calculated. Values are means ± S.D. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control value (2nd column).

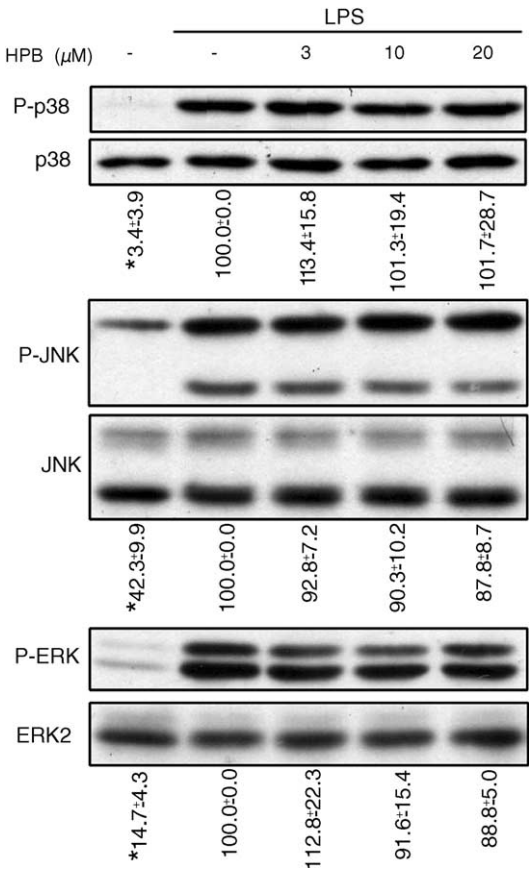

Fig. 6. Effect of HPB on LPS-induced phosphorylation of MAPKs in RAW 264.7 macrophages. Cells were treated with DMSO or the indicated concentrations of HPB for 1 h before stimulation with LPS for 0.5 h. The phosphorylation of MAPKs was detected by immunoblot analysis using antibodies against the corresponding activated forms of MAPKs (dually phosphorylated on Tyr/Thr). The blots above were then stripped and reprobed with antibodies against the corresponding MAPKs. The ratio of immunointensity between the MAPKs and the phosphorylated MAPKs is shown. Values are means ± S.D. of four independent experiments. $^{*}P < 0.01$, compared with the control value (lane 2).

of NO production in RAW 264.7 macrophages, the effect of HPB on *iNOS* gene expression was examined. HPB inhibited the expression of iNOS protein and mRNA as assessed by using Western and Northern blot analyses, respectively, in a similar concentration-dependent manner with IC_{50} values similar to that required for inhibition of NO production. These results imply that HPB may exert its effect through the inhibition of the iNOS mRNA transcriptional step. To examine the effect of HPB on the expression of the *iNOS* gene, cells were transiently co-transfected with a piNOS-LUC reporter construct containing the iNOS promoter sequence linked to the firefly *luciferase* gene [25], and a pRL-TK reporter construct containing the herpes simplex virus thymidine kinase promoter sequence linked to the *Renilla luciferase* gene as an internal control to normalize the reporter gene activity. The finding that HPB inhibited the LPS-induced iNOS promoter activity with IC_{50} value similar to that required for inhibition of iNOS mRNA expression excludes the possibility that HPB affects the stability of expressed iNOS mRNA.

The promoter of the *iNOS* gene is known to contain two transcriptional regulatory regions, an enhancer and a basal promoter [32]. There are a number of binding sites for transcription factors, including κB site, located in both the enhancer and the basal promoter [7], and NF-κB is essential for LPS-mediated NO production. In unstimulated cells, NF-κB is present in the cytosol as homodimer or a heterodimer and is linked to the inhibitory IκB protein. The activation of NF-κB results in phosphorylation, ubiquitination, and proteasome-mediated degradation of IκB proteins, followed by nuclear translocation and DNA binding of the NF-κB [8]. These findings indicate that HPB inhibited the LPS-induced DNA binding activity of NF-κB as assessed in nuclear extract with the EMSA assay and the nuclear translocation of p65, and the transcriptional

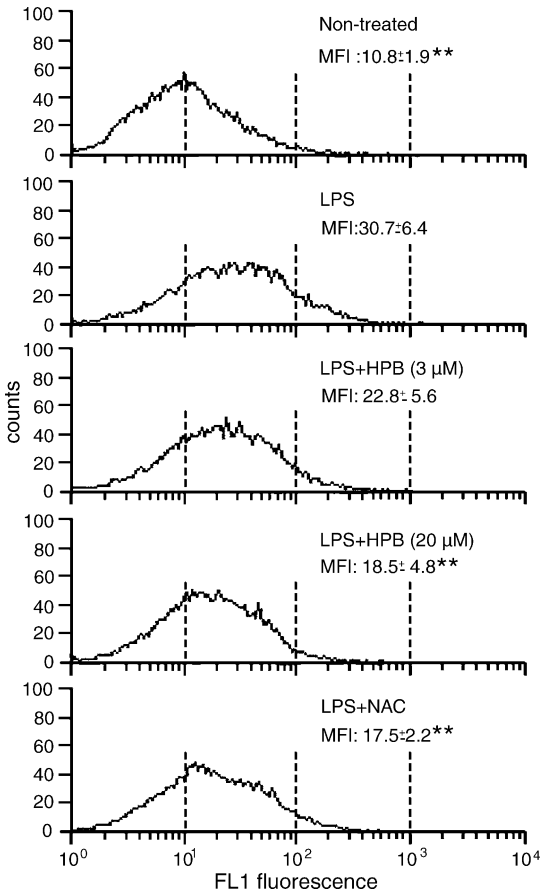

Fig. 7. Effect of HPB on LPS-induced ROS production in RAW 264.7 macrophages. Cells were treated with DMSO, 5 mM NAC or the indicated concentrations of HPB for 1 h before stimulation with LPS for another 3 h. Intracellular ROS levels were determined by labeling with DCFH-DA and the fluorescent intensity was analyzed by a flow cytometry system. Values are means ± S.D. of five independent experiments. $^{**}P < 0.01$, compared with the value of LPS alone-treated group (panel 2).

activity of NF-κB as assessed in cells transiently transfected with a pNF-κB-LUC reporter construct containing four tandem copies of the NF-κB consensus sequence linked to the firefly *luciferase* gene. In addition, HPB inhibited LPS-induced IκBα phosphorylation and degradation. It is conceivable that IκB proteins are phosphorylated by IκB kinase (IKK), and the activation of IKK is a key regulatory step in NF-κB activation [33]. These results suggest that inhibition of IKK activity can be, at least partly, a mechanism responsible for the inhibitory effect of HPB on NF-κB activation.

There are at least three families of MAP kinases (p38 MAPK, JNK, and ERK) that exist in mammalian cells. They play a critical role in the regulation of cell growth and differentiation particularly in response to cytokines and stress [34]. Several studies have demonstrated the implication of MAPKs in LPS-induced iNOS expression [9–12] and the activation of NF-κB [35,36]. Cell stimulation induces a signaling cascade that leads to the activation of MAPKs via phosphorylation on both tyrosine and threonine residues [37], which in turn induces a conformational change that exposes the active site for substrate binding. The results that HPB had no effect on the phosphorylation of the three MAPKs in response to LPS preclude the involvement of MAPK signaling in the inhibition of NO production by HPB.

ROS has been implicated in the activation of NF-κB in various cell types [28,29]. By contrast, treatment with antioxidants, which abrogates the accumulation of intracellular ROS, suppresses NF-κB activation [30–31] and inhibits iNOS expression [38]. Although every step of the NF-κB signaling pathway is redox sensitive, IKK is conceded to be the prime target of redox regulation [39,40]. Phenolics often possess antioxidant activity, which could allow them to inhibit NF-κB activation by reducing ROS [18]. In the present study, exposure of RAW 264.7 cells to LPS strikingly stimulated the accumulation of intracellular ROS. Pretreatment of cells with phenolic HPB, like the thiol-reducing agent NAC, significantly attenuated the LPS-induced ROS production. It is plausible that the antioxidant activity of HPB is the mechanism responsible for its inhibition of NF-κB activation. However, the precise site of action at which HPB inhibits awaits further study.

In summary, we have demonstrated that inhibition of LPS-induced NO production by HPB is attributed to the down-regulation of NF-κB, but not to the cytotoxicity or the inhibition of iNOS activity. The antioxidant activity of HPB, thereby blockade of IκBα phosphorylation, is probably responsible for inhibition of NF-κB activation. The inhibition of NF-κB activation by HPB may be useful for alleviating the symptoms of inflammatory diseases.

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